Human Hexokinase Type I Microheterogeneity Is Due to Different Amino-terminal Sequences*

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Human placenta hexokinase type I was previously shown to be present in two subtypes with similar isoelectric points but different molecular masses of 112 and 103 kDa, respectively. In order to exclude that these subtypes arise by artifact(s) occurring during the protein purification, we have developed a single-step immunoaffinity chromatography for the isolation of microgram quantities of hexokinase. The results obtained confirmed the presence of both hexokinase subtypes in human placenta. By Northern blot analysis a single mRNA species that hybridized with a hexokinase-I cDNA was found to be present in human placenta. Furthermore, in vitro translation of placenta mRNA in a rabbit reticulocyte lysate followed by hexokinase immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography showed that only one hexokinase with apparent molecular mass of about 112 kDa is expressed in this tissue and suggests a post-translational modification as a probable cause of hexokinase I microheterogeneity. To further investigate this point we have purified the high and low M₄ hexokinase and determined their NH₂-terminal sequences. The results obtained show that when compared with the amino acid sequence deduced from a CDNA the high M₄ hexokinase starts at amino acid 11 while the low M₄ hexokinase starts at amino acid 103. Since the first 10 amino acids are involved in the binding of hexokinase to mitochondrial porin these data provide an explanation both for the inability of these hexokinases to bind to mitochondria and for their differences in M₄.

Hexokinase (EC 2.7.1.1), one of the rate limiting enzymes of glycolysis, is present in mammalian tissues as four distinct isoenzymes (type I, II, III, and IV or glucokinase), and each of them is probably the product of a separate gene (1–3). Several reports, however, indicate that the situation is much more complex and that multiple molecular forms of hexokinase type I are present in rat brain (4, 5), pig heart (6, 7), human spleen (8), heart (9), placenta (10), and erythrocytes from many species (11). In many cases this enzyme heterogeneity was found to be due to charge differences among hexokinase subtypes (4–8), but in human heart and placenta two subtypes of hexokinase I that differ in molecular weight have been described (9, 10). This microheterogeneity could be due either to a differential RNA transcription and/or processing or to a post-translational proteolytic modification of a native enzyme.

The work described herein shows that the low and high M₄ hexokinases differ by 92 amino acids at their NH₂-terminal sequences while a single mRNA species and a single translation product were found suggesting a post-translational proteolytic modification for the origin of the low and high M₄ hexokinases type I in human placenta.

EXPERIMENTAL PROCEDURES

Tissue Collection—The placentas for this study were obtained from normal term deliveries. They were collected in a physiological saline solution at 4 °C, washed to remove blood and cleaned of membranous material, and immediately utilized. In the immunoaffinity isolation of hexokinase and in the extraction of RNA, placentas were obtained from caesarean sections and immediately collected in liquid nitrogen.

Isolation of Hexokinase by Immunoaffinity Chromatography—Human placenta hexokinase type I (both the high and low M₄ subtypes) was purified as in Ref. 10. Antisera against the homogeneous enzyme were raised in rabbits. The first injection was with incomplete Freund’s adjuvant followed by two further injections of enzyme at 10-day intervals. Each injection consisted of 80–100 µg of protein. IgG were prepared from preimmune and immune sera by chromatography on immobilized Protein A, and elution by sodium citrate 0.1 M, pH 3.5, 50 µg of anti-hexokinase IgG were found to be able to inactivate 30 million units of hexokinase type I activity and not hexokinase type II. No hexokinase inactivation occurred with IgG from preimmune serum.

IgG from preimmune and immune serum were dialyzed overnight against 0.05 M Hepes¹ buffer, pH 8.0, and coupled to N-hydroxysuccinimide ester of cross-linked agarose (Affi-Gel 10 from Bio-Rad). 40 mg of IgG in 10 ml of 0.05 M Hepes, pH 8.0, were combined with 4.5 ml of Affi-Gel and the gel slurry mixed at 4 °C for 4 h. The Affi-Gel 10 was then washed according to the directions of the manufacturer and packed in 1 × 5-cm columns in the same Hepes buffer. The preimmune and immune IgG coupled were about 4–5 mg/ml of gel. Human placenta (3 g) was homogenized with 9 ml of 0.25 M sucrose, 10 mM glucose, 5 mM sodium potassium phosphate buffer, pH 7.55, containing 3 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 15 µg/ml leupeptin, 5 µg/ml pepstatin (homogenizing buffer). The homogenate was centrifuged at 165,000×g for 1 h. The supernatant was filtered through a 0.45-µm membrane and loaded onto the preimmune IgG-conjugate Affi-Gel 10 column equilibrated in an equilibration buffer that is similar to the homogenizing buffer except it does not contain sucrose. The non-adsorbed proteins were then loaded onto the anti-hexokinase IgG-conjugated Affi-Gel 10 column equilibrated in an equilibration buffer that is similar to the homogenizing buffer except it does not contain sucrose. The non-adsorbed proteins were washed out with 2 ml of the washing buffer and 10 ml of 10 mM Tris-HCl, pH 7.5, 1.5 M NaCl, 0.05% (v/v) Tween 20 and then 20 ml of washing buffer. Hexokinase was eluted with 20 ml of 0.1 M glycine-HCl, pH 3.0, and immediately neutralized by the addition of 1 M solution NaOH. The proteins in the eluate were dialyzed against H₂O, lyophilized, and electrophoresed in SDS-polyacrylamide gels according to Laemmli (12).

