Functional consequences of naturally occurring variants of human hexokinase II

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Summary Hexokinase II (HKII) catalyses a key step in glucose metabolism and can be regarded as a candidate gene for insulin resistance and type 2 (non-insulin-dependent) diabetes mellitus. We observed previously four amino acid substitutions among Finnish type 2 diabetic patients: Gln142His, Ala314Val; 0.9%, Arg353Cys; 2.7% and Arg775Gln; 2.7%. The Arg775Gln mutation was also observed in normal control subjects (2.1%) and the Gln142His substitution was found in both Type II diabetic and normal subjects with similar frequencies (~20%). Since Gln at position 142, Ala at 314 and Arg at 775 are present in human and rat hexokinases and could be im-

portant for structure and function of the enzyme, we generated all four substitutions by site-directed mutagenesis and expressed them in *E.coli*. None of these substitutions had any effect on HKII catalytic activity, K_m or V_{max} for glucose values in vitro. Thus unless these substitutions have an impact on enzyme activity or regulation in vivo, it is unlikely that these substitutions contribute to the aetiology of Type II diabetes. [Diabetologia (1998) 41: 1205–1209]

Keywords Hexokinase II, glucokinase, Type II (non-insulin-dependent) diabetes mellitus, mutagenesis.

Hexokinases (HKI-IV, EC 2.7.1.1) play a central role in intracellular glucose metabolism by catalysing the phosphorylation of glucose to glucose-6-phosphate [1]. Hexokinases I-III are 100 kDa proteins, have a low K_m for glucose and are subject to allosteric inhibition by glucose-6-phosphate (Glc-6-P). The fourth HK, also known as glucokinase, is a 50 kDa protein, has a high K_m for glucose and is not inhibited by Glc-6-P at physiological concentrations. The high sequence similarity between the N- and C-terminal halves of mammalian hexokinases I-III and glucokinase and the yeast hexokinase has lead to the hypothesis that this gene family has evolved from an

ancestral gene by tandem gene duplication and fusion.

The HKII gene encodes a protein of 917 amino acids. The N- and C-terminal halves of human and rat hexokinases are catalytically active and inhibited for feed-back by Glc-6-P [2, 3]. In contrast, the N-terminal half of rat brain HKI contains the allosteric regulatory site for Glc-6-P and the C-terminal half contains the catalytic site [4, 5].

HKII is mainly expressed in tissues sensitive to insulin such as skeletal and cardiac muscle and adipose tissue [6]. It is therefore considered to be a potential candidate gene for type 2 (non-insulin-dependent) diabetes mellitus and insulin resistance [7]. Reduced glucose oxidation and non-oxidation [8, 9], Glc-6-P [10–12] and HKII mRNA and activity levels [13] were observed in skeletal muscle of patients with Type II diabetes.

Both sibling-pair [14] and population association [15] studies in French and Japanese subjects suggested that polymorphic markers at the HKII locus are not associated with Type II diabetes. Another study

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Abbreviations: GK, Glucokinase; HKI-III, hexokinase I-III; HK-B, hexokinase B; PCR, polymerase chain reaction; Glc-6-P, glucose-6-phosphate.

in Pima Indians [16] did not detect either a linkage or an association of HKII with insulin resistance and type 2 diabetes.

The human HKII gene has been screened for variants in the coding region in patients with Type II diabetes and normal subjects [17–22]. A total of seven missense substitutions (Gln142His, Leu148Phe, Ala314Val, Arg353Cys, Arg497Gln, Arg775Gln and Arg844Lys), eight silent base substitutions (Ile201, Asp251, Glu304, Thr331, Leu586, Asn692, Pro736, Leu766) and several intronic nucleotide substitutions were found. The Gln142His substitution was very common and found at an equal frequency (approximately 20%) among type 2 diabetic and normal subjects. This polymorphism was not associated with insulin resistance or type 2 diabetes [19, 20]. The other HKII sequence variants are rare and found in only 1–3% of Type II diabetic patients. The Arg775Gln is also found among control subjects.

In this study, we have assessed the consequences of amino acid substitutions in HKII that we found among the Finnish type 2 diabetic patients and normal subjects on HKII catalytic activity. We observed that none of the substitutions had any detectable effect on catalytic activity in vitro.

