

Hexokinase Mutations that Produce Nonspherocytic Hemolytic Anemia

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ABSTRACT. Among glycolytic enzyme defects, hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1; HK) deficiency is a very rare disease where the predominant clinical effect is nonspherocytic hemolytic anemia. Here we report the characterization at molecular level of the HK type I cDNA from a patient with hemolytic anemia due to hexokinase deficiency. PCR amplification and sequence of the cDNA revealed the presence of a deletion and of a single nucleotide substitution, both in heterozygous form. In particular, the deletion, 96 bp long, concerns nucleotides 577 to 672 in the HK cDNA sequence and was never found in the cDNAs of 14 unrelated normal subjects. The sequence of the HK allele without deletion showed a single nucleotide substitution from T to C at position 1667 which causes the amino acid change from Leu⁵²⁹ to Ser. This heterozygous mutation at nt 1667 was confirmed by direct sequencing of the patient genomic DNA, but when DNAs from 10 normal controls were examined by this technique the substitution at nt 1667 was never found. From these results we concluded that the patient is carrying a point mutation at nt 1667 of one HK allele and a 96 nt deletion in the other allele. In normal subjects two differences from the published cDNA sequence were documented.

INTRODUCTION

Hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1; HK) catalyzes the phosphorylation of glucose to glucose 6-phosphate by Mg-ATP as phosphate donor and is considered one of the rate-limiting enzymes of the glycolytic pathway (1-3). In mammals, there are four isozymes of hexokinase named type I, II, III and IV or glucokinase, which vary in their tissue distribution and kinetic properties (4). Hexokinase deficiency is a rare disease where the predominant clinical effect is nonspherocytic hemolytic anemia. To our knowledge, only 14 cases have been described so far (5), two of which have been studied in our laboratory (6, 7): in one case, designated "HK-Melzo", the residual enzyme activity was heat-unstable while in the other, named

HK-Napoli", an increased K_i for glucose 1,6-bisphosphate was found. Further studies on different cells of the patient carrying the "HK-Melzo" variant showed that the HK deficiency was expressed not only in erythrocytes but also in platelets, lymphocytes (6) and fibroblasts (8). All these types of cells contain HK type I as the predominant glucose phosphorylating enzyme and in particular platelets and erythrocytes share a strict dependence upon glucose utilization for their physiological functions. In this paper we report the characterization of the hexokinase variant named "HK-Melzo" by studying the enzymatic deficiency in a lymphoblastic cell line established from the lymphocytes of the patient. The sequence of the HK coding cDNA was carried out in order to discover

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Table 1. Oligonucleotides used to amplify "HK-Melzo" cDNA

	5' Oligonucleotide	3' Oligonucleotide	
HK 1 (20)	5'-CGCCAGGGCTGCGGAGGACCGA-3'	3'-GTGCCGTACCTTCGGTCGAAA-5'	HK 2 (460)
HK 3 (390)	5'-GAATGTTACATGGAGTCCGAG-3'	3'-CTACTGGACACCGATACTGCTG-5'	HK 4 (744)
HK 5 (667)	5'-CGAGGGGACTATGATGCCAACA-3'	3'-GAGTGGGCTCCCTTCAAATTGT-5'	HK 6 (1087)
HK 7 (1021)	5'-GCCAAGGAGGGCCTCTTATTTG-3'	3'-CCGCAAAGGTGTTCTGAGATTC-5'	HK 8 (1376)
HK 9 (1322)	5'-GATCTCTTTACAAGACGCACCC-3'	3'-AGGCACACGACGACCACTTTTA-5'	HK 10 (1715)
HK 11 (1589)	5'-CGCACAACAATGCCGTGGTTAA-3'	3'-ACCTCCTCTACTTCTTGCACCT-5'	HK 12 (2162)
HK 13 (2101)	5'-GTTGGACTCATTGTTGGGACCG-3'	3'-TGACGAGGTCCAGGCCCGATA-5'	HK 14 (2489)
HK 15 (2433)	5'-CAAGTTTCTCTCTCAGATCGAG-3'	3'-CTTGACAGTGGTTTTACATTGC-5'	HK 16 (2743)
HK 17 (2648)	5'-TGACTGTGGGAGTGGACGGGA-3'	3'-AGGAGTGAACGGGACGGTGAAA-5'	HK 18 (3059)
HK 19 (2947)	5'-GCGGGGAGGAAAGCAAAATC-3'	3'-ACCGTAGCGTAGCACCACACA-5'	HK 20 (3364)
HK 21 (3283)	5'-CTGAAGGCGAGTGTGGGCATAG-3'	3'-CGAAACACTCGGCACAGCATAC-5'	HK 22 (3556)