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¹The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PVDY, polyvinylidene difluoride.
RNA Isolation and Northern Blotting—Total RNA from human placenta was isolated by the acid guanidinium thiocyanate/phenol/chloroform method of Chomczynski and Sacchi (13). Poly(A)+ RNA was purified by affinity chromatography on oligo(dT)-cellulose as in Ref. 14. Electrophoresis in formaldehyde gels, transfer to filters, hybridization, and autoradiography were as in Ref. 14, with very few modifications. The probe used was a pBS plasmid (Stratagene) containing 1,360 base pairs of the coding sequence of human hexokinase 1 starting from nucleotide 940 of the cDNA sequence (HK 16) kindly provided by A. Daniele and L. Silengo of the University of Torino. This probe was labeled with a multiprime labeling system from Amersham Corp. to a specific activity of 3 \times 10^7 cpm/\mu g.

In Vitro Translation of Human Placenta Hexokinase mRNA—In vitro translation of human placenta hexokinase mRNA was carried out in a rabbit reticulocyte lysate, nuclease treated, from Amersham, according to the instructions of the supplier. [35S]methionine was used as a labeled amino acid at a final concentration of 7 μCi/μl translation mixture while oligo(dt)-purified RNA was used at an approximately 0.2 μg/μl. A positive control was made by using tobacco mosaic virus RNA at 0.06 μg/μl. The effectiveness of translation was determined by [35S]methionine incorporation at 15-min intervals over 1 h incubation at 30 °C. At 60 min the ratio of stimulate to endogenous protein synthesis was 25:1. Before precipitating the radiolabeled proteins with 25% (w/v) trichloroacetic acid the [35S]methionyl tRNA complexes were destroyed by treatment with 1 M NaOH containing 5% (v/v) H2O2.

The protein translation products were then analyzed by immuno blotting as described in Ref. 14. To reduce background caused by nonspecific adsorption of irrelevant cellular proteins to staphylococcal protein A or to immunoglobulins, the cell lysate was pretreated with 50 volumes of preimmune IgG with a concentration of 2 mg/ml. After 1 h of incubation at 0 °C, Protein A-agarose was added (0.5 ml of packed beads for each mg of immunoglobulins) and the mixture left overnight at 0 °C under gentle rotation. The supernatant obtained by centrifugation at 12,000 × g for 5 min was treated with 22 μg of monospecific rabbit anti-hexokinase IgG obtained by further affinity purification of the anti-hexokinase IgG described above onto nitrocellulose filter immobilized human hexokinase. Briefly, 100 μg of homogeneous hexokinase type I were loaded in a SDS-polyacrylamide gel, transferred to nitrocellulose filters (16), and while one lane was stained for proteins, the others were blocked by a blocking solution (3% w/v albumin in 20 mM Tris, 500 mM NaCl, pH 7.5). The hexokinase protein bands were identified by comparison with the protein-stained lane, cut using a scalpel blade, and used to affinity purify monospecific anti-hexokinase antibodies. After 5 h at room temperature, the gel was fixed as above and washed, the nitrocellulose filter extensively rinsed in 20 mM Tris, 500 mM NaCl, 0.05% (v/v) Tween 20, pH 7.5, and then in the same buffer without Tween, and finally eluted by sodium citrate 0.1 M, pH 3.5 (20 min) and immediately neutralized with NaOH. These monospecific IgG were diluted to a final volume of 0.5 ml with NET-gel buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 0.25% gelatin, and 0.02% azide).