Materials and methods

Construction of hexokinase II expression vector pET-hHKII. Total RNA from human adipose tissue was prepared with guanidinium isothiocyanate-phenol-chloroform method [23]. Total cellular RNA was used as template to synthesize first strand cDNA by SuperScript kit (BRL, Bethesda Research Laboratories, Life Technologies, Grand Island, N. Y., USA) according to manufacturer's protocol. This cDNA was used as template to amplify human HKII cDNA (nucleotides 108–2876, numbered according to reference [24]) using the Expand Long Template polymerase chain reaction (PCR) system (Boehringer Mannheim, Indianapolis, Ind., USA) according to the manufacturer's protocol. Primers HKII Nde5' and HKII Nde3' (Table 1) were used to create *NdeI* restriction sites on both ends of HKII cDNA. The amplified product was cloned into the *NdeI* site of the expression vector pET28c(+) (Novagen, Madison, Wis., USA), which carries a His-Tag oligohistidine domain for expression in the C-terminal side of the target protein. The entire HKII coding sequence in the resultant clone (pET-hHKII) was verified by sequencing (Sequenase kit, Amersham, Arlington Heights, IL, USA).

Oligonucleotide directed mutagenesis of HKII. Oligonucleotide directed mutagenesis was used to create the HKII mutations Gln142His (CAA- > CAT), Ala314Val (GCC- > GTC), Arg353Cys (CGT- > TGT) and Arg775Gln (CGG- > CAG). HKII cDNA segments containing each of the substitutions were first cloned into vectors pGEM3Zf(+) (Promega, Madison, Wis., USA) or pBSII SK+ (Stratagene, LaJolla, Calif., USA) for production of single-stranded DNA [25]. Mutagenesis was carried out according to Kunkel [26]. The oligonucleotides used to create substitution are given in Table 1. All mutated clones were verified by sequencing and cloned back into their respective positions in plasmid pET-hHKII for expression in *E. coli*.

Table 1. Sequence of primers used in PCR and in vitro mutagenesis

Primer	Sequence (5'-3')
HKII Nde5'	GGAATTCCATATGCGCGGCAGGAT-GATTGCCTC
HKII Nde3'	GGAAGTCCATATGGGGGTTC-TATCGCTGTCCAG
HKII Mut4	ATGGATAAGCTACATATCAAAGACAAG
HKII 8MF	CCTGGTGAAGATGGTCAAGGAG-GAGCTGC
HKII 8MR	GCAGCTCCTCTTGACCATCTTCAC-CAGG
HKII Mut9	CATCCGGAAGGCCTGTGAGGTCCTG
HKII Mut16	CTTGCTCTGAGCTGCTCTGAGATG

One of the clones containing the Ala314Val substitution was found to have three other sequence alterations which had occurred inadvertently during the cloning. One was a 3 bp deletion which resulted in the deletion of Phe²⁹³. The other two were single base substitutions that resulted in Glu294Val and Ile297Val amino acid substitutions. This clone was called 3MUThk8 and was used as a negative control for expression of active HKII in *E. coli*.

The Ala314Val substitution was created by using Quik-Change Site-directed Mutagenesis kit (Stratagene, LaJolla, Calif., USA) basically following manufacturer's protocol. PCR was done using template pET-hHKII, primers HKII 8MF and HKII 8MR (Table 1) and Expand High Fidelity enzyme (Boehringer Mannheim, Indianapolis, Ind., USA). Mutated clones were screened by single strand conformation polymorphism (SSCP) analysis [27] and sequencing.

Expression of HKII in *E. coli*. Plasmids with wild-type or mutant HKII cDNA were transformed into *E. coli* strain HMS174(DE3)pLysS [28]. A 10-ml inoculum of the transformed strain in M9ZB medium [28] supplemented with 30 mg/l kanamycin and 25 mg/l chloramphenicol, was grown overnight and then added to 250 ml of the same medium. The culture was grown at 37°C to an OD at 600 nm of 0.4, and isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 1 mmol/l. Cultures were incubated with vigorous aeration for an additional 2 h at ambient temperature (20–24°C).

Cells were collected by centrifugation and resuspended in lysis buffer (50 mmol/l Tris-HCl, pH 8.0, 5 mmol MgCl₂, 100 mmol/l KCl, 1 mg/ml lysozyme, 0.1% Triton, 2.5 μg/ml leupeptin, 50 μmol/l phenylmethanesulphonyl fluoride [29]). The resuspended cells were incubated on ice for 30 min and subjected to three cycles of freezing and thawing to lyse the cells. Bacterial chromosomal DNA was sheared by sonication. The bacterial lysates were cleared of cellular debris by centrifugation. The supernatant containing the expressed HKII protein was applied to HisBind resin columns (Novagen, Madison, Wis., USA) and purified according to the manufacturer's protocol.