The sequences match those reported by Nishi et al. (10). The numbers in brackets refer to the 5' end of each primer.

the molecular defect responsible for this enzymopathy. This is the first report of mutations in a patient with hemolytic anemia and reduced HK activity.

MATERIALS AND METHODS

Patients

Case reports, laboratory data and the enzymatic properties of "HK-Melzo" have been reported previously (6, 8).

Materials

cDNA cycle kit and TA cloning kit were obtained from Invitrogen. Taq polymerase was purchased from Perkin Elmer Cetus. DynaZyme DNA Polymerase Kit was from Finnzymes (distributed from CELBIO s.r.l.). For DNA sequencing, modifying T7 DNA polymerase (Sequenase, United States Biochemical) was used. [α -³⁵S] dATP, [α -³²P] dCTP and nylon

membranes for hybridization were obtained from Amersham. All other reagents were of biochemical grade.

Oligonucleotides

Oligonucleotides for PCR amplification and sequencing were synthesized by a DNA synthesizer in the laboratory of Prof. L. Silengo (Dipartimento di Genetica, Biologia e Clinica Medica, Università di Torino, Italy). The sequences of the oligonucleotides used in this study are reported in Table 1.

Synthesis of cDNA

Total RNA was extracted from the lymphoblastic cell line by the "Acid Guanidinium Thiocyanate-Phenol-Chloroform Method" as described in (9). mRNA was purified from total RNA by means of an affinity chromatography on oligo-dT cellulose. cDNA synthesis was performed with the cDNA cycle

kit purchased from Invitrogen. Briefly, one microgram aliquots of poly (A⁺) RNA were reverse-transcribed with the following six oligonucleotides, specific for HK I cDNA, as primers:

primer HK 2: 3'-GTGCCGTCACCTTCGGTCGAAA-5';

primer HK 6: 3'-GAGTGGGCTCCCTCAAATTGT-5';

primer HK 10: 3'-AGGCACACGACGACCACTTTTA-5';

primer HK 14: 3'-TGACGAGGTCCAGGCCCGATA-5';

primer HK 18: 3'-AGGAGTGAACGGGACGGTGAAA-5';

primer HK 22: 3'-CGAAACACTCGGCACAGCATAC-5'.

The single strand cDNA obtained was purified following the instructions of the manufacturers and then stored at -20 °C until use for PCR amplification.

Amplification and Cloning of Human Hexokinase I cDNA

Eleven pairs of oligonucleotides (almost all 22-mer), based on the sequence published by Nishi et al. (10), were used to amplify segments of the HK I cDNA by means of the PCR. The PCR conditions for the different pairs of primers were as follows: 92 °C for 45 sec, 62 °C for 30 sec, 72 °C for 30 sec. The extension step at 72 °C was adapted to the length of the PCR product expected. Exceptions were the amplification with primers HK 1-HK 2 where the annealing temperature was raised to 72 °C and that with the couple of primers HK 15-HK 16 where a lower annealing temperature (56 °C) was used. These PCR reactions were performed with both Taq DNA polymerase from Perkin Elmer and DynaZyme DNA polymerase kit (Fynnzymes). The oligonucleotides used for amplification and sequencing of cDNA are shown in Table 1. The different PCR products obtained were ligated

into a plasmid vector supplied with the TA cloning kit (Invitrogen). Transformed colonies were screened by PCR amplification performed directly on boiled bacterial cells, by using the same couple of primers previously utilized to obtain the cloned PCR product.