Rotation was continued for 4 h at 4 °C and Protein A-agarose added as above. The ternary Protein A-antibody-antigen complexes were collected by centrifugation at 12,000 x g for 20 s at 4 °C and then washed three times in NET-gel buffer. The pellet was resuspended in 30 μl of Laemmli sample buffer (12), denatured by boiling for 3 min, and loaded in a SDS-polyacrylamide gel (10% acrylamide). 2 μl of sample were counted in a scintillation counter and the total dpm obtained after the immunoprecipitation procedure ranged between 50,000–80,000 in three different experiments. The gel was then fixed for 30 min in 7% acetic acid and 20 min in ethanol (Amersham Corp.), dried, and exposed to x-ray film at −80 °C. Fluorographs were usually obtained after 30–40 days exposure.

Protein Sequencing—The high and low molecular weight hexokinase I from human placenta were purified as in Ref. 10. Isolation of each subtype was by SDS-polyacrylamide gel electrophoresis, electroblotting onto polyvinylidene difluoride membrane (PVDF) according to Matsudaira (15). The PVDF membrane was washed in double-distilled H2O for 5 min, stained with 0.1% (w/v) Coomassie Blue R-250 in 50% methanol for 5 min, and then destained in 50% methanol, 10% acetic acid for 10–15 min at room temperature. The membrane was washed in destained H2O for 5–10 min, air dried, and the protein bands corresponding to the high and low M, hexokinases excised. 100 pmol of each hexokinase subtype immobilized on PVDF membrane were sequenced on an Applied Biosystems model 477A sequenator equipped with on-line phenylthiohydantoin analysis at the Protein Nucleic Acid Shared Facility of the Department of Biochemistry at the Medical College of Wisconsin (Milwaukee) by Dr. L. Mende-Mueller.

RESULTS

Human Placenta Hexokinase I Is Present in Two Subtypes—We have previously purified human placenta hexokinase type I to homogeneity and found that it is present in two subtypes of molecular mass 112 and 103 kDa, respectively (10). To exclude that these subtypes arise by artifact(s) occurring during the protein purification, we have previously attempted to detect hexokinase in Western blots utilizing the crude placenta homogenate. Unfortunately, by this approach we did not detect any protein band, probably because hexokinase in the homogenate is less than 0.01% of total proteins. As an alternative approach we have developed an immunoaffinity chromatography procedure utilizing rabbit anti-human hexokinase I IgG immobilized on cross-linked agarose. This procedure allows us to isolate in a single step the total hexokinase type I (as determined by enzyme assay) and, as shown in Fig. 1, provides evidence for the presence of both hexokinase subtypes at high and low M,. Some IgG leakage from the column is evident in the electrophoresis experiment. It is worth noting that in order to reduce the risk of proteolytic artifacts this result was obtained by collecting the placenta immediately in liquid nitrogen and by homogenization of the tissue in the presence of many antiproteolytic compounds as specified under "Experimental Procedures." The result shown in Fig. 1 was reproducibly obtained in five different samples using two different columns. The use of a first column with preimmune IgG is essential since several placenta proteins were found to be recognized by rabbit IgG (not shown). Similar results were also obtained by incubation of the placenta homogenate with anti-hexokinase IgG followed by pre-

Fig. 1. Immunoaffinity chromatography of human placenta hexokinase type I. Human placenta hexokinase was purified by immunoaffinity chromatography on immobilized anti-human hexokinase I IgG as described under "Experimental Procedures" (A). The column was loaded with a human placenta homogenate previously chromatographed on immobilized preimmune IgG, then washed, and the bound hexokinase eluted by 0.1 M glycine-HCl, pH 3.0, neutralized, and electrophoresed in SDS-polyacrylamide gel according to Laemmli (12). For comparison, human hexokinase type I was also purified from the same placenta by conventional techniques as described in Ref. 10. B, in addition to the high (112 kDa) and low (103 kDa) molecular mass hexokinases in A, some IgG leakage from the column is evident.
cipitation of the immunocomplexes with Protein A-agarose (not shown).

**Human Placenta Hexokinase mRNA**—To determine the possible existence of multiple mRNAs for hexokinase type I in human placenta, a Northern blot hybridization analysis was performed using poly(A)+ RNA and a 1,360-base pair probe containing the coding sequence of human hexokinase I starting from nucleotide 940 (16). As shown in Fig. 2, a single mRNA of approximately 3.6 kilobases was detected in good agreement with the value expected from human kidney hexokinase I cDNA (16).

