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Metabolic kinases moonlighting as protein kinases

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Abstract

Protein kinases regulate every aspect of cellular activity, whereas metabolic enzymes are responsible for energy production and catabolic and anabolic processes. Emerging evidence demonstrates that some metabolic enzymes, such as pyruvate kinase M2, phosphoglycerate kinase 1, ketohexokinase isoform A, hexokinase (HK), and nucleoside diphosphate kinase 1 and 2 (NME1/2), that phosphorylate soluble metabolites can also function as protein kinases and phosphorylate a variety of protein substrates to regulate the Warburg effect, gene expression, cell cycle progression and proliferation, apoptosis, autophagy, exosome secretion, T-cell activation, iron transport, ion channel opening, and many other fundamental cellular functions. The elevated protein kinase functions of these moonlighting metabolic enzymes in tumor development make them promising therapeutic targets for cancer.

Keywords

metabolic enzymes; protein kinase; phosphorylation

Protein kinases are important regulators of intracellular signal transduction pathways that mediate the development and regulation of both unicellular and multicellular organisms. They play critical roles in cell growth, division, differentiation, adhesion, motility, and death (Brognard and Hunter, 2011; Lu and Hunter, 2009). These protein kinases, more than 500 of which exist in the human genome, can be primarily subdivided into tyrosine (Y)- and serine (S)/threonine (T)-specific kinases based on their catalytic specificity (Manning et al., 2002).

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Disruption of normal protein kinase functions by mutations, altered expression, or dysregulation can cause many human diseases, including cancer and diabetes (Lu and Hunter, 2009).

Cell metabolism comprises the life-sustaining chemical reactions ultimately responsible for all cellular processes; production of ATP and building blocks for proteins, lipids, nucleic acids, and some carbohydrates; and elimination of nitrogenous wastes. Metabolic enzymes catalyze these reactions, facilitating cell growth and proliferation and the response to all intracellular and extracellular signaling and stimuli. Each metabolic enzyme is known to catalyze a unidirectional and/or bidirectional reaction in a specific metabolic pathway. However, recent studies have demonstrated that several metabolic enzymes can also moonlight as protein kinases and phosphorylate multiple protein substrates. These phosphorylations are critical for key cellular functions, including the Warburg effect, a feature of which is a high rate of glycolysis and lactic acid fermentation in the cytosol of cancer cells regardless of oxygen level (Li et al., 2016b; Li et al., 2016d; Yang and Lu, 2015). This review highlights the roles of the unexpected protein kinase activity of the metabolic enzymes pyruvate kinase M2 (PKM2), phosphoglycerate kinase 1 (PGK1), ketohexokinase isoform A (KHK-A, or fructokinase A), hexokinase (HK) and the NME1/2 histidine (H) kinases in regulation of a variety of cellular functions and the impact of this regulation on tumorigenesis.

PKM2

Pyruvate kinase regulates the final rate-limiting step of glycolysis by catalyzing the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) to produce pyruvate and adenosine triphosphate (ATP) (Yang and Lu, 2015). This kinase has four isoforms: PKL, PKR, PKM1, and PKM2. PKL and PKR, which are encoded by the *PKLR* gene, are expressed in the liver and erythrocytes, respectively, whereas PKM1 and PKM2, which are encoded by the *PKM* gene, are expressed in different types of cells and tissues. The heterogeneous nuclear ribonucleoproteins A1 (hnRNPH1) and A2 (hnRNPH2) and polypyrimidine-tract (PPT) binding protein splicing factors regulate alternative splicing of *PKM* pre-mRNA and generate PKM2 via the inclusion of the PKM2-specific exon 10 and exclusion of the PKM1-specific exon 9 (David et al., 2010; Noguchi et al., 1987). An isoform switch from PKM1 to PKM2 and enhanced PKM2 expression have been found in many cancer types (Bluemlein et al., 2011; Desai et al., 2014; Yang et al., 2012a).

