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Mass spectrometric analysis reveals O-methylation of pyruvate kinase from pancreatic cancer cells

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Abstract

Pyruvate kinase (PK) is an important glycolytic enzyme that catalyzes the dephosphorylation of phospho-enolpyruvate to pyruvate. Human PK isozyme M2 (PKM2), a splice variant of M1, is overexpressed in many cancer cells, and PKM2 has been investigated as a potential tumor marker for diagnostic assays and as a target for cancer therapy. To facilitate identification and characterization of PK, we studied the enzyme from pancreatic cancer cells and normal pancreatic duct cells by electrophoresis and mass spectrometry, and identified multiple O-methylated residues from PK. These findings advance our knowledge of the biochemical properties of PK and will be important in understanding its biological function in cells.

Keywords

PKM2; Mass spectrometry; Pancreatic cancer; Metabolism; Methylation

Introduction

Pyruvate kinase (PK) catalyzes the dephosphorylation of phosphoenolpyruvate to pyruvate, the last step in the glycolytic pathway, and is responsible for net energy production within that pathway. There are four isozymes of PK in mammals: L, R, M1, and M2. Type L is the major isozyme in liver and kidney; R is found in red cells; M1 is the main form in muscle and brain. Human PK isozyme M2 (PKM2), a splice variant of M1, is expressed in lung tissues and in all cells with high nucleic acid synthesis, including all proliferating cells, for example embryonic cells, adult stem cells, and, especially, tumor cells [1, 2]. Because elevated levels of PKM2 have been observed in numerous cancer cells, PKM2 has been investigated as a potential tumor marker for diagnostic assays [3, 4]. Notably, Christofk et al. reported that PKM2 is exclusively expressed in tumor tissues, and a change in the expression of PKM1 to PKM2 causes aerobic glycolysis (Warburg effect) during tumorigenesis [5]. Consequently, they suggested that selective targeting of PKM2 by small molecule inhibitors is feasible for cancer therapy [6].

Interestingly, several mechanisms have been proposed for regulation of PKM2 activity in tumor cells:

1. the ratio of the inactive dimeric form PKM2 to the active tetrameric form regulates the proportions of glucose carbon atoms that are channeled to synthetic processes or used for glycolytic energy production [1, 2];
2. PKM2 activity is regulated by phosphotyrosine signaling [7];
3. Anastasiou et al. demonstrated that PKM2 activity is also regulated by oxidative stress. PKM2 is specifically oxidized by hydrogen peroxide (H_2O_2) on cysteine 358 in cancer cells, which reduces PKM2 activity and pyruvate formation and increases flux of glycolytic metabolites into the pentose phosphate pathway [8];
4. Hitosugi et al. showed that PKM2 activity in cancer can be modified by phosphorylation [9];

5. David et al. reported that heterogeneous nuclear ribonucleoprotein (hnRNP) proteins controlled by c-Myc deregulate PK mRNA splicing in cancer, ensuring a high PKM2-to-PKM1 ratio [10, 11];
6. Keller et al. discovered that SAICAR (succinylamino-imidazolecarboxamide ribose-5'-phosphate, an intermediate of the de-novo purine nucleotide synthesis pathway) stimulates PKM2 and promotes cancer cell survival under glucose-limited conditions [12]; and
7. Chaneton et al. revealed that serine is a natural ligand and allosteric activator of PKM2 [13].

In addition to its better-characterized function as a glycolytic enzyme, Yang et al. demonstrated that nuclear PKM2 regulates β -catenin transactivation upon EGFR activation, and PKM2 phosphorylates histone H3 to promote gene transcription and tumorigenesis [14, 15].

Gene regulation and post-translational modifications (PTMs) are essential mechanisms of increased versatility and adaptability of an organism. Today, liquid chromatography-coupled tandem mass spectrometry (LC-MS-MS) is used routinely for large-scale protein identification and global profiling of PTMs from complex biological mixtures [16–19]. Here, we used LC-MS-MS to examine the PTMs of PK from pancreatic cancer cells and revealed O-methylation of PK, extending our knowledge of the biochemical characteristics of this important enzyme.

