

# Expression, Purification and Characterization of a Recombinant Erythroid-Specific Hexokinase Isozyme

Submitted 10/02/98

(communicated by Ernest Beutler, M.D., 10/07/98)

Marzia Bianchi, Giordano Serafini, Elena Bartolucci, Francesco Palma, Mauro Magnani

**ABSTRACT:** Hexokinase type I (HK I; ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1), the predominant glucose-phosphorylating enzyme in red blood cells, exists in human erythrocytes in multiple molecular forms that differ in isoelectric point and are separable by ion-exchange chromatography. The major forms, designated HK Ia, Ib and Ic, have similar kinetic properties but are characterized by different age-dependent decay and different intracellular distribution in reticulocytes. HK Ib, which elutes between HK I and HK II in the DEAE ion-exchange chromatography, appears to be unique to RBCs and different from any other hexokinase isozyme previously described. Indeed, Murakami and Piomelli recently reported the presence of a specific HK isozyme (named HKr) expressed in K562 cells and in human reticulocytes and, moreover, the resolution of the human HK I gene structure provided the direct evidence of an erythroid-specific exon 1. To further investigate the microheterogeneity of HK I in human RBCs we established a prokaryotic expression system for the HKr isozyme, using the pET plasmid, inducible with IPTG. The recombinant HKr, expressed in bacterial cells as a catalytically active enzyme, was purified to homogeneity by a combination of DEAE ion-exchange chromatography followed by hydrophobic interaction chromatography and dye-ligand affinity chromatography. The kinetic and chromatographic properties of the homogeneous recombinant HKr suggest that this erythroid-specific HK isozyme in fact corresponds to the HK isoform previously described in human RBCs and referred to as HK Ib.

© 1998 Academic Press

**Keywords:** erythroid hexokinase, red blood cell enzyme, kinetic properties, (human)

## INTRODUCTION

Four different enzymes with different properties and different tissue distribution are responsible for glucose phosphorylation in humans (hexokinase types I, II, III and IV or glucokinase) (1). In human red blood cells (RBCs), glucose phosphorylation is catalyzed by hexokinase (HK; ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) type I (2). However, using a number of methods including

electrophoresis, column chromatography and immunological techniques it was found that HK is present in multiple forms characterized by different decay during reticulocyte maturation and cell aging and by different intracellular distribution (2-7). Nonetheless, all patients with hemolytic anemia due to hexokinase type I deficiency reported thus far (8-13) have shown no clinical manifestations involving organs or tissues

---

Institute of Biological Chemistry "G. Fornaini", University of Urbino, Italy.

Reprint request to: Prof. Mauro Magnani, Istituto di Chimica Biologica "G. Fornaini", Università degli Studi di Urbino, Via Saffi, 2, 61029-Urbino, Italy, phone 39-722-305211, fax 39-722-320188, email: magnani@bib.uniurb.it

ISSN No. 1079-9796/98 \$25.00  
Copyright © 1998 by Academic Press  
All rights of reproduction in any form reserved

where HK type I is also the major glucose-phosphorylating enzyme, suggesting that HK in red blood cells may be a separate, independently regulated gene product (14). Indeed, Murakami *et al.* (15) reported the presence of a specific HK isozyme (HKr) expressed in K562 cells and human reticulocytes. The resolution of the human hexokinase I gene structure provided unequivocal evidence for an erythroid-specific exon in the HK type I gene (16,17) and suggests that HKr is probably the product of an alternate splicing event involving HK I. In the present paper we report the production, purification and characterization of a recombinant human erythroid-specific HK type I isozyme and of a truncated form lacking the mitochondrial binding domain. Our results indicate that HKr corresponds to the HK isoform previously designated HK Ib (2) or HKr (14) and that other molecular events (post-translational modifications) may be responsible for the further heterogeneity.

## MATERIALS AND METHODS

### *Materials*

Restriction endonucleases, as well as coenzymes, enzymes and substrates used for enzymatic assays, were purchased from Boehringer Mannheim Biochemicals. The expression plasmid pET and the *E. coli* strain BL21(DE3) were from Novagen (Madison, WI). The cDNA Cycle Kit was obtained from Invitrogen. Taq polymerase was purchased from Perkin Elmer Cetus, while the Sequenase Version 2.0 DNA Sequencing Kit (USB) was from Amersham. DEAE Sepharose Fast Flow and Phenyl Sepharose CL-4B were obtained from Pharmacia LKB Biotechnology. Matrex Gel Blue A was from Amicon.

### *Cell Culture and RNA Isolation*

Cells from the human erythroleukemia cell line K562 were grown in RPMI 1640 medium,

supplemented with 10% (v/v) FCS. For isolation of total cellular RNA, approximately  $50 \times 10^6$  cells were collected by centrifugation at  $500 \times g$  for 8 min. The culture medium was removed by aspiration and the cells were washed once in PBS. Total RNA was extracted according to the "Acid Guanidinium Thiocyanate Phenol-Chloroform" (AGPC) method described by Chomczynski and Sacchi (18). The resulting RNA pellet was dried, resuspended in diethylpyrocarbonate (DEPC)-treated water and finally quantitated by O.D. at 260 nm.