Genetic replacement of PKM2 with PKM1 inhibits aerobic glycolysis and tumor growth in mice, although PKM1 has higher glycolytic activity than PKM2 does (Christofk et al., 2008; Guminska et al., 1988; Mellati et al., 1992). One of the fundamental functional differences between PKM1 and PKM2 is that the latter has unique nuclear functions. Specifically, PKM2 contains a nuclear localization signal (NLS) encoded by exon 10, whereas PKM1 lacks an NLS. Activated extracellular signal-regulated kinase (ERK) 1/2 mitogen-activated protein kinases bind to an ERK docking groove encoded by PKM2 exon 10 thus allowing ERK1/2 to phosphorylate PKM2 but not PKM1 at Ser37. Once phosphorylated, the pSer37.Pro38 bond in PKM2 is subject to *cis-trans* isomerization by the phospho-specific peptidyl-proline isomerase protein interacting with never in mitosis A-1 (PIN1), causing a

conformational change in PKM2, conversion of PKM2 from a tetramer to a monomer, and exposure of the PKM2 NLS for importin α5 binding and subsequent nuclear translocation (Yang and Lu, 2013; Yang et al., 2012c). Interaction of PKM2 with JMJD5, a Jumonji C domain-containing dioxygenase, hinders PKM2 tetrameric assembly and facilitates PKM2's nuclear translocation (Wang et al., 2014a). In addition, enhanced nuclear translocation of PKM2 is induced by sumoylation of PKM2 mediated by the SUMO-E3 ligase PIAS3 and by acetylation of PKM2 at Lys433 mediated by the p300 acetyltransferase that prevents the binding of fructose-1,6-bisphosphate to PKM2 and formation of a PKM2 tetramer (Lv et al., 2013; Spoden et al., 2009). This evidence supports the possibility that PKM2 translocates into the nucleus as a monomer.

In the nucleus, PKM2 phosphorylated at Ser37 in the cytosol in response to growth factor receptor (EGFR) activation, is dephosphorylated by the dual-specificity Cdc25A phosphatase, thereby forming a complex with β-catenin that has been phosphorylated by c-Src – at Tyr333, which then binds to β-catenin–regulated target promoter regions of genes, such as *MYC* and *CCND1* (Liang et al., 2016b; Yang et al., 2011). Using PEP as a phosphate donor, nuclear PKM2 phosphorylates histone H3 at Thr11 in nucleosomes associated with gene promoter regions. This phosphorylation is required for the dissociation of histone deacetylase 3 (HDAC3) from the gene promoter regions and the subsequent acetylation of histone H3 at Lys9. PKM2-dependent histone H3 phosphorylation is essential for EGFR-promoted cell proliferation and tumorigenesis as well as expression of specific genes, including c-Myc–regulated genes such as *GLUT1* and lactate dehydrogenase A (*LDHA*), and polypyrimidine-tract binding (*PTB*) protein, which increases PKM2 mRNA expression levels via alternative splicing of *PKM* pre-mRNA. Thus, nuclear PKM2 enhances the Warburg effect by upregulating expression of glycolytic genes, including itself (Yang et al., 2012b; Yang et al., 2012c). PKM2 also binds to other transcription factors, such as hypoxia-inducible factor (HIF) 1α, signal transducer and activator of transcription (STAT)3, and Oct4, and enhances their target gene expression (Gao et al., 2012; Luo et al., 2011; Yang and Lu, 2015). Notably, PKM2 phosphorylates STAT3 at Tyr705 and promotes STAT3 transcriptional activity (Gao et al., 2012). Although one study of the ability of recombinant PKM2 to phosphorylate proteins in PKM2-deficient cell lysates failed to detect PEP-dependent protein kinase activity, possibly due to insufficient levels of key target substrate proteins in the cell lysates and the low concentration of ^{32}P-PEP used in the reactions (Hosios et al., 2015), a later study using similar phosphoproteomic analyses of the proteome of renal cell carcinoma cells demonstrated that recombinant PKM2 in the presence of PEP phosphorylated 974 protein substrates (He et al., 2016). In addition, multiple research groups have demonstrated and validated PKM2's protein kinase activity using both yeast and mammalian cells (Keller et al., 2014; Li et al., 2015). Succinyl-5-aminoimidazole-4-carboxamide-1-ribose-5′-phosphate (SAICA) binds to PKM2 and enhances PKM2-mediated histone H3 phosphorylation at Thr11, as well as phosphorylation of ERK1/2 and more than 100 other proteins (Keller et al., 2014). Another report demonstrated that the yeast PKM2 homolog PYK1 directly phosphorylates histone H3 at Thr11 and regulates the cross-talk between H3 Thr11 phosphorylation and H3 Lys4 methylation in autoregulation of *PYK1* expression (Li et al., 2015). Transgenic mouse studies demonstrated that PKM2 is not required for breast cancer formation promoted by BRCA1 tumor suppressor deficiency but

is essential for BCR-ABL – or MLL-AF9–induced leukemia development (Israelsen et al., 2013; Wang et al., 2014b), suggesting that the roles of PKM2 in tumor suppressor- and oncogene-induced tumorigenesis can differ.