Experimental

CFPAC-1 cells (metastatic cell line derived from pancreatic cancer patients, ECACC ref. no: 91112501) were cultured at 37 °C in Dulbecco modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 20 mmolL⁻¹ glutamine, 10 % fetal calf serum (FCS), and 40 μ molL⁻¹ gentamycin under humidified 5 % CO₂. The cells were harvested and washed with Hank's balanced salt solution (Sigma-Aldrich, Saint Louis, MO, USA). The cell pellet was freeze-dried overnight and stored at -80 °C until use. Normal human pancreatic duct cells were obtained by primary culture of pancreatic duct from a single brain death donor under IRB approval (San Raffaele Scientific Institute, Milano, Italy). The duct cells were cultured in DMEM-F12 (1:1), supplemented with 2 mmolL⁻¹ glutamine, 10 % FCS, 100 U mL⁻¹ penicillin, and 100 μ molL⁻¹ streptomycin. Through a period of suspension culture, epithelial cells were enriched while stromal components were reduced to less than 1 %, confirmed by FACS analysis with markers for epithelial (ESA, Ca19.9) and fibroblast (CD73, CD105, CD90) phenotype. The CFPAC-1 and normal duct cells were resuspended for 1 h in lysis buffer consisting of Tris-HCl (50 mmolL⁻¹, pH7.4), NaCl (150 mmolL⁻¹), Triton X-100 (0.5 % w/v), NP-40 (0.5 % w/v), 80 mmol L⁻¹ dithiothreitol (DTT), 10 μ L mL⁻¹ protease inhibitor cocktails (Sigma-Aldrich), 1 mmolL⁻¹ PMSF, 1 mmolL⁻¹ Na₃VO₄, and PhosStop phosphatase inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA), sonicated for 30 s, and centrifuged at 16,000 $\times g$ for 10 min. The concentration of protein in the supernatant was measured by use of the Bradford Assay (BioRad, Hercules, CA, USA). Four volumes of acetone (Sigma-Aldrich) were then added

to the supernatants which were left overnight at -20°C for precipitation to occur. After centrifugation at $9,000\times g$ for 5 min the pellets were dried by lyophilization (Heto Drywinner, Birkerød, Denmark) for 2 h.

The cell pellets were resuspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. BenchMark pre-stained protein ladder (Invitrogen) and 50 μg proteins from each sample were dispensed into the wells of the Novex 4–20 % Tris-Glycine Gel (Invitrogen) to separate the proteins by SDS-PAGE. Proteins in the bands were in-gel digested and the extracted tryptic peptides were analyzed by high-sensitivity nanospray LC–MS–MS with an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The reversed-phase LC column was a 100 μm i.d. $\times$ 10 cm long piece of fused silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA), with a laser-pulled tip, slurry-packed in-house with 5 μm , 200 Å pore size, C_{18} resin (Michrom BioResources, CA, USA). The mobile phase was a gradient prepared from 0.1 % aqueous formic acid (mobile phase component A) and 0.1 % aqueous formic acid–acetonitrile 1:4 (v/v) (mobile phase component B). After sample injection, the column was washed for 5 min with A; the peptides were then eluted by use of a linear gradient from 0 to 50 % B in 120 min and then to 100 % B in an additional 5 min; the flow rate was 200 nLmin^{-1} . The LTQ-Orbitrap was operated in a data-dependent mode in which each full MS scan (60,000 resolving power) was followed by eight MS–MS scans in which the eight most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) by use of a normalized collision energy of 35 %. “FT master scan preview mode”, “Charge state screening”, “Monoisotopic precursor selection”, and “Charge state rejection” were enabled so that only the 1+, 2+, and 3+ ions were selected and fragmented by CID.

Tandem mass spectra collected by Xcalibur (version 2.0.2) were searched against the NCBI human protein database by use of SEQUEST (Bioworks software from ThermoFisher, version 3.3.1) with full tryptic cleavage constraints, static cysteine alkylation by iodoacetamide, and variable methionine oxidation. Mass tolerance for precursor ions was 5 ppm and mass tolerance for fragment ions was 0.25 Da. The SEQUEST search results were filtered by the criteria “Xcorr versus charge 1.9, 2.2, 3.0 for 1+, 2+, 3+ ions; $\text{Cn}>0.1$; probability of randomized identification of peptide <0.01 ”. For identification of PTMs, variable modifications, for example phosphorylation of Ser, Thr, and Tyr, acetylation of Lys, methylation of Asp and Glu were allowed. Confident peptide identifications were achieved by use of these stringent filter criteria for database match scoring followed by manual evaluation of the results.

Results and discussion

The gel was stained with Coomassie Brilliant Blue R-250, enabling visualization of the separated proteins (Fig. 1). We cut from the gel bands of molecular weight ~ 58 kDa, corresponding to monomer PKM1 (58.0 kDa) and PKM2 (57.9 kDa), and the proteins in the bands were in-gel digested for MS analysis. The SEQUEST search results yielded 136 matched MS2 spectra corresponding to PKM1 (69.2 % protein coverage by amino acid) and 152 matched MS2 spectra corresponding to PKM2 (72.5 % protein coverage by amino acid)

from the CFPAC-1 sample. On the basis of semi-quantitative MS2 spectra count, PKM1 and PKM2 were the most abundant proteins in the gel band. The probability of false discovery of the peptide was estimated as <1 % by searching a combined forward-reversed database, as described by Elias [20]. MS analysis of the gel band from normal duct cells yielded results similar to those from CFPAC-1.

