Control of Glycolysis through Regulation of PFK1: Old Friends and Recent Additions

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Regulation of glucose metabolism is a crucial aspect of cell physiology in normal and disease conditions. Many regulatory events are involved in determining the metabolic fate of glucose and the pathways into which it is directed. The first reaction that commits glucose to the glycolytic pathway is catalyzed by the enzyme phosphofructokinase-1 (PFK-1) and is tightly regulated. One of the most potent activators of PFK-1 is fructose 2,6 bisphosphate (F2,6BP) and its cellular levels are correlated with glycolytic flux. F2,6BP is synthesized and degraded by a family of bifunctional enzymes—the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB). The interplay among F2,6BP levels, the enzymes that generate and degrade it, and PFK-1 activity has important consequences for several different aspects of cell metabolism as well as for systemic metabolic conditions. TIGAR, a recently identified F2,6 bisphosphatase (F2,6BPase), could also contribute to this complexity and participate in shaping the metabolic profile of the cell.

PHOSPHOFRUCTOKINASE-1: ITS ROLE AND REGULATION

Glucose imported into the cell can be utilized by different metabolic pathways depending on the cells' metabolic needs. It can be catabolized through glycolysis or the pentose phosphate pathway (PPP), and in the liver and muscle it can also be used as the building block for glycogen (Fig. 1A). To prevent glucose from escaping the cell, it is readily phosphorylated to glucose-6-phosphate. This is the primary substrate for the oxidative branch of the PPP that generates the ribose backbone of nucleotides as well as reducing agents that can target reactive oxygen species (ROS) (Fig. 1A). Alternatively, glucose-6-phosphate can be utilized in generation of glycogen, following its conversion to glucose-1-phosphate. If glucose is to be directed to glycolytic breakdown, glucose-6-phosphate must first be converted to its isomer, fructose 6-phosphate, in a reversible reaction. Then follows a key step in glycolysis, the phosphorylation of fructose 6-phosphate to fructose 1, 6 bisphosphate by the enzyme PFK-1. This is the first point of commitment of glucose to the glycolytic pathway (Weber 1977) and because this reaction involves the hydrolysis of ATP, it is essentially irreversible. Importantly, PFK-1 activity is rate-limiting and therefore critical in determining glycolytic flux (Yalcin et al. 2009b; Jenkins et al. 2011) with signals that stimulate PFK-1 activity, resulting in an activation of glycolysis.

PFK-1 is encoded by three genes: *PFK-M* (muscle), *PFK-L* (liver), and *PFK-P* (plateletes), each encoding a different isoform. The enzyme functions as a tetramer, and the different isoforms can form homotetramers or heterotetramers depending on the cell type (Moreno-Sanchez et al. 2007). Various mutations in the gene encoding PFK-M cause PFK1 deficiency, which leads to the development of glycogen storage disease type VII (Raben and Sherman 1995). Because the cells cannot commit glucose to glycolysis, glucose is diverted to the glycogenic pathway, leading to glycogen buildup (Garcia et al. 2009). Muscle cells, which express the PFK-M isoforms exclusively, are more severely affected, but even tissues that express two PFK-1 isoenzymes to form heterodimers cannot fully compensate for the inactive PFK-M and cannot commit glucose efficiently to glycolysis (Garcia et al. 2009). The muscle cells of affected patients rely on other carbon sources such as circulating fatty acids or ketone bodies (Haller and Lewis 1991). This testifies to the importance of PFK-1 activity in balancing the alterative fates of glucose.

Indeed, PFK-1 activity has been correlated with changes in cellular metabolism and physiology. It is increased in response to proliferation signals and is correlated with elevated glycolysis in proliferating cells (Yalcin et al. 2009b). Accordingly, elevated PFK-1 activity is also characteristic of cancer cells and is induced in response to oncogenes or following HIF1α activation (Moreno-Sanchez et al. 2007; Yalcin et al. 2009b).

