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Enzymatic properties of the N- and C-terminal halves of human hexokinase II

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Although previous studies on hexokinase (HK) II indicate both the N- and C-terminal halves are catalytically active, we show in this study the N-terminal half is significantly more catalytic than the C-terminal half in addition to having a significantly higher K_m for ATP and Glu. Furthermore, truncated forms of intact HK II lacking its first N-terminal 18 amino acids (Δ18) and a truncated N-terminal half lacking its first 18 amino acids (Δ18N) have higher catalytic activity than other mutants tested. Similar results were obtained by PET-scan analysis using ^{18}F-FDG. Our results collectively suggest that each domain of HK II possesses enzyme activity, unlike HK I, with the N-terminal half showing higher enzyme activity than the C-terminal half. [BMB reports 2009; 42(6): 350-355]

INTRODUCTION

In humans, hexokinase (HK, EC 2.7.1.1) types I, II, III, and IV are four distinct enzymes with different properties and tissue distribution, and are responsible for glucose phosphorylation (1, 2). Besides having a high affinity for glucose, HKs I-III have a molecular mass of 100 kDa and are regulated by feedback-inhibition in response to the physiologic concentration of glucose 6-phosphate (G6P). In contrast, hexokinase IV (HK IV or glucokinase) is a 50 kDa protein with a lower affinity for glucose and is not inhibited by physiologic concentrations of G6P, similar to the 50 kDa yeast enzyme (3, 4). Interestingly, 100 kDa HK isozymes exhibit internal sequence repetition as evidenced by extensive sequence similarity between the N-terminal and the C-terminal halves of the enzymes, which are similar to sequences present in 50 kDa hexokinases (1, 2, 5). Therefore, a widely-accepted hypothesis supported by the high degree of amino acid similarity both between the N- and C-terminal halves of a given HK and between glucokinase and yeast HK (6, 7) is that the 100-kDa isoforms are derived from a 50-kDa precursor through gene duplication and tandem ligation (1).

Many studies confirm marked functional differences between the N- and C-terminal halves of HK I despite sharing similar amino acid sequences. In particular, the C-terminal half of HK I is catalytically active versus an inactive N-terminal half (8, 9). Although G6P binds to both halves of HK I, the G6P regulatory site of HK I is located in the N-terminal half of the intact enzyme whereas the C-terminal binding site is inactive (1, 8). Wilson suggested this latency of high affinity in the G6P binding site of the C-terminal half is due to steric interference caused by the corresponding N-terminal region (10). Gelb *et al*. (11) showed that the 15 amino acids belonging to the N-terminus of hexokinase are sufficient to target a reporter sequence to rat liver mitochondria. Moreover, the first 11 N-terminal amino acids are very hydrophobic and may influence the specific activity of HK I (12, 13). Recent studies show both the N- and C-terminal halves of human and rat HK II (N-HK II and C-HK II, respectively) possess catalytic activity and are inhibited by G6P (14, 15). However, the kinetic characteristics of the N- and C-terminal halves of HK II widely differ: N-HK II has a slightly higher affinity for ATP than does C-HK II, and its *Ki* for G6P is approximately 20-30-fold lower than that of C-HK II (3).

In past years, the high sensitivity and specificity of ^{18}F-FDG PET has promoted its wide use in the field of clinical oncology. Cancer cell growth is heavily dependent on glucose metabolism as a major energy source and therefore the ^{18}F-FDG uptake pattern in PET scans reflects cellular glycolytic activity. In this study, we investigated the enzymatic characteristics of the N- and C-terminal halves of HK II using a series of deletion mutants. We also addressed glucose uptake and metabolism using FDG-PET in transiently-transfected cells expressing mutant HK enzymes.

RESULTS

Expression of truncated human HK II

HKs I-III contain two regions, the N-terminal regulatory region (residues 1-475) and the C-terminal catalytic region (residues 476-917), which share high sequence homology. In HK II, the N-terminal region contains a hydrophobic, 12 amino acid

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chain. To investigate which region is responsible for the enzyme activity of HK II, we compared the full-length enzyme (HK II) with four deletion mutant constructs: a truncated form lacking the first N-terminal 18 amino acids (Δ18), the N-terminal half of hexokinase (N-term), a truncated form of N-term lacking the first 18 amino acids (Δ18N), and the C-terminal half of hexokinase (C-term) (Fig. 1A). Purification was designed