PKM1 and PKM2 are different splicing products of the PK gene (exon 9 for PKM1 and exon 10 for PKM2) and differ only in 45 of 531 amino acids (Electronic Supplementary Material Fig. S1). Notably, two unique tryptic peptides of PKM1, LFEELVR and CLAAALIVLTESGR, and three unique peptides of PKM2, EAEEAIYHLQLFEELR, LAPITSDPTEATAVGAVEASFK, and CCSGAIIVLTK, were identified in both normal duct cells and CFPAC-1 cells, indicating that PKM1 and PKM2 were expressed in both normal duct cells and CFPAC-1 cells (Fig. 2 and Electronic Supplementary Material Fig. S2).

We then searched the MS raw data to identify potential PTMs of PK by changing the SEQUEST search conditions with variable phosphorylation of Ser, Thr, and Tyr, variable acetylation of protein amino terminus and the side chain of lysine, or variable methylation of amino acids Lys, Arg, His, Cys, Asp, and Glu. As a result, 18 unique peptides with O-methylation of the aspartic acid or glutamic acid side chain of PK were identified in both CFPAC-1 and normal duct cells, whereas phosphorylation, acetylation, hydroxylation, S-nitrosylation, N-methylation, or S-methylation of PK were not detected in this study. Among the 18 O-methylated peptides, 14 were present in both PKM1 and PKM2 whereas four were found in PKM2 only (Table 1). Notably, qualitative analysis identified all of the 18 O-methylated peptides from CFPAC-1 cells but only eight O-methylated peptides from normal duct cells. As shown in Fig. 3 and Fig. S3 (Electronic Supplementary Material) of the CID spectra of methylated peptides, most fragmented b-ions and y-ions from the peptides are matched, supporting correct identification. It is noteworthy that the unmodified counterparts of these post-translational-modified peptides were also identified from each gel band, indicating that the PK enzyme was a heterogeneous mixture in each band. It is likely that dozens of different forms of PK, each with different methylation, may occur in the sliced gel. The O-methylated residues of PK identified by LC-MS-MS in this study are presented schematically in Fig. 4.

In our previous proteomic analysis of pancreatic cancer cells by mass spectrometry, we found that PKM2 was an abundant protein in both normal pancreatic duct cells and cancer cells, and that PKM2 was up-regulated in the cancer cells [21, 22]. The MS analysis in this study once again confirmed that PKM2 is expressed in normal pancreatic duct cells, which does not agree with some researchers' observations that PKM2 is replaced by tissue-specific isoforms during tissue differentiation in development and that PKM1 is switched to PKM2 during tumorigenesis [3, 5]. Recently, by use of quantitative MS, Bluemlein et al. demonstrated that PKM2 is the prominent isoform in several analyzed cancer samples and matched control tissues, which also challenges the conclusion that PKM2 is exclusively expressed in cancer cells and there is a switch of PKM1 to PKM2 during development of cancer [23].

By high resolution mass spectrometry with the LTQ-Orbitrap we efficiently profiled the PTMs of PK from CFPAC-1 and normal pancreatic duct cells, and revealed O-methylation of PK. O-Methylation of PK has, to the best of our knowledge, not been reported previously. In addition, the initial results from qualitative analysis showed methylation of PK was different in CFPAC-1 and normal duct cells. Because equal amounts of cell lysates from CFPAC-1 and normal duct cells were loaded for SDS-PAGE, and PK enzyme was up-regulated in CFPAC-1 cells, it is possible that the difference found was because of different amounts of PK in the samples analyzed. The preliminary findings from this comparison, as usual, require further investigations to determine their relevant effects in vivo, and validation of the observed modifications by future immunohistochemical analysis of patient tissue samples.

It is known that PTM of proteins via methylation are important in protein-protein interaction [24]. At physiological pH, the side chains of Asp and Glu are negatively charged. Consequently, methylation of the carboxyl side chain will remove a negative charge and add hydrophobicity. To date, protein O-methylation has been characterized in several cellular processes:

1. O-methylation of the cytoplasmic domain of the trans-membrane receptor is involved in bacterial chemotaxis [25];
2. in eukaryotic cells, the C-terminal COO^- group of prenylated small GTPase is methylated via a prenylated protein methyltransferase, presumably reducing the energy for insertion of the prenylated C-terminus into the membrane compartments of the cell [26];
3. Clarke et al. reported that carboxylate O-methylation occurs when a protein repair methyltransferase acts to repair isoaspartate linkages that arise during the aging process [27].