Besides regulating gene expression, PKM2's protein kinase activity is instrumental for many critical cellular activities. PKM2 binds to the spindle checkpoint protein Bub3 during mitosis and phosphorylates Bub3 at Tyr207 (Jiang et al., 2014a). This phosphorylation leads to recruitment of the Bub3-Bub1 complex to the outer kinetochore protein Blinkin and governs kinetochore-spindle attachment and the mitotic checkpoint, thereby promoting accurate chromosome segregation and proliferation of tumor cells (Jiang et al., 2014a). PKM2 is also involved in cytokinesis. When PKM2 Thr45 is phosphorylated by Aurora B, PKM2 interacts with myosin light chain 2 (MLC2) in the contractile ring regions of mitotic cells and phosphorylates it at Tyr118, and MLC2 Tyr118 phosphorylation is greatly enhanced by EGFR variant III, K-Ras G12V, and B-Raf V600E oncogenic mutant expression (Jiang et al., 2014a). This phosphorylation primes MLC2 phosphorylation at Ser15 mediated by Rho-associated protein kinase 2 (ROCK2) and is required for contraction of the actomyosin complex at the cleavage furrow, completion of cytokinesis, and tumor cell proliferation (Jiang et al., 2014b). In addition, PKM2, whose expression is transcriptionally enhanced by EGFR-dependent activation of nuclear factor (NF)-κB, promotes hormonal and nutrient signal-independent activation of mammalian target of rapamycin complex 1 (mTORC1) via phosphorylation of mTORC1 inhibitor AKT1 substrate 1 (AKT1S1) at Ser202/203 and its release from Raptor (He et al., 2016; Yang et al., 2012a). In renal cell carcinomas and breast cancers, PKM2 overexpression has been correlated with elevated AKT1S1 phosphorylation at Ser202/203, activated mTORC1, and reduced autophagy (He et al., 2016).

The protein kinase activity of PKM2 is also involved in cell apoptosis. In response to oxidative stress, PKM2 translocates to the outer membrane of mitochondria, where heat shock protein (HSP)90α1-mediates a conformational change in PKM2, which then interacts with and phosphorylates Bcl-2 at Thr69. This phosphorylation prevents the binding of a Cul3-based BCR (BTB-CUL3-RBX1) E3 ligase to Bcl-2, thereby stabilizing Bcl-2 and enhancing the resistance of tumor cells to oxidative stress (Liang et al., 2016a). PKM2 also participates in the remodeling of tumor microenvironments by regulating tumor cell exosome secretion. PKM2 acts as a protein kinase to phosphorylate synaptosome-associated protein 23 (SNAP-23) at Ser95, which in turn enables the formation of the SNARE complex to facilitate exosome release (Wei et al., 2017).

In summary, in addition to its originally characterized metabolic function in the glycolytic pathway, it has now been established that PKM2 also acts as a dual-specificity protein kinase that can phosphorylate protein substrates at both serine/threonine and tyrosine residues. PKM2 possesses nonmetabolic functions, acting as a protein kinase and phosphorylating a variety of protein substrates to regulate the Warburg effect, gene expression, mitosis and cytokinesis progression, cell proliferation, apoptosis, and exosome secretion (Fig. 1).