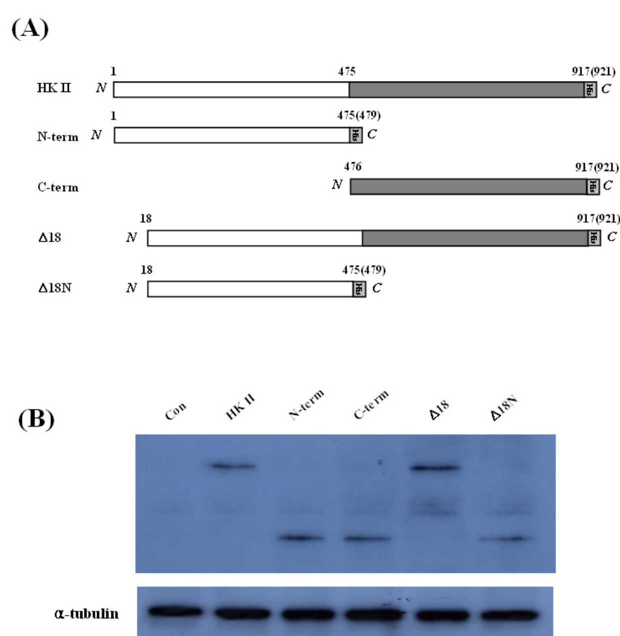

Fig. 1. Expression profile of HK II in SNU449 cell lines. (A) Schematic diagram of HK II and deletion mutant constructs. Full-length (HK II), a truncated form lacking the first N-terminal 18 amino acids (Δ18), the N-terminal half of hexokinase (N-term), a truncated form of N-term lacking the first 18 amino acids (Δ18N) and the C-terminal half of hexokinase (C-term). (B) Western blot analysis of overexpressed HKII and deletion mutants. SNU449 cells were transiently transfected with HKII, N-term, C-term, Δ18 and Δ18N constructs. Cell lysates were prepared 3 days after transfection. Densitometric data: His/α-tubulin ratio: HK II = 1.15 ± 0.04, N-term = 1.17 ± 0.03, C-term = 1.15 ± 0.07, Δ18 = 1.16 ± 0.02, Δ18N = 1.15 ± 0.06; $P < 0.01$, $n = 5$.

based on a 5X histidine motif added to the carboxy-terminus of each protein, which also reduced the degree of conformational change. The protein samples used in this study were found to be highly pure, as determined by SDS-PAGE and western blotting (Supplemental Material 1). We first investigated the enzymatic activity of each purified HK II deletion mutant and found expression of HK II, N-term, and C-term was associated with glucose phosphorylating activity (Table 1). Notable results found enzyme activity of the N-terminal half was higher than the C-terminal half and that Δ18 and Δ18N both have higher specific enzyme activity than their intact counterparts, HK II or N-term. Previous studies showed removal of the hydrophobic N-terminal 11 amino acids from HK I increases its enzyme activity (12). These observations support earlier speculation that the larger isoforms arose originally via duplication and tandem fusion of an ancestral ~50 kDa hexokinase precursor (16, 17). The kinetic properties of these deletion mutants are also shown in Table 1. The C-terminal half of HK II has a lower enzyme activity than the other deletion constructs. Regarding specific activity, N-term, C-term and Δ18N have comparable levels, although still lower than the intact enzyme and the Δ18 construct. In addition, C-term has a slightly lower affinity for glucose compared to the similar K_m values shared by HK II, N-term, Δ18 and Δ18N. C-term also had a significantly lower affinity for ATP than the other mutants. Based on this experiment, we concluded that contrary to HK I the N-terminal region of HK II has higher enzyme activity than in its C-terminal region. In addition, the hydrophobic region of the N-terminus influences enzyme activity.

Kinetic parameters of N-term, C-term, and Δ18N constructs

As noted previously, the N- and C-terminal halves of HK II display a greater degree of similarity than in HK I or III (60% compared with 51% and 47%, respectively), suggesting HK II is more closely related to the original 100-kDa hexokinase than either HK I or III. Therefore, we compared the N- and C-terminal sequences of from each HK and specifically compared each domain (Supplemental Material 2). Sequence similarities between the N- and C-terminal halves of HK II (55.37% identical; 20.21% strongly similar, and 6.11% weakly

Table 1. Kinetic constants of recombinant hexokinase II, N-terminal half, C-terminal half, Δ18 and Δ18N

Over-expressed recombinant protein	Specific activity	*Km* (glucose)	*Km* (ATP)	*Ki* (G6P)
	Units/mg*		mM	
HK II	7.39 ± 0.14	0.37 ± 0.12	0.81 ± 0.11	0.24 ± 0.06
N-term	4.77 ± 0.07	0.4 ± 0.06	0.67 ± 0.07	0.13 ± 0.04
C-term	1.13 ± 0.17	0.64 ± 0.07	3.61 ± 0.1	2.51 ± 0.2
Δ18	7.74 ± 0.09	0.35 ± 0.05	0.79 ± 0.12	0.25 ± 0.04
Δ18N	4.83 ± 0.14	0.38 ± 0.04	0.6 ± 0.14	0.18 ± 0.06