### *cDNA Synthesis*

cDNA was synthesized from total RNA using the cDNA Cycle Kit purchased from Invitrogen, according to the manufacturer's instructions, with the only exception that three oligonucleotides specific for HK I cDNA (16,19) were used as primers instead of oligo-dT or random hexamers. Briefly, 10  $\mu$ g of total RNA were primed for cDNA synthesis with the following anti-sense oligonucleotides:

primer:

HK 37 (540): 5'-CGTGAATCCCACAGGTA ACT TC-3'

primer:

HK 4 (744): 5'-GTCGTCATAGCCACAGGT CATC-3'

primer:

HK 6 (1087): 5'-TGTTAACTTCCCTCGGG TGAG-3'.

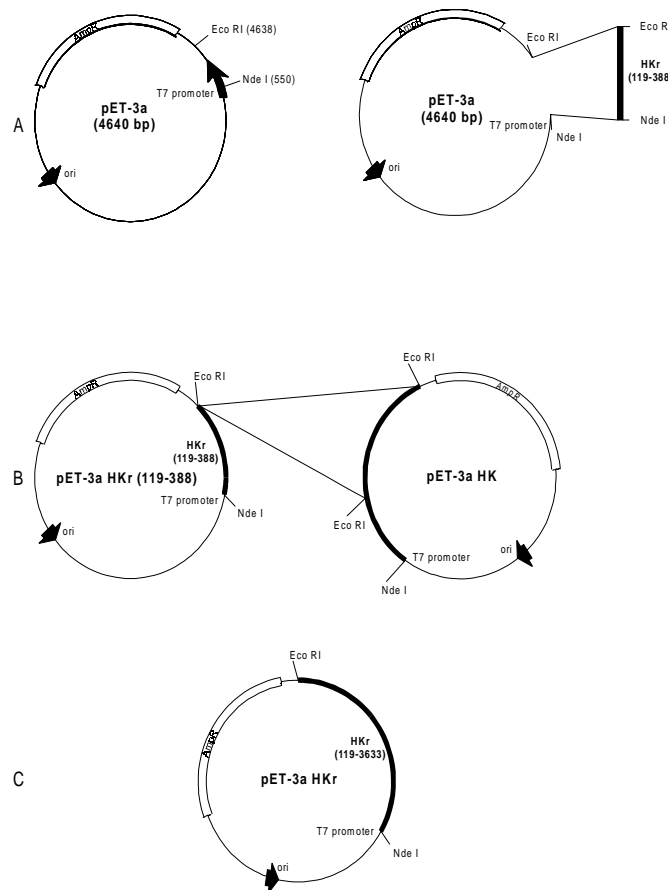
The numbers in brackets refer to the 5' end of each primer.

### *PCR Amplification and Construction of the pET-3a HKr Expression Vector*

The cDNA obtained was used in a PCR reaction aimed at amplifying the 5' portion of HKr cDNA. For this purpose we used a 5' forward primer (referred to as HKr: 5'-TATATCCATATGGACTGTGAGC-3') specific for the erythroid exon 1 (15) and a 3' reverse primer complementary to the conserved exon 4 (HK 37: 5'-CGTGAATCCCACAGGTA ACTTC-

3'). The forward primer was degenerated at the nucleotide underlined in order to create an *Nde I* restriction site at the ATG start codon. PCR amplification was performed in a 50  $\mu$ l volume with the Perkin Elmer Gene Amplification kit (1.5 mM MgCl<sub>2</sub>; 2 U of AmpliTaq) using 15 pmol of each primer. The PCR was carried out for 30 cycles under the following conditions: 94C for 45 sec, 57C for 30 sec, 72C for 30 sec, with a final extension at 72C for 10 min. The 465 bp PCR product was made blunt-ended using the Klenow enzyme and then cloned into the Bluescript KS<sup>-</sup> plasmid (Stratagene) at the *Sma I* site. The cloned HKr cDNA was digested with the restriction enzymes *Nde I* and *Eco RI* to obtain the fragment encoding the N-terminal 90 amino acids. This fragment was subsequently

ligated with a pET-3a expression plasmid which had been previously cut with *Nde I* and *Eco RI*. The 3245 bp *HK I* cDNA fragment, which included the coding region for amino acid residues 91-916 as well as the 3' untranslated region, was obtained by digestion of the pET-3a HK [the expression vector for the full-length HK I; (20)] with the restriction enzyme *Eco RI*. This 3245 bp *Eco RI* fragment was then inserted into the pET-3a plasmid that already contained the HKr N-terminal sequence. The maintenance of the correct orientation of the two partial HK cDNA fragments was verified by nucleotide sequence analysis. The expression vector for the HKr isozyme was named pET-3a HKr and its construction is detailed in Figure 1.