SDS-PAGE and protein determination. The *E. coli* cell lysates and column purified HKII protein preparations were subjected to sodium dodecyl sulphate (SDS)-polyacrylamide (8%) gel electrophoresis [30]. Gels were stained with Coomassie Brilliant Blue. The protein concentration in lysates and column fractions was determined by the Bradford assay [31] using bovine serum albumin as a standard.

Fig. 1. SDS-polyacrylamide gel electrophoresis of total cellular proteins and purified HKII. Lane 1, total proteins expressed from HMS174DELysS-HKIIcDNA in *E. coli* without induction with IPTG. Lane 2, proteins from HMS174DELysS-HKIIcDNA culture after 2 h induction with IPTG. Lane 3, protein standards. Lanes 4–9, normal HKII, variant HKII Gln142His, Ala413Val, Arg353Cys, Arg775Gln, 3MUThk8 proteins purified from *E. coli* extracts on His-Bind columns. HKII protein is marked with an arrow

Table 2. Kinetic measurements of the wild-type and variant hexokinases. Human hexokinase II purified from extracts of *E. coli* transformed with cDNAs encoding either wild-type or variant HKII was assessed for various kinetic parameters. The mean $\pm$ SE was determined from three to four separate preparations of each protein

Enzyme	Specific activity mU/ μ g	K _m for glucose mmol/l	V _{max} nmol/min
normal	4.0 $\pm$ 0.9	0.11 $\pm$ 0.03	23.4 $\pm$ 3.0
Gln142His	3.5 $\pm$ 0.8	0.10 $\pm$ 0.03	19.6 $\pm$ 3.4
Ala314Val	3.6 $\pm$ 1.2	0.08 $\pm$ 0.02	20.5 $\pm$ 6.7
Arg353Cys	2.6 $\pm$ 0.8	0.14 $\pm$ 0.05	17.3 $\pm$ 8.0
Arg775Gln	4.3 $\pm$ 0.7	0.13 $\pm$ 0.01	24.8 $\pm$ 1.7

Assay of catalytic activity and heat stability. 5–10 μ g of column purified wild-type and variant hexokinase protein preparations were assayed immediately after elution of the HisBind column for hexokinase activity at ambient temperature in a total volume of 1 ml of assay buffer (40 mmol/l Tris-HCl pH 7.5, 20 mmol/l MgCl₂, 0.9 mmol/l NADP, 2 mmol/l ATP, 2 mmol/l EDTA, 0.2–2.5 mmol/l glucose, 1 U glucose-6-phosphate dehydrogenase) (modified from reference [32]). The reaction was followed by measuring the increase in absorbance at 340 nmol/l for 5 min. One unit of hexokinase activity is defined as the amount of enzyme which catalyses the formation of 1 μ mol of NADPH in 1 min at ambient temperature. The K_m and V_{max} for glucose were determined from double-reciprocal (Lineweaver-Burk) plots.

Heat stability of the wild-type and exon 9 (Arg353Cys) variant was tested by treating 5–10 μ g of column purified protein for 10, 20 or 30 min at room temperature or at 30 °C.

Results and discussion

Wild-type and mutant human HKII proteins were expressed in *E. coli*. A large fraction of the expressed HKII proteins were found to accumulate as insoluble aggregates, perhaps as a result of overexpression. However, significant amounts were also found in the supernatant. Soluble HKII was highly purified by affinity chromatography.

Induction of cells containing pET-hHKII resulted in accumulation of a 100 kDa protein that was not present in the non-induced cell culture (Fig. 1, lanes 1 and 2), and thus this protein can reasonably be assumed to correspond to hexokinase II. Mutant HKII proteins were purified to the same degree and showed the same electrophoretic mobility on sodium dodecyl sulphate (SDS) polyacrylamide gels as the wild-type protein (Fig. 1, lanes 4 to 8). The co-elution of some bacterial proteins seen also in Figure 1 is a well-known phenomenon with this expression system (protocol manual, Novagen, Madison, Ind., USA). These proteins did not contribute to HKII activity measurements as evidenced by the lack of detectable activity in extracts of induced *E. coli* cells transformed with HKII mutant 3MUThk8 (Fig. 1, lane 9) which produced an inactive protein.

The results of kinetic measurements on wild-type and mutant enzymes are given in Table 2. The wild-type enzyme had a K_m value of 0.11 $\pm$ 0.03 mmol/l for glucose which is comparable with that determined previously (0.14–0.2 mmol/l [6], 0.23–0.28 mmol/l [33], 0.19 mmol/l [29], 0.34 $\pm$ 0.06 mmol/l [2]). None of the mutant HKII enzymes showed a statistically significant difference in specific activity, K_m and V_{max} for glucose values from the wild-type enzyme. Since the expressed proteins had some bacterial proteins, comparison of specific activities is uncertain.