Because PFK-1 functions as the gatekeeper to glycolysis, its activity is tightly regulated. Localization to the actin filaments of the cytoskeleton has been demonstrated to enhance PFK-1 activity (Real-Hohn et al. 2010). Lactate, an end product of glycolysis, induces dissociation of the tetramers to dimers, which reduces enzymatic activity, providing negative feedback for glycolytic rate (Costa Leite et al. 2007). PFK-1 is also subject to

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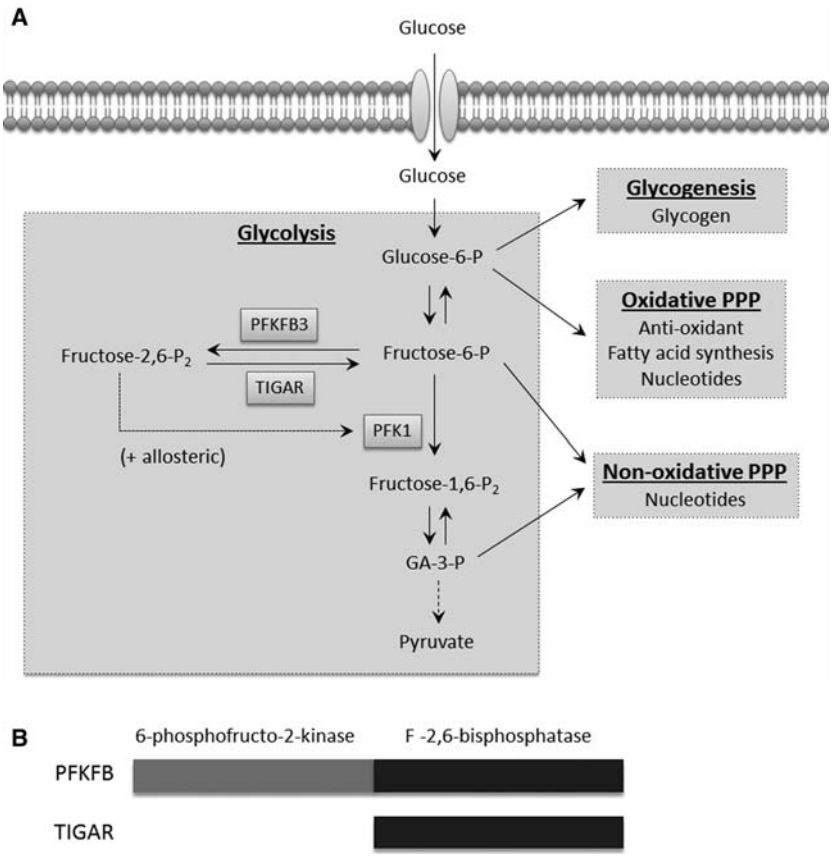

Figure 1. (*A*) Alternate pathways for glucose metabolism. Glucose entering the cell can be directed to different pathways that will generate different metabolites. Phosphofructokinase-1 (PFK-1), which catalyzes the first committed step of glycolysis in an irreversible reaction, is activated by fructose 2,6 bisphosphate (F2,6BP). F2,6BP levels in the cell are determined by the rate of its generation and degradation by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB) and TIGAR. (*B*) Schematic representation of the functional organization of PFKFB and TIGAR. PFKFB is a bifunctional enzyme where the kinase and bisphosphatase active sites are found in tandem. TIGAR displays partial similarity and possesses only the bisphosphatase active site. PPP, Pentose phosphate pathway.