Sequence of the Cloned HK I cDNA

Sequences of the HK I cDNA fragments ligated into the TA cloning vector were performed with the "Sequenase Version 2.0 DNA Sequencing Kit" (United States Biochemical), according to the instructions of the manufacturers. The oligonucleotides used for sequencing analyses were the same as for PCR amplifications but in addition we used also the M13 (-20) Forward primer and the M13 Reverse primer to sequence the 5' and 3' ends of the inserts.

Direct Sequencing of PCR Amplified Genomic DNA from Patient and Controls

The production of single-stranded DNA suitable for sequencing, using PCR technique, in order to verify mutations found in the cloned cDNA fragments, was obtained as described in (11).

RESULTS

The patient with the HK variant named "HK-Melzo" we investigated in the past (6, 8) showed HK deficiency in red blood cells, platelets, lymphocytes and fibroblasts. Therefore a lymphoblastic cell line from the lymphocytes of the patient was established in order to study the enzymatic defect at the molecular level. These lymphoblastic cells, original lymphocytes, present a hexokinase activity of about 50%

respect to the value of the controls: patient 31.3 ± 4.7 and controls 61.0 ± 8.6 munits per mg of protein. Moreover the residual hexokinase activity in the patient cells is not immunoinactivated by an anti-hexokinase type I antibody (not shown) and thus must be contributed by a different isozyme.

However, northern blotting analysis performed with a cDNA probe, 1,341 bp long, specific for hexokinase type I, revealed that the mRNA for this HK isozyme is expressed in the lymphoblastic cells and shows by electrophoretic mobility an apparent dimension of approximately 3.6 kb.

Identification of a 96 nt Deletion in Heterozygous Form in the "HK-Melzo" cDNA

All the PCR amplifications performed as described in the "Materials and Methods" section produced a single band of the expected dimension, except for the couple of primers HK 3-HK 4 which gave two PCR products in the same ratio: the first one was 354 bp long (as expected) and the second one was 258 bp long. Sequencing of these amplified cDNAs showed that the shorter one is lacking nucleotides 577 to 672 in the HK cDNA sequence. The same PCR was performed on 15 cDNAs from different normal controls showing that none of them contained the nt 577-672 deletion (Fig. 1).

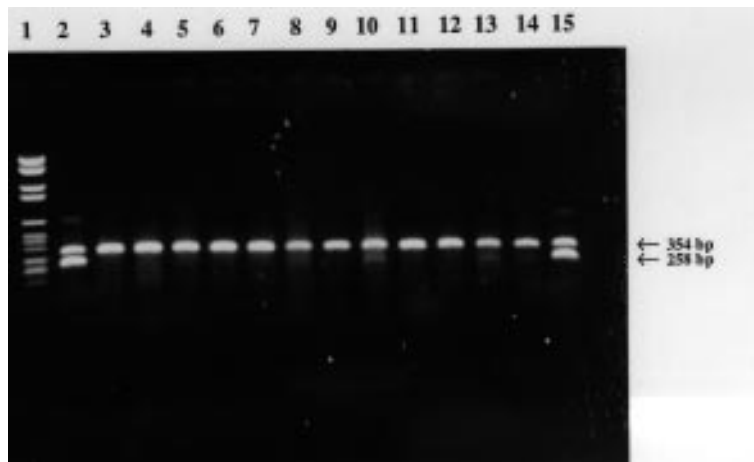


Figure 1. Electrophoretic analysis of PCR products obtained with primers HK 3 and HK 4 from "HK-Melzo" and control cDNAs. PCR amplifications were performed as described under "Materials and Methods" section. Aliquots of 10 μ l of each PCR product were loaded on a 1.3% agarose gel in 0.5 x TBE. Lane 1 shows DNA molecular weight markers. Lanes 2 and 15 were loaded with the sample obtained from the amplification of "HK-Melzo" cDNA, showing two bands of 354 and 258 bp, respectively. Lanes 3 to 14 show the single PCR product of 354 bp obtained by amplification of different normal cDNAs.