Heat stability of the in vitro expressed HKII with the Arg353Cys substitution was examined for heat

Table 3. Comparison of the residues 142, 314, 353 and 775 of human HKII with the corresponding residues in the hexokinase family

Codon	HKI		HKII		HKIII		GK		GK yeast	HK-B yeast
	human	rat	human	rat	human	rat	human	rat		
142	Lys	Lys	Gln	Gln	Gln	Tyr	Gln	Gln	His	Gln
314	Ala	Ala	Ala	Ala	Ala	Ser	Val	Val	His	Tyr
353	Lys	Lys	Arg	Tyr	His	His	Tyr	His	Gly	Asp
775	Thr	Pro	Arg	Arg	Cys	Cys	Gln	Gln	Gln	Cys

stability relative to the wild-type enzyme expressed under the same conditions. Activity of both the wild-type and the variant enzyme decreased by 90 % and 70 % after a 10 min incubation at 30 °C or 30 min incubation at room temperature, respectively.

With the exception of the polymorphic Gln142His, the amino acid substitutions investigated in this study were observed only in the Finnish population [17]. The Ala314Val substitution was found in 0.9 %, the Arg353Cys and the Arg775Gln mutation in 2.7 % of Type II diabetic patients. The Arg775Gln mutation was also found in control subjects (2.1 %) [17].

Based on amino acid sequence comparisons among various hexokinases and on structure/function studies that have been carried out on the yeast hexokinase and on X-ray crystallographic studies [34, 35], the putative glucose and ATP binding domains have been assigned [2, 3, 36, 37]. None of the amino acid substitutions examined in this study fall within any of these domains.

Glutamine at position 142 is conserved in human and rat HKII, glucokinase and human HKIII, suggesting an important role in structure and function of the enzyme. Residue 142 is in close proximity to the Ser at position 155 (conserved in all hexokinases), which is postulated to hydrogen bond with glucose in the yeast hexokinase [35]. Mutagenesis studies in rat HKI [38] and tumour hexokinase [37], however, indicated that substitutions of this residue has no effect on catalytic activity. The human and rat HKI have lysine at position 142 (Table 3) and the yeast glucokinase has histidine at the corresponding position (residue 145) [1]. All these amino acids residues have quite similar hydrophobicity indices of between -3.2 and -3.9 [39]. These results are consistent with our finding that the Gln142His substitution has no impact on catalytic activity.

Alanine at position 314 is conserved among the human and rat HKI, HKII and human HKIII, but not in the human and rat glucokinases, which have valine at the corresponding position. It appears that having a hydrophobic residue at position 314 is evolutionarily conserved among all hexokinases and, therefore, may have structural (stability, for example) or functional significance. It is possible, however, that the larger and more hydrophobic valine side chain is more appropriate for HKI-HKIII than for glucoki-

nase. Our results indicate that at least in the HKII synthesized in *E. coli*, the advantage of having Val, if any was not detectable.

Argine at position 353 is conserved in both halves of human HKII but not in the rat enzyme which has Tyr. Moreover, human and rat HKI and HKIII have Lys and His, respectively; human and rat glucokinases have Tyr and His, respectively at the corresponding position. The Arg, Lys, His and Tyr residues have similar hydrophobicity indices and, therefore the above differences among hexokinases may be well tolerated. The Arg353Cys, however, is not a chemically conservative substitution (a change in hydrophobicity index from -4.5 to +2.5). In addition, the new Cys residue could potentially form a disulphide bond with other Cys residues at positions 368, 377 or 385, which would result in significant conformational changes. Therefore, it was surprising to find that the in vitro protein with this substitution exhibited the kinetic properties and stability of the wild-type protein expressed in the same manner.

Arginine at position 775 is common to only human and rat HKIIs (Table 3). The arginine to glutamine substitution is relatively conservative, having hydrophobicity indices of -4.5 and -3.5, respectively [39]. The human and rat glucokinases have glutamine at the corresponding position. Our results are consistent with the notion that this substitution has no effect on catalytic activity of the enzyme.

In conclusion, based on results of expression of the variant and wild-type HKII enzymes in vitro, it seems unlikely that the amino acid variants we observed among Finnish Type II diabetic patients contribute significantly to the aetiology of this disease.

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