PGK1

Pyruvate kinase and PGK1 are the only two ATP-generating enzymes in the glycolysis pathway. PGK1 is the first ATP-generating enzyme in this pathway and is highly expressed in many types of cancer (Li et al., 2016d). It catalyzes the reversible conversion of 1,3-diphosphoglycerate and ADP to 3-phosphoglycerate and ATP, respectively (Li et al., 2016d). Nuclear PKM2 enhances the Warburg effect by upregulating the expression of glycolytic enzymes, including PKM2 itself, to increase glucose uptake and lactate production (Yang et al., 2011), but how this enhanced aerobic glycolysis coordinates with suppression of mitochondrial pyruvate metabolism is a central question in understanding the Warburg effect. Our recent work demonstrated that EGFR activation, oncogenic K-Ras G12V and B-Raf V600E mutant expression, and hypoxic stress all result in ERK activation-dependent mitochondrial translocation of a small portion of cytosolic PGK1 (Li et al., 2016a). Activated ERK1/2 phosphorylates PGK1 at Ser203. This phosphorylation recruits the PIN1 prolyl isomerase, leading to isomerization of the Ser203.Pro204 bond and subsequent exposure of the presequence of PGK1 (38-QRIKAA-43) on its surface. The exposed PGK1 presequence is then recognized by the mitochondrial translocase of the outer membrane (TOM) complex, leading to translocation of PGK1 into the mitochondria. In the mitochondria, PGK1, acting as a protein kinase, interacts with and directly phosphorylates pyruvate dehydrogenase kinase isozyme 1 (PDHK1) at Thr338 using ATP as a phosphate donor. This phosphorylation activates PDHK1 and enhances PDHK1-mediated pyruvate dehydrogenase E1α phosphorylation at Ser293, which inactivates the pyruvate dehydrogenase complex preventing conversion of pyruvate and coenzyme A (CoA) to acetyl-CoA and CO_2 in the mitochondria. This PGK1-mediated phosphorylation event suppresses mitochondrial oxidative phosphorylation and thereby increases extracellular acidification and lactate production by shunting pyruvate from the mitochondria into the cytosol, thereby promoting tumorigenesis (Li et al., 2016a). Thus, tumor cells promote the Warburg effect via nuclear PKM2-dependent upregulation of glycolytic gene expression and through mitochondrial PKG1-mediated PDHK1 activation and subsequent inhibition of mitochondrial pyruvate metabolism. Thus, the protein kinase activities of both PKM2 and PGK1 play critical roles in this regulation.

Rapid tumor growth results in outgrowth of the existing vasculature and ischemia, and the tumor inevitably encounters metabolic stress, which induces autophagy to maintain cellular homeostasis (Lin et al., 2012). During the initiation of autophagy, autophagosome nucleation requires functional Beclin1, which recruits the class III phosphatidylinositol (PI) 3-kinase VPS34 into a complex with VPS15 and ATG14L to generate PI 3-phosphate (PI(3)P) (Funderburk et al., 2010). PI(3)P recruits proteins with PI(3)P–binding domains to modulate intracellular trafficking and autophagosome formation (Kim et al., 2013). Recent studies demonstrated that PGK1 plays a critical role in initiation of autophagy. In response to glutamine deprivation and hypoxia, mTOR-mediated phosphorylation of acetyltransferase ARD1 at Ser228 is inhibited, leading to an association of ARD1 with PGK1 and subsequent PGK1 acetylation at Lys388. Acetylated PGK1 interacts with Beclin1 and phosphorylates it at Ser30. This phosphorylation alters VPS34 conformation and dramatically enhances its ability to bind to PI, thereby significantly increasing VPS34 activity and PI(3)P production.

As a consequence, PGK1-mediated Beclin1 phosphorylation at Ser30 is required for initiation of autophagy, which is instrumental for tumor development. In addition, Beclin1 phosphorylation at Ser30 positively correlates with poor prognosis for glioblastoma (Qian et al., 2017).

These studies revealed that PGK1 can function as a protein kinase to regulate mitochondrial function and cellular stress-induced autophagy initiation and that integrated regulation of glycolysis, mitochondrial metabolism, and autophagy by PGK1 is instrumental to promotion of tumor cell proliferation and maintenance of cell homeostasis (Fig. 1).