*Units/mg is the protein amount that was used after calculating for molar ratio of the protein's molecular weight.

similar) are more extensive than for either HK I or III. Kinetic parameters showed the N-term and Δ18N constructs contain significantly higher affinities for ATP than the C-term construct (Table 1) yet also significantly lower *Ki* values for G6P. In addition, the first 18 N-terminal amino acids influence the specific activity of HK II.

Different truncated forms of human HK II in intact cells

The positron-labeled glucose analogue ^{18}F-FDG, which is transported into cells, phosphorylated, but undergoes little further metabolism, is utilized in cancer imaging to identify increased glucose uptake in tumor cells by detecting accumulated FDG with positron emission tomography (PET) (18). Therefore, to investigate activity of the HK mutants within cells we inserted the mutant genes into eukaryotic expression vectors and added a histidine motif at the carboxy-terminus of each protein to confirm successful transfection. We first identified a cell line with low levels of HK II expression. As shown in Fig. 1B, successful transfection of each mutant was confirmed by western blot analysis. We then investigated the enzyme activity of the various mutants by comparing the ^{18}F-FDG uptake patterns on media containing various glucose concentrations after transient transfection. As shown in Fig. 2A, the FDG phosphorylation efficiency of HK II appears to be slightly different than the other mutants. Only small differences in efficiency between the mutants were observed under high glucose conditions, and similar results were obtained with the purified recombinant proteins. However, more significant differences in ^{18}F-FDG uptake were observed between the mutants under conditions of low glucose. Uptake of ^{18}F-FDG was highest in HK II, followed by the N-term, Δ18 and Δ18N constructs. Both Δ18 and Δ18N demonstrated higher intensity compared to their respective intact forms. In contrast, despite high FDG phosphorylation efficiency *in vitro*, the C-term mutant displayed decreased ^{18}F-FDG uptake compared with the other mutants. Similar results were obtained in FDG-PET scans (Fig 2B).

These results indicate the enzyme activity of the C-terminus *in vivo* is lower than the N-terminus. Furthermore, the 18 amino acids at the end of the HK II N-terminus slightly increased enzyme activity *in vivo*.

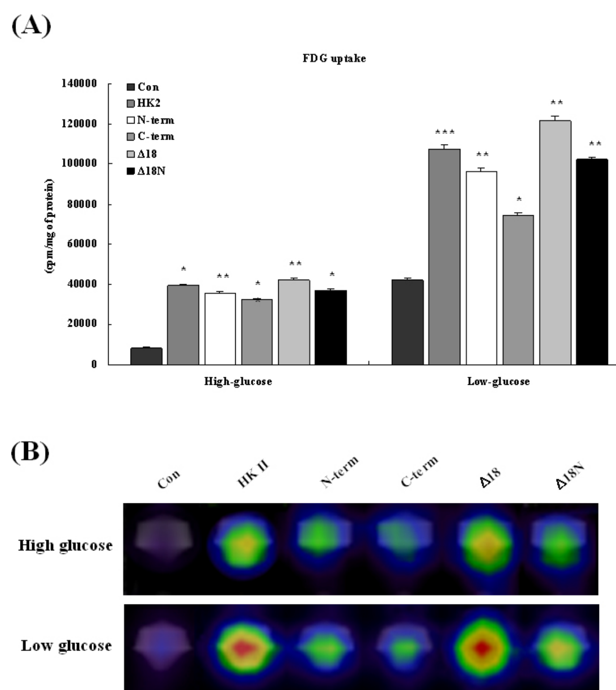

Fig. 2. Measurement of ^{18}F-FDG uptake levels in transiently transfected cells. (A) Uptake of ^{8}F-FDG by cells expressing HKII, N-term, C-term, Δ18 and Δ18N in comparison with wild-type cells (con). Uptake values are expressed as mean ± standard deviation of three independent experiments (n = 4). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (B) Cellular uptake of ^{18}F-FDG.