**Figure 1.** Construction of the pET-3a HKr expression vector. (A) The pET-3a plasmid, previously cut with *Nde I* and *Eco RI*, was ligated with the *Nde I*-*Eco RI* fragment of the amplified HKr cDNA, encoding the 90 N-terminal amino acids (shown as 119-388 HKr cDNA). (B) The pET-3a HK recombinant plasmid [used to express the full-length HK I; (20)] was digested with *Eco RI* to obtain a 3245 bp HK I cDNA fragment including the coding region for amino acid residues 91-916. (C) The 3245 bp *Eco RI* cDNA fragment was ligated with the pET-3a plasmid that already contained the HKr N-terminal sequence to obtain the final expression vector referred to as pET-3a HKr. More details concerning this strategy are reported in the "Materials and Methods" section.

### *Expression and Purification of the Recombinant HKr Isozyme*

Using the pET-3a HKr construct, we transformed *E. coli* strain BL21(DE3) and single transformed colonies were cultured in LB medium containing 50 µg/ml ampicillin until the optical density at 600 nm reached 0.4 O.D. Then, recombinant human HKr was induced by 0.4 mM IPTG for approx. 15 h at 22 C. The optimization of human hexokinase expression in bacterial cells as well as the preparation of bacterial extracts for enzymatic activity and protein assays are detailed in (21). Recombinant HKr enzyme was further purified to homogeneity by a combination of DEAE ion-exchange chromatography followed by Hydrophobic Interaction Chromatography (HIC) and Blue A dye-ligand chromatography, performed as described for the full-length HK I (20) with slight modifications in the last purification step. In detail, the enzyme eluted from the HIC column was first dialyzed, concentrated and then directly loaded onto the Blue A column. The washing and elution buffers for the Blue A column contained 8 mM KCl rather than the 90 mM solution used for full-length HK.

### *Hexokinase Assay*

Hexokinase activity was measured at 37C spectrophotometrically in a system coupled with glucose 6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) or, in the Glc 6-P inhibition studies, in a coupled enzyme system with pyruvate kinase (PK; EC 2.7.1.40) and lactate dehydrogenase (LDH; EC 1.1.1.28) as described in (22). One unit of hexokinase activity is defined as the amount of the enzyme which catalyzes the formation of 1 µmol of Glc 6-P or ADP/min at 37C. Kinetic studies were performed in 100 mM Tris-HCl, pH 7.2 (22).

### *Determination of Protein*

Protein concentration was determined using

the Bradford method (23) with bovine serum albumin as standard, or spectrophotometrically by measuring the absorbance of solutions at 280 nm against appropriate blanks.

### *SDS-PAGE and Immunoblotting*

SDS-PAGE of the expressed human HKr was performed in 10% polyacrylamide gels according to the method of Laemmli (24), while Western blot analysis was carried out exactly as described in (25).

### *Chromatographic Separation of Recombinant Forms of Human Hexokinase I*

Chromatographic separations were performed in a Toyopearl DEAE 650 S column (15 cm x 1 cm i.d.) (26). The column was packed using a peristaltic pump and was equilibrated with 5 mM sodium-potassium phosphate buffer containing 3 mM 2-mercaptoethanol, 3 mM potassium fluoride and 5 mM glucose. 0.5 IU of full-length hexokinase, hexokinase-11aa and RBC-specific hexokinase were analyzed separately or as a mixture. The samples were loaded onto the column and the elution of the enzyme was obtained using a 200-200 ml linear gradient of KCl, from 40 to 200 mM, in the same equilibrating buffer. Elution was achieved operating at 4C using a flow rate of 10 ml/h. Fractions of 0.7 ml were collected and assayed for hexokinase activity. The recovery of hexokinase activity was higher than 95%.

