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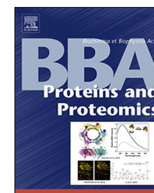

Kallikrein-related peptidase 6 can cleave human-muscle-type 6-phosphofructo-1-kinase into highly active shorter fragments

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ABSTRACT

Purpose: Cancer cells consume more glucose than normal human cells and convert most glucose into lactate. It has been proposed that deregulated glycolysis is triggered by the posttranslational modification of 85 kDa muscle-type 6-phosphofructo-1-kinase (PFK-M) which is cleaved by a specific protease to form shorter, highly active, feedback-inhibition-resistant PFK-M fragments.
Principal results: To find the protease involved in PFK-M modification, analyses of the protease target sites on the human PFK-M enzyme yielding 45–47 kDa fragments were performed in silico. The results suggested that an enzyme in the kallikrein (KLK) family may be involved. Kallikreins can be self-activated in the cytosol and are often overexpressed in cancer cells. After incubating the internally quenched FRET peptide with a sequence characteristic of the target site, along with the active KLK6, the cleavage of the peptide was observed. The ability of KLK6 to cleave native PFK-M and form highly active citrate-resistant 45 kDa fragments was further confirmed by enzymatic tests and SDS-PAGE. A role of KLK6 in the posttranslational modification of native PFK-M was ultimately confirmed in vivo. A yeast strain that encoded native human PFK-M as the only PFK1 enzyme was additionally transformed with pro*KLK6* or *KLK6* genes under the control of an inducible promoter. The transformants growth rate was found to increase after the induction of proKLK6 gene expression as compared to the strain with the native PFK-M enzyme.
Conclusion: KLK6 may be the key protease involved in the modification of PFK-M and trigger deregulated glycolytic flux in cancer cells.

1. Introduction

A reprogrammed energy metabolism is one of the hallmarks of cancer, and it is characterized by the deregulated control of cell division and proliferation [1]. Enhanced glycolysis is at the root of this cancer energy metabolism and appears to be triggered by oncogenic mutations in the PI3K/Akt/mTOR signaling pathway [2]. The activation of this pathway stimulates metabolic activities via two major events. Firstly, the synthesis of the sugar transporter Glut1 is induced, facilitating glucose uptake by the cells. Secondly, the activity of transcription complex HIF-1α is increased [3], which in cooperation with transcription factor c-Myc, enhances the synthesis of the majority of glycolytic enzymes [4]. Increased levels of the wild-type enzymes consequently result in increased specific activities. However, in mammalian organisms, glycolytic flux is tightly controlled by allosteric enzymes that retain their regulation via feedback inhibition in spite of the elevated activities of intermediary enzymes. Therefore, important changes in the kinetic characteristics of some glycolytic regulatory enzymes must be made to trigger deregulated glycolytic flux. Such changes occur during the transformation of normal cells into cancer cells. The three regulatory glycolytic enzymes are hexokinase, 6-phosphofructo-1-kinase, and pyruvate kinase. In the cancer cells, embryonic isoenzymes of hexokinase (HK2) and pyruvate kinase (PKM2) prevail. Although hexokinase (HK2) shows a high affinity for glucose, it is inhibited by glucose-6-phosphate [5]. On the other hand, the activity of pyruvate kinase isoform M2 is repressed unless elevated levels of fructose-1,6-bisphosphate [6] and serine [7] are present in cells.

One of the events that results in increased glycolysis in cancer cells seems to be the posttranslational modification of 6-phosphofructo-1-kinase (PFK1), the key regulatory enzyme of glycolysis [8]. The native PFK1 enzyme catalyzes the phosphorylation of fructose-6-phosphate (F6P) into fructose-1,6-phosphate (FBP) using Mg-ATP as a phosphoryl donor. This enzyme is stimulated by fructose-2,6-bisphosphate (F2,6BP), ADP/AMP, and ammonium ions, whereas citrate and ATP act as strong inhibitors [9]. Some glycolytic enzymes, including PFK1, have been reported to associate with the cytoskeleton, which may impact

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their activities by changing the quaternary structures of these enzymes. PFK1 can become attached to f-actin in a form of fully active tetrameric holoenzyme, while only dimers exhibiting low catalytic activity were associated with microtubules [10]. In human cells, three PFK1 genes are present, and these are expressed differently in individual tissues. The protein products are designated as muscle-type (PFK-M), liver-type (PFK-L) and platelettype (PFK-P). Individual isoforms have been observed to be differently expressed in cancers. In breast cancer cell lines of different aggressiveness levels, different ratios of PFK1 isoforms have been detected [11].

In several cancer cell lines, the C-terminus of the human 85 kDa native muscle-type PFK-M protein has been found to be post-translationally modified via the cleavage of a specific protease, forming a shorter fragment. The newly formed enzyme retains catalytic activity. However, it becomes resistant to feedback inhibition by citrate and ATP, whereas some effectors increase its activity to a level higher than that of the native enzyme. Only the short 45–47 kDa fragments, not the native 85 kDa PFK-M, have been detected in four tumorigenic cell lines [8].