complex allosteric regulation that allows tight control over glycolytic flux and coordination of glucose entry to glycolysis with the overall metabolic needs of the cells. PFK-1 is inhibited by ATP and activated by AMP (Hers and Van Schaftingen 1982), providing a mechanism through which its activity can respond to the energetic status of the cell, preventing superfluous glucose degradation when ATP levels are ample. PFK-1 is also regulated by other metabolites that indicate the flux through metabolic pathways found downstream from glycolysis. For example, PFK-1 is inhibited by citrate generated in the TCA cycle and by long-chain fatty acids (Jenkins et al. 2011). The most potent allosteric activator of PFK-1 is fructose 2,6 bisphosphate (F2,6BP), which can elevate enzymatic activity even in the presence of ATP (Hers and Van Schaftingen 1982; Moreno-Sanchez et al. 2007). Because F2,6BP is also generated from glucose-6-phosphate (discussed below), it functions to enhance glycolysis when glucose is present. By allowing PFK-1 regulation to bypass the product inhibition effect of ATP, F2,6BP uncouples the link between cellular bioenergetics and glycolytic flux, suggesting that it could play a role in the elevated PFK-1 activity detected in many cancer cells (Moreno-Sanchez et al. 2007).

THE ROLE OF FRUCTOSE 2,6 BISPHOSPHATE IN CELL METABOLISM

F2,6BP is produced in a reaction catalyzed by phosphofructokinase-2 (PFK-2), utilizing the same substrate as PFK-1: fructose 6-phosphate derived from glucose 6-phosphate (Fig. 1A). Cellular levels of F2,6BP are determined by the balance between its production and its hydrolysis to fructose-6-phosphate by F2,6BPase. F2,6BP stimulates glycolysis in the presence of glucose in various tissues (Okar et al. 2001; Rider et al. 2004). In the liver, F2,6BP plays a role in synchronizing the utilization of glucose in glycolysis and its regeneration by gluconeogenesis. Because the reaction catalyzed by PFK-1 is irreversible, the conversion of F1,6BP to fructose-6-phosphate is catalyzed by a different enzyme—F1,6BPase, which functions as a rate-limiting enzyme in gluconeogenesis. F2,6BP regulates the dynamics between

glycolysis and gluconeogenesis in the liver by activating PFK-1 and inhibiting F1,6BPase (Rider et al. 2004).

Interestingly, both the kinase and phosphatase reactions generating and degrading F2,6BP are catalyzed by the same protein, the 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (PFKFB; Okar et al. 2001; Rider et al. 2004). The two catalytic domains are localized on one polypeptide chain with the kinase domain in the amino-terminal region and the phosphatase at the carboxyl terminus (Fig. 1B; Rider et al. 2004). In mammals, there are four PFKFB genes (annotated *PFKFB1–4*) encoding various isoenzymes that all share the same overall domain organization (Okar et al. 2001). Each of the different PFKFB isoenzymes have both kinase and phosphatase activity, with the exception of PFKB3, which essentially functions only as a kinase. One isoform of PFKFB3 is expressed ubiquitously (Okar et al. 2001; Rider et al. 2004), whereas an inducible isoform of PFKFB3 with a different carboxy-terminal sequence is expressed in rapidly proliferating cells as well as in tumors (Chesney et al. 1999). Tissue-specific expression of the PFKFB variants as well as alternative splicing and alternative promoter usage coupled with various enzymatic regulatory mechanisms (covalent and allosteric) each contribute to the regulation of these bifunctional enzymes (Okar et al. 2001; Rider et al. 2004).