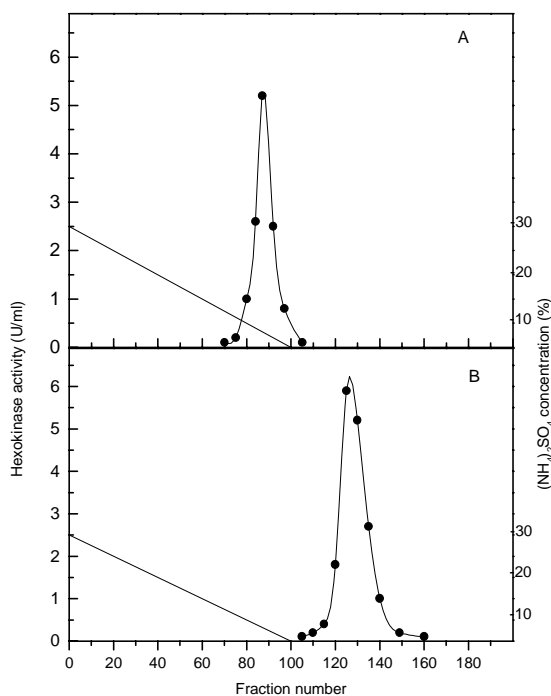
## RESULTS

### *Expression and Purification of Recombinant HKr*

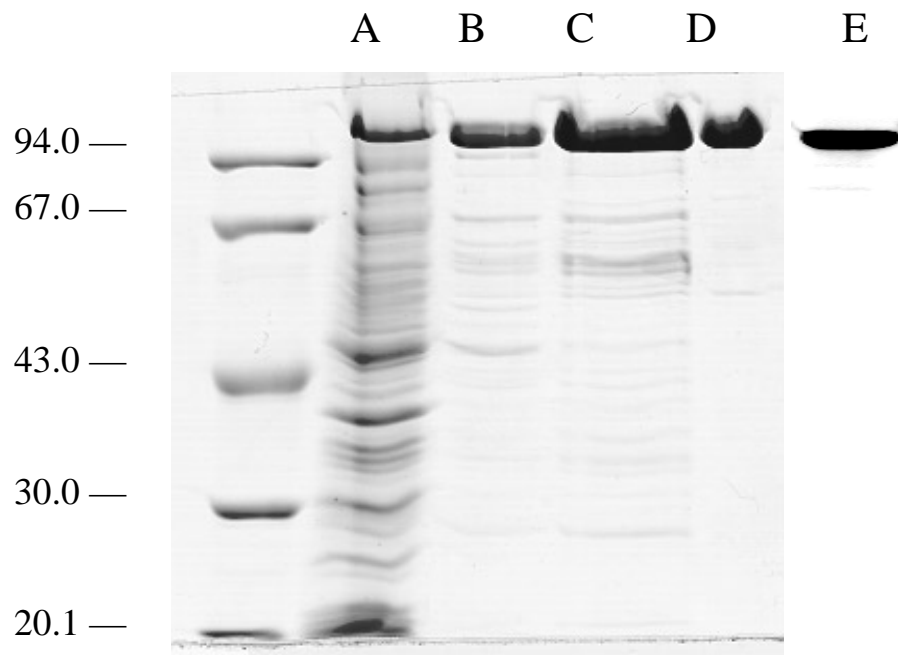
*E. coli* strain BL21(DE3) cells transformed with pET-3a HKr plasmid, obtained as described in the "Materials and Methods" section, were used for the expression of recombinant human HKr, induced by adding 0.4 mM IPTG to the growing bacterial culture. The determination of specific activity of HK, measured in the bacterial

lysates, revealed that HKr is expressed in bacterial cells as a soluble protein in a catalytically active form (HK specific activity: 5.5 U/mg for induced pET-3a HKr transformed cells and 0.088 U/mg for induced control cells). The catalytically active recombinant HKr was purified to homogeneity using a combination of DEAE ion-exchange chromatography, followed by hydrophobic interaction chromatography and dye-ligand affinity chromatography, as already done for the recombinant full-length HK I enzyme (20). It is noteworthy that the results obtained by HIC revealed different affinity of the recombinant HKr for the Phenyl Sepharose CL-4B resin compared to those observed for the full-length HK and the HK-11aa. This fact is demonstrated by the different elution patterns of the three recombinant HKs when using the linear gradient of ammonium sulfate from 30 to 0%. In fact, as shown in Figure 2, the full-length HK I as well as the HK lacking the first 11 amino acids eluted

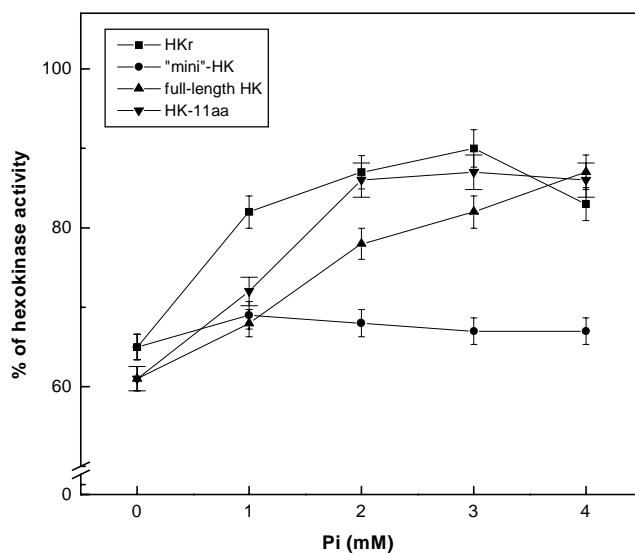
from the column by 3 to 0% ammonium sulfate while the HKr isozyme eluted later, beyond the end of the gradient. This behavior can probably be explained by the different N-terminal sequences of the human HKs investigated. Table 1 summarizes the purification of recombinant HKr; the values are the means of three preparations. The average yield of purified HKr was about 2.8 mg from 1.2 liters of bacterial culture. The specific activity of the final homogeneous enzyme (130 U/mg), corresponding to a 23-fold purification, was comparable to the values obtained for the recombinant human HK type I [150 U/mg; (20)] and the recombinant human HK lacking the 11 N-terminal amino acids [180 U/mg; (27)], both purified to homogeneity. Figure 3 shows the SDS-PAGE of samples taken at the various steps in the purification procedure as well as Western blotting of the recombinant homogeneous HKr, detected with a monospecific rabbit anti-human hexokinase type I antibody.