KHK-A

Like glycolysis, fructose metabolism is a critical component of cell metabolism, with aberrant fructose metabolism leading to development of liver diseases (Ishimoto et al., 2012). In the fructose metabolic pathway, KHK, also known as fructokinase, the first rate-limiting metabolic enzyme, catalyzes the conversion of fructose and ATP to fructose 1-phosphate (F1P) and ADP, respectively. F1P is metabolized into dihydroxyacetone phosphate and glyceraldehyde by aldolase and subsequently converges on the glycolysis pathway (Li et al., 2016b). Mutually exclusive splicing of adjacent exons 3A and 3C in the KHK precursor RNA results in expression of KHK isoform A (KHK-A) or isoform C (KHK-C). KHK-A and KHK-C have low and high fructose phosphorylation activity, respectively, because only KHK-C has high binding affinity for fructose, whereas KHK-A has low fructose-binding affinity and a high Km for phosphorylation of fructose (~7 mM) (Asipu et al., 2003; Bonthron et al., 1994).

KHK-C is predominantly highly expressed in the liver, kidneys, and pancreas. In contrast, KHK-A is ubiquitously expressed at low levels (Ishimoto et al., 2012). In hepatocellular carcinoma (HCC) cells, KHK-C expression switches to KHK-A expression as a result of c-Myc–induced high level expression of hnRNPH1 and hnRNPH2, which bind to a motif located in the intron close to the 3′ end of exon 3C resulting in alternative splicing of the *KHK* pre-mRNA and expression of KHK-A. KHK-A expression results in much lower fructose catabolism rates, ATP consumption, and reactive oxygen species production in HCC cells than in normal hepatocytes (Li et al., 2016c). Importantly, instead of binding fructose, KHK-A interacts with the rate-limiting enzyme phosphoribosyl pyrophosphate synthetase 1 (PRPS1) in the *de novo* nucleic acid synthesis pathway. This allows KHK-A to function as a protein kinase and directly phosphorylate PRPS1 at Thr225 (Km, ~0.2 μM), which lies in the binding region for PRPS1's allosteric inhibitor ADP. This phosphorylation abrogates the feedback inhibition of PRPS1 by blocking the binding of ADP to PRPS1, leading to elevated *de novo* nucleic acid synthesis via constitutive activation of PRPS1 in HCC cells, and thereby promotes HCC proliferation and growth in the livers of mice. Immunohistochemical staining of human HCC specimens demonstrated that KHK-A and PRPS1 pThr225 were positively correlated with each other and that the levels of KHK-A and PRPS1 pThr225 staining were inversely correlated with survival duration, supporting a pivotal role for KHK-A–dependent PRPS1 phosphorylation in HCC progression (Li et al., 2016b; Li et al., 2016c).

Thus, in addition to the identified protein kinase activity of PKM2 and PGK1 in the glycolytic pathway, KHK-A in the fructose metabolic pathway acts as a protein kinase that plays an instrumental role in *de novo* nucleic acid synthesis and promotion of HCC development (Fig. 1).

Hexokinase

Hexokinase (HK) catalyzes the phosphorylation of glucose to produce glucose 6-phosphate, using ATP as a phosphate donor. Four isoforms of hexokinase (HK 1–4) exist in mammalian tissues [1]. Incubation of HK-PII, an isoenzyme from *Saccharomyces cerevisiae*, with γ-^{32}P ATP shows that HK-PII can autophosphorylate itself (Fernandez et al., 1988). Phosphoamino acid analyses reveal that HK1 purified from rat brain can autophosphorylate at serine, threonine, and tyrosine residues (Adams et al., 1994). In addition, *in vitro* phosphorylation assays show that HK1 can phosphorylate purified histone H2A, and that this phosphorylation and the autophosphorylation activity of HK1 is inhibited in the presence of its normal substrate glucose (Adams et al., 1991). Although HK1 exhibits protein kinase activity *in vitro*, whether HK1 as well as other HK isoforms can act as protein kinases *in vivo* and the physiological role of such phosphorylation activity in the regulation of HK and cellular activities remain to be determined.

Histidine Kinases

In prokaryotes and lower eukaryotes (yeast, fungi, and plants), protein-histidine kinase activity of metabolic enzymes was identified in two-component and multicomponent signaling systems and this activity plays an important role in sugar phosphorylation (Hess et al., 1988; Kennelly and Potts, 1996; Swanson et al., 1994). In the PEP/sugar phosphotransferase system, enzyme I, which converts phosphoenolpyruvate to pyruvate, is autophosphorylated at His189, with PEP being used as the phosphate donor. The phosphate group in enzyme I is then transferred sequentially to a histidine in the HPr enzyme and then to a histidine in enzyme IIA before being transferred to a cysteine residue in enzyme IIB, which, in complex with enzyme IIC, phosphorylates intracellular glucose (Attwood and Wieland, 2015). Thus, the histidine kinase activity of enzyme I plays a critical role in glucose metabolism.