Our previous experiments have suggested that a specific serine protease may be required for the modification of PFK-M [8]. The protease involved should be active in the cytosol, where the enzyme is located, and should be expressed at higher levels in cancer cells. Among serine proteases, high gene expression levels for human tissue kallikreins (KLKs) are associated with a variety of cancers [12–14]. The increased expression of these genes results in higher cellular concentrations of KLKs, which may provoke an auto-activating process. In many trypsin-like serine proteases, including KLKs, the auto-lytic activation of zymogen precursors via the proteolytic cleavage of the pre-pro region has been described [15]. Beside, in studies of the expression of several human tissue kallikreins (KLKs) using immunohistochemical reactions, cytoplasmic expression was always detected, suggesting the cytosolic activity of some KLKs [16].

Human tissue kallikreins (KLKs) belong to the PA clan and S01 subfamily of serine proteases, which are known to mediate important physiological functions in human cells [15]. In humans, there is a family of 15 tissue KLKs that are closely related (KLK1-KLK15). These 15 homologous proteases are encoded by the largest protease gene cluster. The majority of KLKs have an Asp residue in their binding pocket, suggesting that they have trypsin-like substrate specificity. Others (KLK3, 7, 9) show chymotrypsin-like activity, and some show a mixed-type activity (KLK11 and KLK14) [17]. KLKs are implicated in a wide range of normal physiological processes, from the regulation of cell growth to tissue remodeling [18].

In the present paper, we report on the ability of human KLK6 to post-translationally modify the human-muscle-type PFK-M enzyme and form highly active shorter PFK-M fragments.

2. Materials and methods

2.1. Enzymes, protease, and peptide

Recombinant native human PFK-M was synthetized in the n*PFKM* *Saccharomyces cerevisiae* strain [19], and the enzyme was partially purified according to the protocol published previously [8]. Human trypsin and mouse endoproteinase Arg-C were purchased from Sigma-Aldrich (Steinheim, Germany). Recombinant proKLK4 (PHC9344), in zymogen form, was purchased from Invitrogen (Carlsbad, CA, USA), and proKLK6 (MBS343204) was purchased from Mybiosource (San Diego, CA, USA). The internally quenched FRET peptide was tailor-made and purchased from Bachem (Bubendorf, Switzerland).

2.2. Purification of active KLK6

Active KLK4 and KLK6 were obtained after the incubation of recombinant proKLK4 or proKLK6 with enterokinase (Sigma-Aldrich, Steinheim, Germany), as suggested by Blaber et al. [20]. ProKLK4 or proKLK6 (1 μg) was incubated with enterokinase (0.01 μg) in 200 μL of 50 mM Tris-HCl buffer (pH = 8.0) for 60 min. After digestion, both enzymes were separated on a Superose 12™ size exclusion column (GE Healthcare, Piscataway, NJ). The collected fractions were tested for KLK6 activity using the internally quenched peptide Abz-AFRFSQ-EDDnp (Peptides International, Louisville, KY), which was recommended for testing KLK6 activity by the supplier (Mybiosource, San Diego, CA).

2.3. Proteolytic degradation of human PFK-M and measurements of enzyme activity

Purified native human PFK-M (100 μg/mL) was incubated with trypsin, endoproteinase Arg-C, and the active and inactive forms of KLK4 and KLK6 (1 μg/mL) in 200 μL of phosphate buffer with 0.15 M glycerol (pH 7.8) at 37 °C, unless stated differently. At the end of the incubation period, the proteolytic activities were blocked by adding 2 μL of cOmplete™ Protease inhibitor cocktail (Roche, Basel, Switzerland). The total reaction mixtures were then transferred into HEPES buffer with 0.15 M glycerol (pH 7.8) and 5 mM citrate, and the PFK-M activities were recorded as previously described [21]. As a control, purified native human PFK-M was incubated under identical conditions without the proteolytic enzyme, followed by enzyme activity measurement.

2.4. Proteolytic cleavage of the internally quenched FRET peptide (Abz-STVRI-EDDnp)

The proteolytic activities of proKLK6 and KLK6 were assessed by measuring the cleavage rate of a specific amino acid sequence as a target. The Abz-STRVI-EDDnp intrinsically quenched FRET peptide was synthesized and purchased from Bachem (Bubendorf, Switzerland). In 1 mL of 50 mM Tris-HCl buffer with a pH of 8.0, 200 μM of FRET peptide, and 1 μg of individual protease were mixed and incubated at 37 °C. Increases in fluorescence were continuously measured for 120 min using an LS 55 fluorescent spectrophotometer (Perkin Elmer, Boston, MA, US), with excitation at 320 nm and emission at 420 nm.

2.5. Detecting the proteolytic cleavage products of the native PFK-M via SDS-PAGE

Purified native human PFK-M (100 μg/mL) was incubated with activated KLK6 (0.1 μg/mL) in 200 μL of phosphate buffer (pH = 7.8) at 37 °C. After 15 and 120 min, the proteolytic activity was blocked via adding 20 μL of cOmplete™ Protease inhibitor cocktail (Roche, Basel, Switzerland). Aliquots of 20 μL were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gel with 0.1% SDS under reducing conditions and, finally, stained with silver nitrate. A pre-stained leader for proteins with specific molecular weights (Lane 1) PageRuler™ Plus (00070238) was purchased from Fermentas Life Sciences (Thermo Fisher Scientific, Waltham, MA, US).

2.6. Construction of nPFKM/proKLK6 and nPFKM/KLK6 transformants

Described in Supplementary material.

3. Results

3.1. Posttranslational modification of human native PFK-M with some trypsine-like serine proteases, including kallikreins

Initially, some commercially available serine proteases that belong to the subfamily of trypsin-like proteases were randomly tested for their ability to cleave the native 85 kDa human PFK-M into highly active,

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