Mutation Analysis

The sequence of the entire cloned cDNA revealed three nucleotide substitutions: a T-C mutation at nt 1667 which caused a single amino acid substitution Leu⁵²⁹-Ser; a A-G mutation at nt 2269 which caused the amino acid variation Asn⁷³⁰-Asp; a A-C mutation at nt 2407 which caused the amino acid substitution Met⁷⁷⁶-Leu. The nucleotide changes at positions 2269 and 2407 were found in all the clones sequenced (about 20) and also in 28 alleles from 14 normal subjects as verified by restriction analyses. As regarding the position 2269 we used the restriction endonuclease Hph I which cleaves only if an adenine is present. All the 28 alleles examined had a guanine at nt 2269. For the nucleotide 2407 we used the endonuclease Hga I: in this case cleavage occurs when a cytosine is present. Each of the 28 alleles was completely cleaved indicating that a cytosine was always present at position 2407. So these two nucleotide substitutions were concluded to be discrepancies from the published sequence (10) and not polymorphisms. The mutation at nt 1667 which caused the amino acid change from leucine to serine was found in 16 of the 37 clones sequenced (43%) from the cDNA of "HK-Melzo" patient suggesting a heterozygous condition. To confirm this conclusion we did a direct sequencing of the patient genomic DNA. Briefly, the HK I gene was amplified with oligonucleotides HK 11-HK 10 as follows: 94° C for 1 min, 66° C for 1 min and 72° C for 2 min. The PCR product obtained, about 1,700 bp long, was sequenced with an internal primer based on an intron sequence (unpublished): the result obtained, reported in Fig. 2, confirmed the heterozygosis at the position 1667. The DNAs from 10 normal subjects (corresponding to 20 alleles) were also examined for this mutation by

direct sequencing and in every case the substitution at nt 1667 was not found. The problem that remained to be solved was to verify that the deletion and the nucleotide change at position 1667 were present in the two different alleles. For this purpose we decided to amplify the "HK-Melzo" cDNA with a 5' primer placed within the deleted sequence and a 3' primer placed below the mutation with the aim of amplifying only the HK allele without the nucleotide deletion. The two oligonucleotides used for this amplification were: HK 38 5'(619)-GTGGAAGGAG-CAGATGTGGTCA-3'(640) and HK 32 3' (1 8 8 1) - G T G C A A G A G - TAAAGGGACGGTC-5'(1902). Direct sequencing of this PCR product with primer HK 11 revealed the presence of the T¹⁶⁶⁷-C substitution in homozygous form (sequence not shown). So we conclude that the nucleotide mutation is situated in one chromosome and the deletion in the other chromosome. Thus, the patient is a double heterozygote with the result of a complete absence of hexokinase type I activity.

DISCUSSION

Among glycolytic enzyme defects, HK deficiency is a rare disease where the predominant clinical manifestation appears to be hemolytic anemia (12, 13). This is probably due to the fact that the HK reaction is catalyzed by four isozymes and therefore only tissues strongly dependent on only one isozyme could be affected by such a mutation. In this paper we investigated a case of HK variant, named "HK-Melzo", where the enzymatic defect was expressed in RBC, platelets, lymphocytes and fibroblasts (6, 8) of the patient. We previously found (6) that the lym-

phocytes had a hexokinase activity of about 50% of controls and the residual enzyme was not of type I. The aim of the present work was the study of this enzymopathy at the molecular level. The sequence of the entire cDNA obtained from the lymphoblastic cell line revealed the presence of a heterozygous deletion 96 bp long. The three-dimensional structure of hexokinase I and the organization of its gene are not known. However by analogy with others and due to the high homology of human HK I, human glucokinase and yeast HK we have tentatively placed the 96 bp deletion within amino acids 162 to 193 of human glucokinase for which the gene structure has been reported (14). This deletion, when placed in the glucokinase gene, includes all exon 6 (14). In other words, it may occur by aberrant splicing. The derived amino acid sequence for this deletion corresponds in the crystallographic structure of yeast hexokinase (15-17) to α -helix 4. This helix is important for hexokinase activity having loops at both ends that connect to active site residues (18).