PEP can be used as a phosphate donor to phosphorylate proteins by enzyme I in prokaryotes and lower eukaryotes and by PKM2 in eukaryotes. The low sequence homology between enzyme I and PKM2 and their different structures suggest that they will have different substrates for phosphorylation. Moreover, enzyme I is a histidine kinase that autophosphorylates itself and then transfers this phosphate onto its protein substrates, whereas PKM2 does not use a phosphoenzyme intermediate, and transfers phosphate from PEP onto both ADP and protein substrates in a concerted reaction. In addition, although PKM2 can phosphorylate several different proteins, these phosphorylations are regulated by posttranslational modification, oligomerization state or alteration of PKM2's subcellular compartmental localization (Jiang et al., 2014a; Yang et al., 2012b; Yang et al., 2012c).

In mammals, nucleoside diphosphate kinase (NDPK, or NDK) is a ubiquitous enzyme that catalyzes the conversion of nucleoside diphosphates (NDPs) into nucleoside triphosphates (NTPs) by transferring terminal γ-phosphate groups from 5′-triphosphate-nucleotides to 5′-diphosphate-nucleotides. NDPKs are encoded by the NME gene family, which is comprised of 10 family members (Boissan et al., 2009). Besides their NDPK activity, NDPK-A (also known as NME1) and NDPK-B (also known as NME2) function as protein-histidine kinases (Attwood and Wieland, 2015). Similar to the phosphoenolpyruvate/sugar phosphotransferase system in lower organisms, the NDPK catalytic mechanism involves NTP (usually ATP)-dependent autophosphorylation of a highly conserved histidine residue in its active site and this phosphate group is then transferred from the phosphohistidine either to an NDP molecule or to the histidine in substrate proteins (Attwood and Wieland, 2015).

Several proteins have been identified as substrates of NDPK-A and NDPK-B protein kinase activity. Specifically, NDPK-A phosphorylates the histidine at the catalytic site of ATP citrate lyase (ACLY), a cytosolic enzyme that converts citrate to acetyl-CoA and is critical for fatty acid synthesis (Attwood and Wieland, 2015). Autophosphorylated NDPK-A transfers the phosphate group from NDPK-A to ACLY. Citrate then binds to ACLY, and the phosphate is transferred to citrate to generate citryl-phosphate, an intermediate product of the reaction that forms acetyl-CoA (Wells, 1991). Like NDPK-A, NDPK-B can also act as a histidine kinase, forming a complex with G protein βγ dimers and phosphorylating His266 in the Gβ subunit. The phosphate on His266 is highly energetic and can be transferred onto guanosine diphosphate (GDP), leading to formation of guanosine triphosphate (GTP) and contributing to G-protein activation (Cuello et al., 2003). NDPK-B also binds directly to and activates the Ca^{2+}-activated K^+ channel KCa3.1 by phosphorylating H358 in the cytoplasmic C-terminal tail of KCa3.1, thus relieving copper-dependent inhibition of KCa3.1 channel function, and promoting subsequent activation of $CD4^+$ T cells (Srivastava et al., 2006; Srivastava et al., 2016). In addition, NDPK-B phosphorylates His711 in the C-terminal tail of transient receptor potential-vanilloid-5 (TRPV5), which regulates urinary Ca^{2+} excretion by mediating active Ca^{2+} reabsorption in the distal convoluted tubule of the kidney. His711 phosphorylation activates TRPV5 channel activity (Cai et al., 2014).