PFK-2 activity is also subject to hormonal regulation. It is enhanced in response to insulin signaling, leading to elevated F2,6BP levels and enhancing glycolytic rate (Rider et al. 2004; Atsumi et al. 2005). The different isoforms of PFK2 respond to additional hormonal stimuli such as adrenalin and androgen, leading to elevation in the kinase function and increasing cellular F2,6BP levels (Moon et al. 2010). This activation, mediated by hormonal signaling, allows the cells to respond to increased glucose by elevating glycolytic rate. Elevation of PFKFB3 levels, which results in elevated F2,6BP and thus increased glycolysis, is also involved in adipocyte lipogenesis and triglyceride synthesis (Atsumi et al. 2005). Thus, F2,6BP is an important agent in adaptation of different tissues to changes in metabolic requirements. Furthermore, these changes in cellular metabolism could consequently lead to systemic metabolic conditions. A reciprocal consequence has been described in which PFKB3 is involved in insulin resistance, obesity, and diabetes. Long-term exposure to insulin has been shown to reduce inducible-PFKFB3 mRNA (messenger RNA) levels in an adipocyte cell line, whereas short-term doses have been shown to activate the enzyme, suggesting a negative feedback mechanism (Atsumi et al. 2005). Mice with reduced levels of the inducible-PFKFB3 in adipose tissue displayed the expected reduced glycolysis but also increased insulin resistance (Huo et al. 2010). The reduction in glycolysis was accompanied by a compensatory increase in fatty acid oxidation that resulted in reduced weight gain when the mice were fed a high-fat diet and possibly elevated oxidative stress. This study demonstrated the importance of PFKFB regulation to the adequate function of adipose tissue.

Because it is tightly connected to metabolism, the cell cycle has also been shown to be involved in regulation of F2,6BP levels. The inducible isoform encoded by PFKFB3 is constantly degraded through ubiquitination in resting cells. Preceding the G_1-to-S transition, the E3 ubiquitin ligase APC/C-Cdh1 is inactivated and PFKFB3 levels increase, leading to the enhanced glycolysis required for cell proliferation (Almeida et al. 2010). The continuous degradation of PFKFB3 throughout the cell cycle strictly limits its function to a unique stage. This mechanism, which is classically known for other proteins regulating cell cycle progression (cyclins), suggests that adequate timing of PFK-2 activity and elevated glycolysis could be equally important to the control of cell cycle progression.

F2,6BP REGULATION BY ONCOGENES AND TUMOR SUPPRESSORS AND ITS ROLE IN CANCER CELL METABOLISM

The link between glucose metabolism and cancerous transformation has long been known (Warburg 1956), although the mechanisms underlying this connection are still being explored. Various glycolytic enzymes are commonly elevated in cancer cells, including PFK-1, leading to the elevated glycolytic flux characteristic of these cells (Moreno-Sanchez et al. 2007). There is also a link between the regulation of cellular F2,6BP levels and various processes involved in cancer. The inducible PFK-2 isoform PFKFB3 is often highly expressed in human cancers of brain astrocyles, colon, prostate, breast, ovary, and thyroid when compared with adjacent normal tissues (Atsumi et al. 2002; Bando et al. 2005; Kessler et al. 2008). The tight regulation of PFKFB3, which coordinates its levels with cell cycle progression, suggests a role for F2,6BP in cell proliferation (Almeida et al. 2010; Tudzarova et al. 2011). Moreover, when PFKFB3 is silenced in HeLa cells, this prevents the sharp and short increase in protein levels in late G_1, and the cells fail to progress to S phase (Tudzarova et al. 2011). It should be noted that, in these cells, elevation of PFKFB3 coincided with a rise in lactate production, supporting its contribution to glycolytic flux. Another study in HeLa cells demonstrated that cell viability and anchorage-independent cell growth are also compromised by PFKFB3 silencing (Calvo et al. 2006). Heterozygotic genomic deletion of PFKFB3 in *ras*-transformed mouse fibroblasts reduced the invasive capacity of these cells (Telang et al. 2006). Reciprocally, cells transformed by oncogenes such as *v-src*/*vfps* or *ras* showed elevated F2,6BP as well as increased glycolytic flux (Bosca et al. 1986; Kole et al. 1991), demonstrating the link between oncogenic transformation and metabolic adaptation conferred by F2,6BP. However, there is evidence that in some cells the relationship between PFKFB3 expression and intracellular F2,6BP levels is not so straightforward, with the elevated PFKFB3 expression associated with immortalization being accompanied by a decrease in intracellular F2,6BP (Telang et al. 2006). Interestingly, in this case F2,6BP levels were

also not directly correlated with glycolytic flux. The authors speculate that this could be the outcome of elevated glycolysis leading to negative feedback compensation or increased use of F2,6BP as glycolytic substrate following its conversion to F6P.