It remains to be determined which protein-methyltransferase is responsible for the observed modification of PK enzyme and how the methylation can affect its catalytic activity, location in the cell, protein stability, and the ability to form tetramers or complex with other molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LC–MS–MS Liquid chromatography–coupled tandem mass spectrometry

PK Pyruvate kinase

PKM2 Pyruvate kinase isozyme M2

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Fig. 1. SDS-PAGE of cell lysates from CFPAC-1 and normal duct cells. A total of 50 μ g proteins from each sample was dispensed into the wells of the Novex 4–20 % Tris-Glycine Gel to separate the proteins by SDS-PAGE. Protein bands corresponding to PK were excised from the gel and in-gel digested with trypsin for MS analysis

Fig. 2.

Identification of unique peptides of PKM1 and PKM2 by LC-MS-MS. (a) example CID spectrum of the identified peptide LFEELVR (2+ ion m/z 453.2580) of PKM1 in normal duct cells; (b) CID spectrum of the identified peptide EAEAAIYHLQLFEELR (2+ ion m/z 966.5012) of PKM2 in normal duct cells. These two peptides were also identified in CFPAC-1 cells. The spectrum (*left panel*) is labeled to show b ions, y ions, and neutral loss of water from parent ions. The *right panel* is the table of the fragment assignments of the peptide, in which the matched b ions are colored with *red* and y ions with *blue*

Fig. 3.

Identification of O-methylated PK by LC-MS-MS. **(a)** example CID spectrum of the identified peptide CD#ENILWLDYK (2+ ion m/z 741.8510) in normal duct cells. The O-methylated peptide was also identified in CFPAC-1 cells. **(b)** CID spectrum of the identified peptide EAEAAIYHLQLFEE#LR (2+ ion m/z 973.5053) from PKM2 in CFPAC-1 cells. The spectrum (*left panel*) is labeled to show b ions and y ions. The *right panel* is the table of the fragment assignments of the peptide, in which the matched b ions are colored with *red*, y ions with *blue*, and ions containing modified residues with *cyan*

Fig. 4.

Schematic diagram of post-translational modification sites of PK. The primary sequences of PKM1 and PKM2 from normal duct cells are presented with O-methylated Asp/Glu highlighted in *yellow* and CFPAC-1 cells in *red*. The unique amino acids of PKM1 and PKM2 are *underlined*. (a) O-methylation at Asp-153, Glu-131, 223, 234, 300, 373, and 480 were identified from PKM1 in both CFPAC-1 and normal duct cells. O-Methylation of Asp-101, 357, 476, Glu-81, 284, 304, and 332 were identified from PKM1 in CFPAC-1 cells but not in normal duct cells; (b) O-Methylation at Asp-153, Glu-131, 223, 234, 300, 373, 410, and 480 were identified from PKM2 in both CFPAC-1 and normal duct cells. O-methylation of Asp-101, 357, 476, Glu-81, 284, 304, 332, 386, 397, and 418 were identified from PKM2 in CFPAC-1 cells but not in normal duct cells

Table 1

O-Methylated peptides identified by use of the LTQ-Orbitrap

Protein and peptides	Position ^a	Normal duct (ratio) ^b	CFPAC-1 (ratio) ^b
PKM1 and PKM2			
LNFSHGTHE#YHAETIK	73–89	(0/3)	(1/4)
TATESFASD#PILYR	93–106	(0/3)	(1/5)
GSGTAE#VELK	126–135	(1/4)	(2/5)
CD#ENILWLDYK	152–162	(2/4)	(2/6)
GVNLPGAADLPAVSE#K	208–224	(1/5)	(2/6)
FGVE#QDVDMVFASFIR	231–246	(1/3)	(1/5)
RFDEILE#ASDGIMVAR	278–294	(0/3)	(1/4)
GDLGIE#IPA EK	295–305	(1/5)	(2/7)
GDLGIEIPAE#K	295–305	(0/5)	(1/7)
PVICATQMLE#SMIK	322–336	(0/3)	(1/4)
AEGSDVANAVLDGAD#CIMLSGETAK	343–367	(0/4)	(2/6)
GDYPLE#AVR	368–376	(1/5)	(3/8)
D#PVQEAWAEDVDLR	476–489	(0/6)	(2/9)
DPVQE#AWAEDVDLR	476–489	(1/6)	(1/9)
PKM2			
EAE#AAIYHLQLFEELR	384–399	(0/3)	(1/5)
EAEAAIYHLQLFEE#LR	384–399	(0/3)	(1/5)
LAPITSDPTE#ATAVGAVEASFK	401–422	(1/5)	(2/8)
LAPITSDPTEATAVGAVE#ASFK	401–422	(0/5)	(1/8)

^aPositions of the initial and final peptide amino acids in the protein sequence^bRatio of the spectra count from the methylated peptide to the spectra count from the corresponding non-methylated peptide