DISCUSSION

One of the most important functions of mammalian HK enzymes I-IV is the phospholylation of glucose during glycolysis. The extensive amino acid sequence similarity between the N- and C-terminal halves of the 100-kDa hexokinases indicates that these proteins evolved from an ancestral 50-kDa, yeast-like hexokinase by a process of gene duplication and gene fusion (16, 19). Interestingly, despite sharing high sequence homology and very similar glucose and ATP binding domains, the two halves differ in enzymatic activity, K_m values for ATP and glucose and *Ki* values for G6P. Indeed, evidence has accumulated in recent years proposing the N- and C-terminal halves of HK I have a marked functional difference despite the similarity of their amino acid sequences. Although, the N- and C-terminal halves of HK I have individually retained the ability to bind both glucose and G6P at distinct sites (8, 20), the catalytic activity of HK I is functionally restricted to its C-terminal half (9, 11, 21). In contrast, both the N- and C-terminal halves of HK II possess intrinsic catalytic activity (3, 15, 22). In addition, the intact and N-terminal half of HK II possess significantly higher affinities for ATP and G6P than the C-terminal half does. In this study, we demonstrated the N-terminal half (amino acid residues 1-475) provides the major enzyme activity of HK II while the C-terminal half (amino acid residues 476-917) has lower enzyme activity. Similar to HK I, specific activity of HK II appears mediated by the mitochondrial binding domain (amino acid residues 1-18) at its N-terminus. Furthermore, the N-terminal and Δ18N constructs show higher affinity for ATP than the C-terminal half.

Many lines of evidence suggest either GST or the GST-domains of fusion proteins have the potential to mediate oligomerization both *in vitro* and *in vivo* (23, 24). In particular, Tudyka and Skerra mentioned that one should remember many proteins require an intact N-terminus for their bio-

chemical activity when considering the use of GST as a permanent rather than transient fusion partner (24). Regarding hydrophobicity, previous studies have shown the first 12 N-terminal amino acids of HK II (the mitochondrial binding domain) along with the first 11 N-terminal amino acids of HK I are very hydrophobic and may influence the kinetic properties of the enzyme (12). Therefore, difficulties are associated with measuring the biochemical activity of either HK II or its mutants using the GST fusion system. In addition, the shape of the active site that binds the substrate is very important and could be problematic when masking or conformational changes occur during GST fusion in enzyme function studies. TO minimize such effects, we therefore decided to add a 5X histidine to the C-terminus of each recombinant protein to maintain the native conformation of the enzyme. We found that during protein purification the hydrophobic moieties of HK II and its N-terminal half were very unstable and denatured rapidly compared with the C-terminal half, Δ18, or Δ18N contructs. These results suggest the first 18 amino acids not only affect the enzyme activity of HK II, but also contribute to the stability of the enzyme.

^{18}F-FDG PET is becoming a routine clinical procedure for tumor detection by exploiting the enhanced utilization of glucose exhibited by tumor cells compared with normal cells (25). HKs are the first rate-limiting enzymes of the glycolytic pathway and therefore play a pivotal role in ^{18}F-FDG uptake and glycolytic pathways in malignant cells. We examined ^{18}F-FDG uptake in SNU-449 HCCs transiently transfected with full-length HK II and each mutant construct. Additionally, we controlled the glucose level in the media and investigated the sensitivity of each mutant. The FDG uptake pattern within the cell correlates with the results obtained for the purified protein. *In vitro* examination of the C-terminal half showed relatively high levels of FDG phosphorylation, likely due to additional FDG uptake mediated by Glut-1.

In contrast to HK I, each domain of HK II possesses enzyme activity with the N-terminal half showing higher enzyme activity than the C-terminal half. In addition, the first 18 N-terminal amino acids influence the specific activity of HK II.

MATERIALS AND METHODS

Materials

Leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF) and imidazole were purchased from Boehringer Manheim (Manheim, Germany). Glucose, *Leuconostoc mesentroides* glucose-6-phosphate dehydrogenase (G6PD) and adenosine triphosphate (ATP, disodium salt) were obtained from Roche Chemical Company (Mannhein, Germany). Nicotinamide adenine dinucleotide (NAD+, disodium salt) was obtained from Fluka Chemical Company (Buchs, Switzerland), and Ni-NTA resin, b-D-thiogalactopyranoside (IPTG), 4-(2-hydroxyethyl)-1- piperazineethane sulfanic acid (HEPES) buffer and dithioerythritol (DTE) were purchased from Sigma Chemical Company (St. Louis, MO).