Studies in different cancer cell lines have demonstrated that the increased levels of PFK-2 activity are achieved through various mechanisms, including elevated transcription, activation of the enzyme through posttranslational modification, and reduced proteosomal degradation (Okar et al. 2001; Rider et al. 2004; Bando et al. 2005; Almeida et al. 2010). For example, hypoxia-inducible factor 1 (HIF1), which is commonly stabilized in cancer cells, induces increased transcription of the PFKFB genes but to a different extent depending on the cell type (Minchenko et al. 2003). PFKFB3 phosphorylation, which enhances enzymatic activity, is increased in human tumor cells and is correlated with increased proliferation of COS7 cells in culture (Bando et al. 2005). Phosphorylation of the different isoforms of PFKFB may be achieved by several kinases, including AKT and AMP-activated protein kinase, protein kinase A, and protein kinase C, among others (Marsin et al. 2000, 2002; Rider et al. 2004; Mukhtar et al. 2008; Moon et al. 2010), allowing the regulation of glycolysis in response to various signaling pathways. Whereas several isoforms of PFK-2 are expressed in cancer cells, it is assumed that the inducible FKBFB3 has the dominant effect on cellular F2,6BP levels and glycolytic flux rate because of its high kinase activity (Telang et al. 2006; Yalcin et al. 2009b). Indeed, a small-molecule inhibitor designed to specifically target PFKFB3 reduced F2,6BP levels, glucose uptake, and tumor growth when administered to tumor-bearing mice (Clem et al. 2008). As an efficient glycolytic activator, which is tightly regulated in normal cells and highly expressed in transformed ones, PFKFB3 thus provides an attractive target to cancer therapy. Development of specific inhibitors that would directly inhibit the metabolically modified cancer cells and spare the normal cells could provide an effective method to utilize the central role of F2,6BP levels for cancer treatment.

Interestingly, another link between PFKFB3 and cell cycle regulation in transformed cells has been suggested because one of its splice variants harbors a nuclear localization signal (Yalcin et al. 2009a). This variant is the dominantly expressed splice variant in several tumor cell lines, and it is the only one that localizes to the nucleus. Both kinase activity and nuclear localization were found to be important for the induction of cell proliferation and resulted in elevated expression of cell cycle proteins. This study suggests that the effects of F2,6BP on cancer cells can be mediated at many levels, requiring correct localization as well as timing.

TIGAR F2,6BPase: WHAT CAN WE EXPECT?

TIGAR (*T*P53-*i*nduced *g*lycolysis and *a*poptosis *r*egulator), is a p53-inducible protein that functions as fructose-2,6-bisphosphatase and fructose-1,6-bisphosphatase, reducing glycolytic rate and promoting the PPP (Bensaad et al. 2006; Li and Jogl 2009). Although TIGAR shares only partial structural similarity with the PFKFB family (Fig. 1B), its ability to degrade F2,6BP has been shown to participate in the balance of F2,6BP cellular levels (Bensaad et al. 2006; Li and Jogl 2009; Derdak et al. 2011). Consistent with increased activation of the PPP, cells expressing TIGAR have higher NADPH levels and a concomitantly enhanced ability to regulate levels of cellular ROS and thus reduce oxidative stress (Bensaad et al. 2006, 2009; Lui et al. 2011). Like PFKFB, TIGAR levels are also elevated in response to hypoxia (Kimata et al. 2010).