Histidine phosphorylation, like serine, threonine, and tyrosine phosphorylation, is reversible. Using monoclonal antibodies that specifically recognize phosphorylated histidine in proteins (Fuhs et al., 2015), phosphoglycerate mutase family 5 (PGAM5) was identified as a phosphohistidine phosphatase that specifically associates with and dephosphorylates the catalytic pHis118 on NDPK-B and negatively regulates $CD4^+$ T cell activity by inhibiting NDPK-B–mediated histidine phosphorylation and activation of the K^+ channel KCa3.1, which is required for T-cell receptor (TCR)-stimulated Ca^{2+} influx and cytokine production (Panda et al., 2016). On the other hand, NDPK-phosphorylated ACLY, Gβ, and KCa3.1 are substrates for a phosphohistidine-specific phosphatase, PHPT1, which dephosphorylates the phosphorylated active site histidine and reverses the functional consequences of NDPK-dependent phosphorylation (Klumpp et al., 2003; Maurer et al., 2005; Srivastava et al., 2008). In addition, phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP), a conserved phosphatase that hydrolyzes P-N bonds in synthetic substrates *in vitro*, is potentially a third phosphohistidine protein phosphatase, but LHPP is otherwise poorly characterized.

Thus, in addition to regulating nucleoside triphosphate production, NDPK-A and NDPK-B function as histidine kinases and regulate a spectrum of cellular activities in a cooperative manner with phosphohistidine phosphatases.

How can one establish unequivocally that a metabolic kinase acts as a protein kinase?

The recent finding that many metabolic kinases can also act as protein kinases is unexpected and raises a number of mechanistic questions. The most obvious is how the active site of an enzyme designed to recognize ATP/ADP and a second small metabolite molecule for phosphate transfer is also able to recognize a specific Ser, Thr or Tyr residue in a selected protein substrate and phosphorylates it. This in turn raises the issue of whether the protein substrate occupies the same binding site as the small molecule substrate that is normally phosphorylated. One way to answer this is to show that the small molecule substrate can compete with the protein substrate for phosphorylation *in vitro*, which has only been demonstrated in a few cases. The ultimate way to establish how a protein substrate binds will be to solve a structure of the protein bound to the metabolic kinase as an enzyme intermediate complex. So far this has not been achieved for any metabolic kinase and its proposed protein substrate. Detailed structural analyses will also elucidate how posttranslational modifications of metabolic enzymes, such as Aurora B-mediated PKM2 Thr45 phosphorylation and ARD1-mediated PGK1 Lys388 acetylation (Jiang et al., 2014a; Qian et al., 2017), enable these metabolic enzymes to bind to and phosphorylate their protein substrates.

Another concern is that the observed protein kinase activity could be due to contamination with an authentic protein kinase, particularly if the metabolic kinase is isolated from a eukaryotic cell. To address this issue, recombinant forms of the metabolic kinase and its substrates should be used, and ideally a kinetic analysis of protein substrate phosphorylation should be carried out to demonstrate that the protein kinase activity has properties consistent with this activity being of physiological relevance in cells.

To establish unequivocally that the protein kinase activity of a metabolic kinase is an intrinsic and physiologically activity, we propose a number of criteria that should be met. Table 1 lists these criteria and indicates which of them have been satisfied for the different metabolic kinases with reported protein kinase activities that we have reviewed.

Prospective

The original characterization of the human kinome identified more than 500 protein kinases (Manning et al., 2002). Characterization and demonstration of the protein kinase activities of metabolic enzymes expands the kinome. Identification of additional metabolic enzymes, which are able to transfer phosphate groups during their metabolic reactions, as protein kinases is a reasonable expectation. Demonstration of multiple roles of metabolic enzymes, such as PKM2, PGK1, KHK-A, HK1, and NDPK-A/B, in plural cellular functions provides new insights into integrated regulation of cell metabolism and many other important cellular activities, such as cell growth, proliferation, survival, autophagy, and apoptosis.

The existence of non-canonical functions for PKM2, PGK1, and KHK-A in tumor development make these enzymes promising targets for new therapeutic interventions for human cancer. Structural elucidation of the binding and phosphorylation of protein substrates of these metabolic enzymes will facilitate identification of specific interventions that can selectively inhibit their protein kinase activity rather than their metabolic enzymatic activity. Disruption of processes involved in their subcellular localization will inhibit their important subcellular compartment-specific protein kinase activity that is critical for tumor development. In addition, cancer type-specific expression of some of these metabolic enzymes, such as HCC-specific splicing of the *KHK* gene and expression of KHK-A (Li et al., 2016c), afford the possibility of cancer type-specific treatment by targeting the protein kinase activity of these metabolic enzymes. Advances in understanding the protein kinase activity of metabolic enzymes could lead to the development of new and specific therapeutic cancer interventions.

