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Isoenzyme pattern and partial characterization of hexosaminidases in the membrane and cytosol of human erythrocytes

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Abstract

Objectives: Hexosaminidase activity is present in lysosomes, plasma membrane and cytosol of many human cells. Plasma membrane and cytosolic hexosaminidase is not well characterized, particularly as regards their isoenzyme forms and their relationship with the lysosomal ones.

Design and methods: Erythrocyte hexosaminidase isoforms were chromatographically separated, characterized and compared to those in the plasma of healthy individuals and in the erythrocytes of a Tay–Sachs patient.

Results: Hexosaminidase isoenzymes were found in plasma membrane and cytosol and were composed of the same α- and β-subunits as the lysosomal and plasma hexosaminidase A and B isoenzymes, though with some structural and kinetic differences. In addition, the cytosol contained a hexosaminidase that is a specific *N*-acetyl-β-D-glucosaminidase, the one involved in the removal of *N*-acetylglucosamine residues *O*-linked to proteins, named *O*-GlcNAcase.

Conclusions: This work provides an additional step in the characterization of hexosaminidases helping better understand their role in non-lysosomal compartments and their involvement in physiological or pathological situations.
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Keywords: Hexosaminidases; Erythrocytes; Plasma membrane; Cytosol; Tay–Sachs disease; *N*-Acetyl-β-D-glucosaminidase

Introduction

Hexosaminidase (Hex), one of the most widely studied glycohydrolases, is a ubiquitously distributed enzyme found in the lysosomes, plasma membrane and cytosol of many mammalian cells and tissues, as well as in blood plasma and other body fluids [1–6].

The lysosomal form of the enzyme, which is responsible for the degradation of complex glycoderivatives – both *N*-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) derivatives – has been extensively studied for its involvement in different lysosomal inborn errors of metabolism such as Tay–Sachs and Sandhoff diseases [7,8], and in specific acquired pathologies [9–11]. The protein structure, isoenzymatic pattern, kinetic characteristics and gene coding of the lysosomal Hex have all been defined [12,13]. The enzyme exists as two major isoforms, an acidic “A” form (Hex-A) and a basic “B” form (Hex-B), and several minor isoenzymes, including the “S” form (the only form present in Sandhoff disease) and the so-called “intermediate forms” I_1 and I_2. All these forms are derived from different combinations of two polypeptide chains, α- and β-subunits, from two different genes, *HEXA* and *HEXB*, with additional heterogeneity conferred by post-translational modifications [12].

In the traffic from the site of biosynthesis to the lysosomes, part of lysosomal Hex is released into the extracellular compartment [12]. In blood plasma Hex-A, Hex-B and the intermediate forms are present as differently sialylated isoforms, with a more acidic isoelectric point than the corresponding lysosomally located isoenzymes, and are sensitive to sialidase treatment [12]. These isoenzymes increase markedly during pregnancy where a new form appears, named “P” and related to Hex-B [12,14]. The plasma I_2 isoform is related to Hex-B and the lysosomal one to Hex-A [15].

In contrast, there are fewer studies on the membrane-bound and cytosolic Hex. With regard to the former, the post-translational

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modifications that enable it to be anchored to the membrane are still not known and its physiological role is unclear. Plasma membrane Hex could be involved in glycosyl epitope changes of glycoproteins and glycosphingolipids implicated in specific cell–cell recognition events during development or neoplastic transformation [16]. Red blood cell membranes from diabetic patients have alterations to their physicochemical, dynamic, conformational and functional properties and their glycohydrolase pattern [10].

Erythrocyte membrane-bound Hex and other erythrocyte glycohydrolases have been proposed as tools to study healthy and pathological senescence [17,18]. For the cytosolic Hex, two forms, "D" and "C", have been characterized [19–22]. The "C" form from human brain [20], which is now called *O*-GlcNAcase on account of its specificity of action on GlcNAc residues *O*-linked to proteins, has a different polypeptide composition from the lysosomal Hex and is coded by a separate gene [20,23]. Interest in this enzyme was aroused by the observation that a class of cytosolic glycoproteins carries single residues of *O*-linked GlcNAc, and undergoes functional modifications after insertion or detachment of GlcNAc [24]. The possible involvement of GlcNAcase in certain pathological conditions such as Alzheimer's disease, diabetes mellitus and breast cancer was suggested [25–27].