Because TIGAR has no kinase domain, studies on PFKFBs in which the kinase activity has been abolished can also teach us about the role of TIGAR in cells. Overexpression of a kinase-deficient PFKFB in the heart led to reduced glycolysis, elevated glycogenesis, and reduced insulin sensitivity (Donthi et al. 2004). Overexpression of kinase-dead PFKFB3 in cultured HeLa cells eliminated the proliferative effect detected following overexpression of the wild-type enzyme (Yalcin et al. 2009a), although in this case the effect was not attributed to direct proglycolytic function but to nuclear localization of a specific PFKFB3 splice variant and activation of cell-cycle-related proteins by generation of F2,6BP in the nucleus. In a rat model for alcoholic liver disease, reduction of hepatic F2,6BP levels as a result of TIGAR induction by p53 led to reduced glycolysis and increased insulin resistance (Derdak et al. 2011). This demonstrates that TIGAR, like PFKBP, can contribute to the regulation of systemic glucose metabolism. Maintaining low F2,6BP levels could also play a role in normal cell physiology. Reduced PFK-2 kinase activity is characteristic of neuronal cells as compared with neighboring astrocytes, because PFKFB3 is actively removed in these cells through proteosomal degradation. Ectopic expression of PFKFB3 demonstrated the importance of diverting glucose from glycolysis to the maintenance of neurons (Herrero-Mendez et al. 2009). By elevating glycolysis, less glucose was shunted to the PPP pathway and the resulting reduction in reduced glutathione levels caused neurons to be more sensitive to oxidative stress (Herrero-Mendez et al. 2009). It is therefore tempting to speculate that TIGAR may also contribute in this response.

Like the PFKFBs, TIGAR may also play a role in cancer metabolism. It should be noted that the effect of glycolytic inhibition and PPP elevation on cell fate is cell-type dependent and probably reflects the metabolic background of the cell (Bensaad et al. 2006; Kimata et al. 2010). The effect of TIGAR in a particular cell type may also depend on the activities of all the other PFKFBs present, which all together will determine the total amount of F2,6BP and thus the rate of glycolysis. Therefore, whereas TIGAR might be predicted to function in the opposite way to PFKFB3, it is not clear that we can deduce whether TIGAR inhibits or promotes tumor cell growth. On the one hand, TIGAR expression would be detrimental for cells that are highly dependent on glycolysis for survival—or under conditions where the promotion of glycolysis (as may be mediated by expression of

PFKBP3) contributes to tumorigenesis. TIGAR expression would help to limit glycolysis and so shift the balance away from aerobic glycolysis, which is so commonly seen in cancers. Each of these activities would be consistent with TIGAR as a downstream mediator of p53's tumor suppressor activity. The ability of TIGAR to shift at least some glucose into the PPP will result in the generation of NADPH and precursors of nucleotide synthesis. Again, these activities could help to limit cancer development by lowering ROS and allowing effective DNA repair. However, unregulated expression of TIGAR may also help aberrantly proliferating tumor cells to survive toxic levels of oxidative stress. Factors that determine the ultimate response of the cell are unknown, but may partially reflect the different proliferative capacities, which will lead to different requirements for glycolysis versus ROS protection by the PPP. For example, in several cancer cell lines, down-regulation of TIGAR resulted in reduced NADPH levels and growth inhibition (Bensaad et al. 2006; Lui et al. 2010, 2011). In addition, a high expression of TIGAR protein is observed in tissues from human breast cancer patients (Won et al. 2011). On the other hand, increased TIGAR levels resulted in enhanced apoptosis in a lymphocytic cell line that correlated with decreased glycolysis (Bensaad et al. 2006). Additionally, in primary cardiomyocytes, enhanced glycolytic flux following TIGAR down-regulation can maintain mitochondrial membrane potential and protect these noncancerous cells from hypoxia-induced cell death (Kimata et al. 2010). This bimodal effect can also be relevant to different stages of cancerous transformation as the cell's metabolic profile gradually changes.

As a new member of the family, the role of TIGAR in cancer formation and later in tumor progression and maintenance is still to be determined. Likewise, its importance to the metabolism of different tissues is yet to be described. However, because regulation of F2,6BP levels clearly has complex and widespread implications, it will be interesting to further study TIGAR's contribution to the regulation of metabolism in health and disease.

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