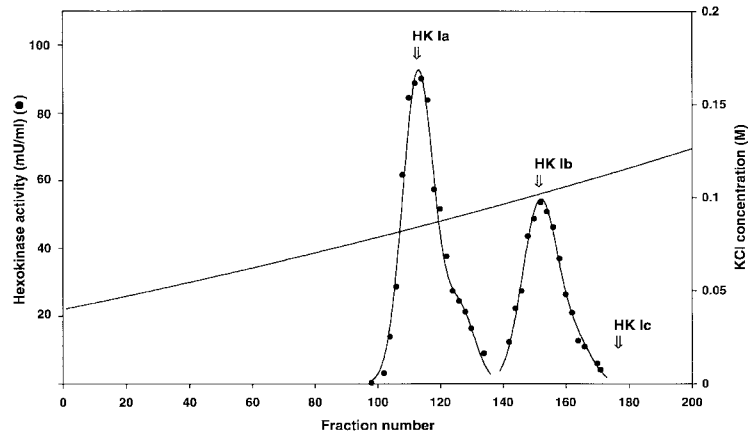
**Figure 2.** Separation of different recombinant HK I isoforms using Hydrophobic Interaction Chromatography. Pooled fractions obtained after DEAE ion-exchange chromatography of full-length HK and HK-11aa (A) or HKr (B) were loaded onto a Phenyl Sepharose CL-4B column (1.8 x 13 cm). Elution of hexokinase activity was performed with a linear gradient of ammonium sulfate from 30 to 0% in 5 mM sodium potassium phosphate buffer, pH 7.55, containing 5 mM glucose and 3 mM 2-mercaptoethanol (500 ml in each chamber) and then with buffer alone. The flow-rate was 1ml/min and fraction volume was 10 ml. (•), hexokinase activity; (—),  $(\text{NH}_4)_2\text{SO}_4$  concentration.



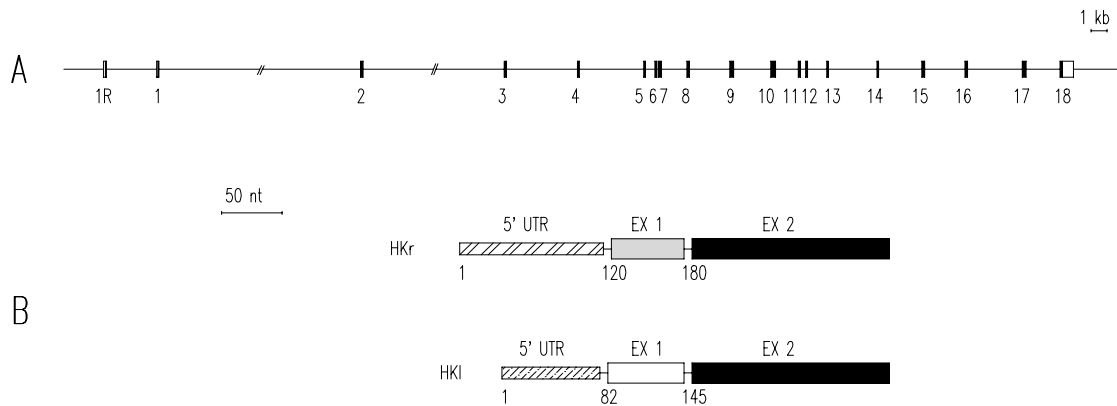
**Figure 3.** SDS-PAGE of human recombinant HKr at different purification steps (lanes A-D) and Western blot analysis of the purified enzyme (lane E). The recombinant human erythroid hexokinase (HKr) was electrophoresed in a 10% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. The positions of the molecular weight markers (with corresponding  $M_r$  in kDa) are indicated on the left. Lane A, 12  $\mu$ g of soluble post-induction cell proteins from BL21(DE3) cells transformed with the pET-3a HKr plasmid; lane B, 8  $\mu$ g of ammonium sulfate precipitate of the DEAE pool; lane C, 12  $\mu$ g of sample after HIC chromatography; lane D, 6  $\mu$ g of the purified recombinant HKr, obtained after the Blue A dye-ligand chromatography. Lane E, loaded with the same sample as lane D, shows the immunochemical detection of the homogeneous HKr, performed using immunoaffinity-purified rabbit anti-human HK type I IgG. The immunoreactive band was visualized using horseradish-peroxidase-labelled goat anti-rabbit IgG antibodies, followed by ECL chemiluminescence detection.



**Figure 4.** Effectiveness of *ortho*-phosphate ( $P_i$ ) in reversing hexokinase inhibition by glucose 1,6-bisphosphate. The experiments were performed in 100 mM Tris-HCl, pH 7.2, at 37°C. The glucose concentration was 2.5 mM, that of free  $Mg^{2+}$  was 5 mM while MgATP was used at 1 mM. Glucose 1,6-bisphosphate concentration was 100  $\mu$ M and  $P_i$  concentrations tested ranged from 0 to 4 mM. (■), HKr; (●), "mini"-HK; (▲), full-length HK; (▼), HK-11aa.