The mutation Leu⁵²⁹-Ser corresponds to a change from a non-polar to a polar amino acid residue. This Leu⁵²⁹ residue is conserved in all the hexokinases so far sequenced and is placed in the β -sheet of the small domain of yeast hexokinase (17). The small domain of hexokinase which contains the β -sheet has been shown to undergo major conformational changes during substrate binding and to undergo the greatest deformation when hexokinase is in the closed conformation. Based on these considerations the substitution of even only one residue may be important both for catalysis and enzyme stability. Two other amino acid differences have been found during

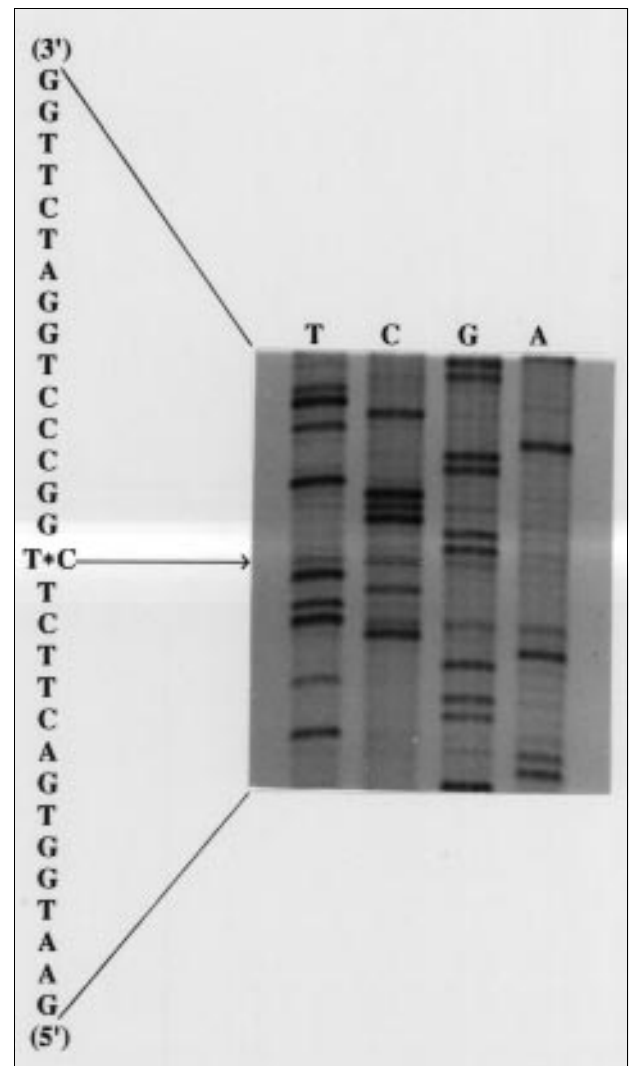


Figure 2. Direct sequencing of the "HK-Melzo" genomic DNA amplified with primers HK 11 and HK 10 (see "Materials and Methods"). The partial nucleotide sequence of the variant HK, shown in the figure, is written on the left: the asterisk marks the position 1667 where both T and C are present in a heterozygous state.

our studies compared to the published original sequence. We have investigated the possibility that the mutation A→G at nt 2269 and the mutation A→C at nt 2407 were polymorphic

sites. However, the presence of these two substitutions was found in all the alleles investigated.

The fact that two different mutations have been found in a single patient with hemolytic anemia due to hexokinase deficiency is not surprising since, as stated in the introduction, the defect is apparently very rare and the parents of this patient were not related. It would be of interest to study other patients with hexokinase I deficiency to understand whether the mutation and the deletion found here are common or occur randomly among hexokinase deficient patients. This work adds new information to the molecular basis of erythrocyte enzyme defects that are becoming an area of great interest and provide the basis for other enzymopathy studies.

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