**In Vitro Translation of Human Hexokinase I**—In vitro translation of human placenta hexokinase mRNA was carried out in a rabbit reticulocyte lysate, nuclease-treated. Since we expect the hexokinase mRNA to be one minor component we have tried to optimize the immunoprecipitation of synthesized hexokinase to be as specific as possible. In this respect we have used preimmune IgG to reduce nonspecific adsorption. Furthermore, the rabbit anti-human hexokinase I IgG isolated by Protein-A chromatography were further purified on nitrocellulose immobilized homogeneous human placenta hexokinase I. Notwithstanding all these precautions several protein bands are evident in the fluorographs of SDS-polyacrylamide gel of the translation immunoprecipitates (Fig. 3). However, the 112-kDa protein band was constantly seen in three translation experiments only when these were conducted in the presence of mRNA. Several of the other bands also occurred in the absence of mRNA and changed in intensity from experiment to experiment, and some were still present in immunoprecipitation experiments performed at very short incubation time. In other words, the 112-kDa component was concluded to be hexokinase on the basis of its electrophoretic mobility and by comparison of gel fluorographs of minus mRNA tracks. Many attempts to improve the results shown in Fig. 3 were also done by changing the mRNA concentration and magnesium, potassium ion concentration without success. It is worth noting that the 103-kDa compound of the low Mr, hexokinase I was never seen in these fluorographs.

**Amino-terminal Sequences of High and Low Mr, Hexokinases**—Homogeneous human placenta hexokinase I purified as in Ref. 10 was submitted to SDS-polyacrylamide gel electrophoresis to separate the high and low Mr, components, followed by electroblotting onto PVDF membranes (15). The high and low Mr, hexokinases were then excised and separately collected. 100 pmol of each subtype were directly sequenced in an Applied Biosystem sequenator. The results obtained show that each component has a free terminal amino group and that when each sequence is aligned with the deduced cDNA amino acid sequence of human hexokinase type I (17) the placenta high Mr, hexokinase I starts from amino acid 103 while the low Mr, hexokinase I starts from amino acid 10 (Fig. 4). This difference that corresponds to a polypeptide of about 10 kDa is in good agreement with the difference in molecular mass determined by SDS-gel electrophoresis (9 kDa). In some experiments the high Mr, hexokinase was found to start with Thr instead of Phe suggesting a further possible microheterogeneity.

**DISCUSSION**

The presence of protein isoforms is a fundamental characteristic of many cells. The molecular mechanism responsible for this protein diversity includes gene selection among multigene families, differential RNA transcription and/or processing, and post-translational protein modifications. Hexokinase in mammalian tissues is present as four distinct isoen-
zymes and recent reports have shown that the study of hexokinase isoenzymes expression involves several of the above-mentioned processes. Each isoenzyme is the product of a separate gene (1–3), a different RNA splicing and the use of different promoters (18, 19) are responsible for the differences among the β cell and hepatic glucokinases (hexokinase type IV), and the results reported in this paper suggest that a post-translational modification is responsible for the hexokinase type I heterogeneity in human placenta.

In fact, while only a single mRNA was found to be present and a single in vitro translation product was obtained, the determination of amino acid NH₂-terminal sequences provided evidence for the existence of two proteins that differ by 92 amino acids. This difference is likely to be due to a proteolytic cleavage of the native enzyme. Compared with the predicted amino acid sequence obtained from the cDNA of the human kidney hexokinase type I, the high Mₙ, placenta hexokinase does not contain the first 10 amino acids at its NH₂-terminal. This NH₂-terminal has been shown to be important for the binding of hexokinase to the external mitochondrial membrane and found to be present in the rat brain (20, 21), and mouse hepatoma hexokinase I (22), but not in rat liver glucokinase (hexokinase type IV) (23) known to be a soluble cytoplasmic enzyme. These considerations provide an explanation for the results we have previously obtained on the inability of both the high and low Mₙ, placenta hexokinases to bind to mitochondria. This binding confers new kinetic properties to the enzyme (a reduced sensitivity to product inhibition, and an increase in the apparent affinity for Mg-ATP; see Ref. 24) and it is also important in the turnover of the enzyme protein (25).

In this respect, the presence in human placenta of hexokinase I subtypes that, lacking their hydrophobic NH₂-terminal sequence, do not bind to mitochondria, can be important for the regulation of glucose metabolism in this tissue (26). The presence of human hexokinase I subtypes has also been reported in human heart (9), but not in human erythrocytes (27), suggesting that the same isoenzyme can possess different kinetic and regulatory properties in different tissues depending on the tissue-specific post-translational modifications occurring to the native protein.

Since a hexokinase I isoenzyme with a propensity for binding to mitochondria in mouse hepatoma cells has been described (22) and found to contain the hydrophobic NH₂-terminal sequence it will be interesting to further investigate the mechanism(s) regulating the post-translational modifications of hexokinase in different tissues and particularly in normal and tumor cells.

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