**Figure 5.** Separation of different recombinant forms of human hexokinase I using a Toyopearl DEAE 650 S column. A mixture of full-length HK, HK-11aa and RBC-specific HK (0.5 IU of each isoform) was analyzed as described in the “Materials and Methods” section. Full-length HK and HK-11aa coelute in a peak corresponding to red blood cell HK Ia, while HKr behaves exactly like red blood cell HK Ib. No correspondence was found for red blood cell HK Ic (2). Chromatography of single isozymes confirmed the elution position shown above. The elution position of human erythrocyte hexokinase isozymes Ia, Ib and Ic is indicated by an arrow.



**Figure 6.** (A) Structure of the human hexokinase type I gene and (B) comparison of somatic HK type I with red blood cell-specific HK type I cDNA species. (A) Exons are represented by boxes and introns by a thin line. Black boxes indicate coding sequences; white boxes mean non-coding sequences. (B) 5' UTR sequences are represented by narrow boxes and are different in somatic and RBC-specific HK I mRNAs. Also exon 1 (light grey) of the RBC-specific hexokinase isoform (HKr) differs from exon 1 (white) of the somatic hexokinase type I (HK I). Exon 2 (black) is common to the two isoforms.

**Table 1.** Purification of recombinant human HKr expressed in *E. coli*

Purification step	Total activity (Units)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Cell-free extract	1870	5.5	100	1
Ammonium sulfate (70%) of DEAE peak	800	9.0	42.8	1.6
Hydrophobic interaction chromatography	760	28	40.6	5.1
Blue A dye-ligand chromatography	540	130	28.9	23.6

Typical results of a purification procedure for recombinant HKr (starting from 1.2 liters of induced bacterial culture) are shown. Similar values (within 10%) were obtained in three different experiments.

**Table 2.** Kinetic properties of human recombinant HKr

Property	Recombinant HKr
$K_m$ (mM) for Mg-ATP	$0.65 \pm 0.10$
$K_m$ ( $\mu$ M) for glucose	$41.1 \pm 3.0$
$K_i$ ( $\mu$ M) for Glc 6-P	$39.4 \pm 3.8$
$K_i$ ( $\mu$ M) for Glc 1,6-P <sub>2</sub>	$60.0 \pm 5.8$

The  $K_m$  for Mg-ATP was determined at saturating glucose concentration (5mM), while the  $K_m$  for glucose was derived at saturating Mg-ATP concentration (5 mM). The  $K_i$  values were determined with Mg-ATP as the varied substrate (0.5-2.0 mM) at a glucose concentration of 5 mM by a replot of slopes, obtained from double reciprocal plots, versus inhibitor concentrations. The patterns all indicated competitive inhibition. All the experiments were performed in 100 mM Tris-HCl, pH 7.2, at 37C. The values are means  $\pm$  S.D. of three experiments.

### *Kinetic Properties of Recombinant HKr*

The homogeneous recombinant HKr was also characterized for its kinetic and regulatory properties, in comparison with the recombinant full-length HK (20), recombinant truncated HK I lacking the first 11 amino acid residues at the N-terminus (27) and human placental HK I (28). The kinetic parameters of recombinant HKr are reported in Table 2. The kinetic values obtained suggest that, in terms of its kinetic properties, the

erythroid-specific isozyme belongs to HK I, in accordance with the previously reported data on the multiple isoforms of red blood cell HK (2). The effectiveness of *ortho*-phosphate in reversing HKr inhibition by Glc 1,6-P<sub>2</sub> was also investigated. The results obtained indicate that P<sub>i</sub>, in the 0-4 mM range, is able to antagonize the inhibition of HKr by Glc 1,6-P<sub>2</sub> (Figure 4), while at higher concentrations it behaves as a competitive inhibitor with ATP (data not shown).

### *Chromatographic Separation of Different Recombinant HK I Isoforms*

To confirm the expectation that HKr indeed corresponds to the HK isoform HK Ib, previously described in human RBCs, we performed DEAE ion-exchange chromatography of the recombinant full-length HK I, the truncated form lacking the mitochondrial binding domain (HK-11aa) and the HKr isozyme. As shown in Figure 5, HK I and HK-11aa coeluted in the same peak corresponding to the erythrocyte HK Ia. HKr, instead, was eluted exactly as HK Ib. Thus, HKr indeed corresponds to HK Ib while HK Ia could either be contributed by the full-length isozyme or a truncated form of it.