Characterization of erythrocyte Hex will enable us to establish their role in physiological and pathological situations such as diabetes and aging. Our specific objectives were: a) to study the isoenzymatic pattern of plasma membrane and cytosolic Hex; b) to define the properties and kinetic characteristics of the different isoenzymes; c) to investigate possible structural and functional relationships between the different erythrocyte forms of Hex and the lysosomal forms. We also compared the behaviour of erythrocyte Hex in a case of Tay–Sachs disease, with mutation of the *HEXA* gene, and deficient Hex-A activity.

Materials and methods

Chemicals and other products

The purest available commercial chemicals were used. Water was freshly redistilled in a glass apparatus. 4-Methylumbelliferone (4-MU), purchased from Fluka GmbH (Bucks, Switzerland) was recrystallized three times from ethanol; 4-MU-2-acetamido-2-deoxy-β-D-glucopyranoside (4MU-GlcNAc), 4-MU-2-acetamido-2-deoxy-β-D-galactopiranoside (4MU-GalNAc) and 4-MU-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside (4MU-GlcNAc-6*S*) were purchased from Melford (Suffolk, UK); bovine serum albumin (BSA), Triton X-100, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, saponin, β-NADH, haemoglobin, *N*-acetylgalactosamine and cytochrome *c* were from Sigma (St. Louis, MO, USA). Polybuffer exchanger PBE-94 and polybuffer 74–HCl were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), *Clostridium perfringens* sialidase from Boehringer GmbH (Mannheim, Germany) and CM cellulose from Whatman Ltd (Maidstone, UK).

Subjects

Blood (5–6 mL) was taken from 20 adult volunteer blood donors, males and females, aged 20–50 years, from the blood bank of the National Cancer Institute of Milan, Italy, when they attended for scheduled blood chemistry tests. All the specimens were carefully checked to make sure there were no antibodies indicative of infectious diseases such as AIDS or hepatitis.

We also studied one patient with infantile GM2–gangliosidosis, Tay–Sachs variant, due to *HEXA* mutations, admitted to the Department of Paediatrics, Center for Metabolic Diseases, University of Padua, Italy. He presented psychomotor retardation and an eye lesion consisting of a dark red spot surrounded by a very pale ring. The biochemical diagnosis was done on leukocytes and no Hex-A activity was detected.

All the individuals involved or their parents/relatives were informed about the purpose of the investigation and gave consent.

Specimen preparation

Erythrocytes and plasma were prepared from heparinised venous blood [2]. In brief, after collection, the blood sample was immediately centrifuged for 15 min at 3000×*g* and plasma withdrawn and stored with glycol ethylene. The buffy coat aspirated from the surface of the pellet was discarded and the residual material was diluted (1:1, v/v) with phosphate buffer solution (PBS) at pH 7.4 and filtered with Leucostop 4LT-B filters (Baxter, Mirandola, Italy). All platelet and leukocyte contaminants were completely removed, as previously described [2] and their absence was verified by microscopic examination. The filtered material, containing only erythrocytes, was centrifuged for 5 min at 1200×*g* and the pellet was washed twice (1200×*g* per 5 min) with PBS at pH 7.4.

Unsealed erythrocyte membranes (ghosts), right-side-out vesicles (ROV) and inside-out vesicles (IOV) were prepared according to the method of Steck et al. [28]; haemoglobin-depleted cytosol (CYT) was obtained using the method of Perrella et al. [29]. The specimens were assayed immediately. Hex loosely bound to erythrocyte membranes was released by treatment with 1 M NaCl followed by centrifugation, according to Goi et al. [2].

Hexosaminidase assay

Hex activity was determined fluorimetrically using 4MU-GlcNAc and 4MU-GalNAc as substrates. In order to determine the activity only toward GlcNAc derivatives the assay was done with 4MU-GlcNAc as substrate in the presence of GalNAc (50 mmol/L) [19]. Under these conditions any residual activity after addition of GalNAc indicates the presence of a Hex specifically acting on GlcNAc-derivatives, that is an *N*-acetyl-β-D-glucosaminidase activity. Hex activity involving the α-subunit was assessed using 4MU-GlcNAc-6*S* as substrate [30].

Hex assays in ghosts, CYT, ROV, IOV and the effluents from chromatofocusing or anion exchange columns were run according to Goi et al. [2]. The incubation mixture for determining

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