Construction of expression vectors for HK II and the deletion mutants

The protein-coding regions of full-length (HK II) and the N-terminal half (N-term) of HK II were amplified by PCR using the 5'-oligonucleotide primer CGAGCTC**CATATG**ATTGCCTCGC ATCTGCT, containing the underlined *Nde* I restriction site, and the 3'-oligonucleotide primers CG**GAATTC**TTAATGATGA TGATGATGTCGCTGTCCAGCCT and CG**GAATTC**TTAATGA TGATGATGATGCTCTAATGT, containing the underlined *Eco*RI restriction site. Truncated protein-coding regions of HK II lacking the first N-terminal 18 amino acids (Δ18) and its N-terminal half lacking the first 18 amino acids (Δ18N) were amplified by PCR with the 5'-oligonucleotide primer ATTC**CATATG**GTG CAGAAGGTTGACC, containing the underlined *Nde* I restriction site, and the 3'-oligonucleotide primers CG**GAATTC**TTAA TGATGATGATGATGTCGCTGTCCA and CG**GAATTC**TTAAT GATGATGATGATGCTCTAATGT, containing the underlined *Eco*RI restriction site. The protein-coding region of the HK II C-terminus (C-term) was amplified by PCR with the 5'-oligonucleotide primer ATTC**CATATG**GTGCAGAAGGTTGACC, containing the underlined *Nde* I restriction site, and the 3'-oligonucleotide primers CG**GAATTC**TTAATGATGATGATGATGTC GCTGTCCA, containing the underlined *Eco*RI restriction site. PCR amplification products were gel purified, digested with the appropriate restriction enzymes and cloned into the pRSET A bacterial expression vector (Invitrogen). To generate mammalian expression plasmids, DNA corresponding to full length HK II and each deletion mutant was isolated from pRSET A using *Nde* I/Klenow and *Eco*RI and ligated into pcDNA 3.1/Myc-His (Invitrogen, CA, USA), followed by digestion with *Eco*RV and *Eco*RI. All constructs were confirmed by DNA sequencing.

Bacterial expression and purification of mutant proteins

HK II deletion mutants were overexpressed in *Escherichia coli* BL21 followed by purification of recombinant proteins to apparent homogeneity using Ni-NTA resin, according to the manufacturer's instructions (Sigma). Protein concentration was determined with the BCA assay kit (Pierce, Rockford, IL) according to the manufacturer's recommendations, using bovine serum albumin as a protein standard. Protein samples were stored at -30°C until use.

Cell culture

Human hepatocelluar carcinoma (HCC) cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Cells were maintained in low (1 g/L) glucose medium for 16 h and then cultured in either low or high (4.5 g/L) glucose medium for an additional 24 h.

Transient transfection

Transient transfections were performed using the cationic lipid

Lipofectamine 2,000 (Invitrogen) according to the manufacturer's specifications. Expression of recombinant protein was confirmed by western blot analysis with anti-HisTag antibodies. Cells were allowed to recover in RPMI 1,640 for 24-72 h after transfection before use in experiments.

Western blot analysis

Western blot analyses were performed using an anti-HK II antibody (AB3279 Chemicon, Temecula, CA, USA) and an anti-HisTag antibody (Qiagen, Hilden, Germany), respectively, and visualized using an ECL-detection system (Pierce, Rockford, IL), followed by exposure to photographic film. Relative protein expression levels were calculated based on X-ray film densitometry data (ChemiImager 5500 software, Alpha Innotech) after normalization with the respective housekeeping α-tubulin signals.

^{18}F-FDG uptake

For radiotracer uptake experiments, cells were plated in 24 well plates at a density of 5×10^4 cells/well (Greiner, Frickenhausen, Germany). ^{18}F-FDG uptake was determined by incubation with fresh medium containing 185 kBq (5 μCi) ^{18}F-FDG/mL for 30 min at 37°C. PET imaging was performed using a whole-body PET camera (Advance Tomograph; GE Healthcare). Cell lysates were counted with a Cobra II gamma counter (Canberra-Packard, Meriden, CT, USA).

Measurement of hexokinase activity

Hexokinase activity was determined spectrophotometrically in which glucose 6-phosphate formation is coupled to NADPH production in the presence of excess glucose 6-phosphate dehydrogenase. The process is monitored at 340 nm. One unit of HK activity is defined as the amount of enzyme that catalyzes the production of 1 μmol of G6P or ADP per minute at 30°C. Kinetic parameters were determined using Lineweaver-Burke and Eadie-Hofstee analyses, with K_m determined using at least five experiments, as described previously (26, 27).

Measurement of total cellular hexokinase activity

Total cellular hexokinase activity was measured using the Vinuela method as modified by Waki *et al* (28, 29). Hexokinase activity was determined from a standard curve, with 1 unit defined as the enzyme activity that phosphorylates 1 μmol/L of glucose per minute at 20°C. Aliquots were removed to measure protein content, and the enzyme activity was expressed as mU/mg protein.

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