### DISCUSSION

It has long been known that hexokinase, one of the rate-limiting enzymes of glycolysis, is present in mammalian tissues as four distinct isozymes (type I, II, III and IV or glucokinase), each of which is probably the product of a separate gene (1,29,30). Numerous reports also revealed that the situation is much more complex and that multiple molecular forms of hexokinase type I (the almost ubiquitous HK isozyme) are present in rat brain (31,32), pig heart (33,34), human spleen (35), heart (36), placenta (28,37) and erythrocytes from many species (3). In particular, HK I in human erythrocytes exists in multiple molecular forms that are separable by ion-exchange chromatography. We (2,5) and others (4) have clearly shown that this glucose-phosphorylating activity consists of at least three different subtypes, designated HK Ia, Ib and Ic (based on their elution order), that differ in isoelectric point, intracellular distribution and, moreover, show pronounced cell age-dependent modifications (2,14). Of these isoforms, only hexokinase Ia corresponds to hexokinase type I from human liver, while the others differ from every other previously reported hexokinase isozyme. Hexokinase Ib, also known as HKr (14), is the main form found in the youngest RBCs but

decreases rapidly with cell age, suggesting that HKr may be an RBC-specific isozyme that is predominant in the reticulocytes and is then rapidly degraded. In any case, the origin of HK I microheterogeneity in human RBCs remained an open question for a long time: in fact, it could be explained either by differential RNA transcription and/or processing or by post-translational proteolytic modification of a native enzyme. Indeed, Murakami and Piomelli recently reported the presence of a unique cDNA for hexokinase encoding for a specific HK isozyme (HKr) expressed in K562 cells and in human reticulocytes (15). Its nucleotide sequence was identical to that of HK I cDNA (19) except for the 5' extreme end causing the substitution of the porin-binding domain (PBD) with a different stretch of 20 amino acid residues, thus explaining the exclusive cytoplasmic localization of HKr. The resolution of the human HK I gene structure provides direct evidence for an erythroid-specific exon 1 in the HK type I gene, located approx. 3.1 kb upstream from the somatic exon 1 (16; Figure 6). The aim of the present work was to characterize the HKr isozyme described by Murakami and Piomelli (15) in order to demonstrate its correspondence with the isoform HK Ib previously described in human RBCs. To shed more light on this matter, we studied the expression, purification and characterization of the recombinant human HKr and compared its kinetic properties with the data already available for the recombinant full-length human HK type I (20) and for the truncated form of the enzyme lacking the mitochondrial binding domain at the N-terminus (27). The results obtained indicate that, in terms of its kinetic properties, HKr belongs to HK I. Moreover, the chromatographic experiments (i.e. DEAE ion-exchange chromatography) performed by using the three different recombinant HKs mentioned above confirmed that HKr indeed corresponds to the isoform HK Ib described in human RBCs. These results would explain all the evidence previously reported for the isoform HK Ib. First of all, HKr lacks the porin-binding domain at the N-terminus,

thus explaining its exclusive cytoplasmic localization. Moreover, HKr is an erythroid-specific gene product resulting from alternate gene splicing (16) and alternate usage of promoters, and is thus independently regulated. The separate genetic control of the RBC-specific HK isozyme explains the clinical reports of two types of HK deficiency: one in which HK activity is reduced exclusively in the RBCs [HKr defect; (38)] and another with generally decreased HK I activity involving most tissues in which HK I is the predominant glucose-phosphorylating enzyme [HK I defect; (9,10,13)]. Thus, we argue that HKr is generated by an alternative splicing mechanism, but other molecular events (i.e. post-translational proteolytic modifications) may be involved in the further HK I heterogeneity described in human RBCs.

#### ACKNOWLEDGMENTS

This work was supported by M.U.R.S.T. funds.

#### REFERENCES

1. Wilson JE. Hexokinases. *Rev Physiol Biochem Pharmacol* 126: 65-198, 1995.
2. Stocchi V, Magnani M, Canestrari F, Dachà M, Fornaini G. Multiple forms of human red blood cell hexokinase. Preparation, characterization, and age dependence. *J Biol Chem* 257: 2357-2364, 1982.
3. Fornaini G, Dachà M, Magnani M, Stocchi V. Molecular forms of red blood cell hexokinase. *Mol Cell Biochem* 49: 129-142, 1982.
4. Rijksen G, Jansen G, Kraaijnshagen RJ, Van Der Vlist MJM, Vlug AMC, Staal GEJ. Separation and characterization of hexokinase I subtypes from human erythrocytes. *Biochim Biophys Acta* 659: 292-301, 1981.
5. Magnani M, Serafini G, Stocchi V. Hexokinase type I multiplicity in human erythrocytes. *Biochem J* 254: 617-620, 1988.
6. Stocchi V, Stulzini A, Magnani M. Chromatographic fractionation of multiple forms of red blood cell hexokinase. *J Chromatogr* 237: 330-335, 1982.
7. Gahr M. Different biochemical properties of foetal and adult red cell hexokinase isoenzymes. *Hoppe-Seyler's Z Physiol Chem* 361: 829-837, 1980.
8. Miwa S. Hereditary disorders of red cell enzymes in the Embden-Meyerhof pathway. *Am J Haematol* 14: 381-391, 1983.
9. Rijksen G, Akkerman JWN, van den Wall Bake AWL, Hofstede DP, Staal GEJ. Generalized hexokinase deficiency in the blood cells of a patient with nonspherocytic hemolytic anemia. *Blood* 61: 12-18, 1983.
10. Magnani M, Stocchi V, Cucchiarini L, et al. Hereditary nonspherocytic hemolytic anemia due to a new hexokinase variant with reduced stability. *Blood* 66: 690-697, 1985.
11. Magnani M, Stocchi V, Canestrari F, et al. Human erythrocyte hexokinase deficiency: a new variant with abnormal kinetic properties. *Br J Haematol* 61: 41-50, 1985.
12. Beutler E, Dymment PG, Matsumoto F. Hereditary nonspherocytic hemolytic anemia and hexokinase deficiency. *Blood* 51: 935-940, 1978.
13. Bianchi M, Magnani M. Hexokinase mutations that produce nonspherocytic hemolytic anemia. *Blood Cells Mol Dis* 21: 2-8, 1995.
14. Murakami K, Blei F, Tilton W, Seaman C, Piomelli S. An isozyme of hexokinase specific for the human red blood cell (HKr). *Blood* 75: 770-775, 1990.
15. Murakami K, Piomelli S. Identification of the cDNA for human red blood cell-specific hexokinase isozyme. *Blood* 89: 762-766, 1997.
16. Ruzzo A, Andreoni F, Magnani M. Structure of the human hexokinase type I gene and nucleotide sequence of the 5' flanking region. *Biochem J* 331: 607-613, 1998.
17. Ruzzo A, Andreoni F, Magnani M. An erythroid-specific exon is present in the human hexokinase gene. *Blood* 91: 363-364, 1998.
18. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159, 1987.
19. Nishi S, Seino S, Bell GI. Human hexokinase: sequences of amino- and carboxyl-terminal halves are homologous. *Biochem Biophys Res Commun* 157: 937-943, 1988.
20. Bianchi M, Crinelli R, Serafini G., Giammarini C, Magnani M. Molecular bases of hexokinase deficiency. *Biochim Biophys Acta* 1360: 211-221, 1997.
21. Bianchi M, Serafini G, Corsi D, Magnani M.