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Highlights

- In addition to their originally identified metabolic enzymatic activities, PKM2, PGK1, KHK-A, HK, and NDPK-A/B can act as protein kinase.
- The protein kinase activity of metabolic enzymes phosphorylates a variety of protein substrates and regulates critical cellular activities.
- Metabolic enzymes are often posttranslationally modified, which changes their subcellular localization and allows them to phosphorylate protein substrates.
- The protein kinase functions of metabolic enzymes make them promising therapeutic targets for cancer.

Outstanding Questions

In addition to PKM2, PGK1, KHK-A, HK1, and NDPK-A/B, are there any other metabolic enzymes that function as protein kinases?

How is the active site of a metabolic enzyme designed to recognize ATP/ADP and a second small metabolite molecule for phosphate transfer also able to recognize a specific Ser, Thr or Tyr residue in a selected protein substrate and phosphorylate it?

How can specific targeting of the protein kinase activity of key metabolic enzymes be achieved without affecting their metabolic kinase activity?

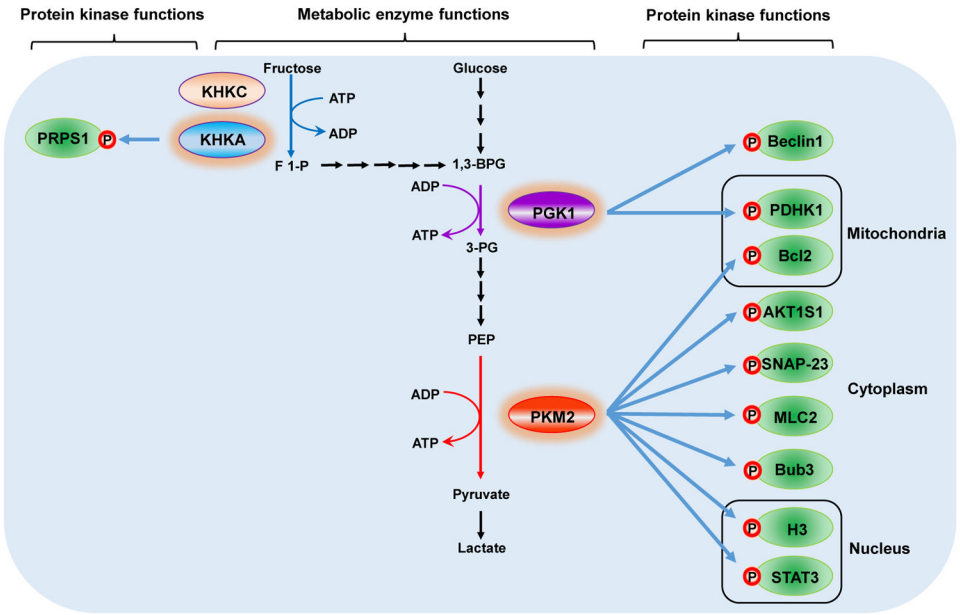

Figure 1.

PKM2, PGK1, and KHK-A possess both metabolic enzyme functions and protein kinase functions.

Table 1

Criteria that need to be satisfied to establish that a metabolic kinase can act as a protein kinase

	Criteria	Kinases					
		PKM2	PGK1	KHKA	HK2	NDPK-A	NDPK-B
1	Demonstrate that purified recombinant metabolic kinase from bacteria can phosphorylate itself or a purified recombinant substrate protein *in vitro*.	•	•	•	•	•	•
2	Demonstrate that a purified kinase dead-mutant of the metabolic kinase cannot phosphorylate the purified protein substrate *in vitro*.	•	•	•			
3	Demonstrate by mass spectrometry/mass spectrometry (LC-MS/MS) analyses that the metabolic kinase exhibits site-specific phosphorylating activity towards its protein substrate *in vitro*.	•	•	•			
4	Determine a co-crystal structure of the metabolic kinase bound to its protein substrate to define how a target amino acid in the substrate protein can be presented/accommodated in the active site for phosphate transfer.						