- High-level expression and purification of a human "mini"-hexokinase. *Protein Expression Purif* 7: 58-66, 1996.
22. Magnani M, Stocchi V, Dachà M, Fornaini G. Regulatory properties of rabbit red blood cell hexokinase at conditions close to physiological. *Biochim Biophys Acta* 804: 145-153, 1984.
  23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.
  24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685, 1970.
  25. Magnani M, Bianchi M, Casabianca A, et al. A recombinant human "mini"-hexokinase is catalytically active and regulated by hexose 6-phosphates. *Biochem J* 285: 193-199, 1992.
  26. Stocchi V, Masat L, Biagiarelli B, et al. High resolution of multiple forms of red blood cell enzymes using a Toyopearl DEAE 650 S. *Prep Biochem* 22: 11-40, 1992.
  27. Bianchi M, Serafini G, Bartolucci E, Giammarini C, Magnani M. Enzymatic properties of overexpressed human hexokinase fragments. *Mol Cell Biochem*, in press, 1998.
  28. Magnani M, Stocchi V, Serafini G, Chiarantini L, Fornaini G. Purification, properties, and evidence for two subtypes of human placenta hexokinase type I. *Arch Biochem Biophys* 260: 388-399, 1988.
  29. Katzen HM, Schimke RT. Multiple forms of hexokinase in the rat: tissue distribution, age dependency, and properties. *Proc Natl Acad Sci USA* 54: 1218-1225, 1965.
  30. Colowick SP. The hexokinases. In: Boyer P B, eds. *The enzymes*. New York: Academic, vol 9, pp. 1-48, 1973.
  31. Polakis P, Wilson JE. Purification of highly bindable rat brain hexokinase by high performance liquid chromatography (HPLC). *Biochem Biophys Res Commun* 107: 937-943, 1982.
  32. Needels DL, Wilson JE. The identity of hexokinase activities from mitochondrial and cytoplasmic fractions of rat brain homogenates. *J Neurochem* 40: 1134-1143, 1983.
  33. Easterby JS, O' Brien MJ. Purification and properties of pig heart hexokinase. *Eur J Biochem* 38: 201-211, 1973.
  34. Vowles DT, Easterby JS. Comparison of type I hexokinases from pig heart and kinetic evaluation of the effects of inhibitors. *Biochim Biophys Acta* 566: 283-295, 1979.
  35. Neumann S, Falkenburg F, Pfliederer G. Purification and immunological characterization of the human hexokinase isozymes I and III (ATP-D-hexose 6-phosphotransferase EC 2.7.1.1). *Biochim Biophys Acta* 334: 328-342, 1974.
  36. Haritos AA, Rosemeyer MA. Isolation and glucose-6-phosphate-mediated dimerization of hexokinase from human heart. *Biochim Biophys Acta* 830: 113-119, 1985.
  37. Magnani M, Serafini G, Bianchi M, Casabianca A, Stocchi V. Human hexokinase type I microheterogeneity is due to different amino-terminal sequences. *J Biol Chem* 266: 502-505, 1991.
  38. Altay C, Alper CA, Nathan DG. Normal and variant isoenzymes of human blood cell hexokinase and the isoenzyme patterns in hemolytic anemia. *Blood* 36: 219-227, 1970.