Lysosomal α-mannosidases of mouse tissues: characteristics of the isoenzymes, and cloning and expression of a full-length cDNA

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Lysosomal α-D-mannosidase from mouse tissues was separated into its constituent isoenzymes by DEAE-cellulose chromatography. Forms corresponding to the human isoenzymes B and A were present in testis, brain, spleen and kidney, whereas in epididymis and liver only the B form was present. Murine α-mannosidases A and B are glycoproteins and have pH optima, thermal stabilities and molecular masses similar to those of the human isoenzymes. A full-length cDNA (3.1 kb) containing the complete coding sequence for α-mannosidase was isolated from a mouse macrophage cDNA library. Comparison of the deduced amino acid sequences of human and mouse α-mannosidases showed that they had 75% identity and 83% similarity. Expression of this cDNA in COS cells showed that both the A and the B isoenzymes can arise from a single transcript. Northern blotting analysis showed a 10-fold range in the abundance of α-mannosidase mRNA in mouse tissues, with the highest levels found in epididymis, and the lowest in liver.

INTRODUCTION

Lysosomal enzymes such as α-mannosidase (E.C. 3.2.1.24) are regarded as having 'housekeeping' functions because they are widely distributed in nature and are expressed in all mammalian cell types and tissues. However, levels of these enzymes are not uniform throughout the various tissues of the body and there are especially high activities in, for example, the reproductive tract of mature males [1,2]. In certain pathological conditions there may be altered activities of lysosomal enzymes so that leukaemic cells of the macrophage/monocyte lineage, for example, have characteristically high activities of α-mannosidase [3].

The most intensively studied lysosomal α-mannosidases are those of human tissues, which can be separated on the basis of charge into an acidic form B and a basic form A [4,5]. That both forms are the product of a single gene (MANB) is demonstrated in the lysosomal storage disease mannosidosis in which both A and B are lacking [6]. However, the mature forms extracted from tissues differ in their polypeptide composition, indicating that there are alternative patterns of proteolytic processing. It is possible that the primary differences between A and B reside in different glycoforms, but an alternative possibility is that they have differences in primary structure arising from differential splicing of the RNA transcript. The MANB gene has been mapped proximal to the centromere on the long arm of chromosome 19 [7].

A cDNA encoding the human mannosidase has been isolated and sequenced [8]. Its identity has been confirmed by analysis of the N-terminal sequence of a 30 kDa subunit [3] and by expression in mammalian cells [9]. Although some of the questions concerning the nature, functions and expression of lysosomal α-mannosidases have been answered by studying the human enzymes and their genes, the mouse offers greater prospects for understanding the biochemistry of these enzymes. There are a number of reasons for this. A greater range of fresh tissues from the same animal of known strain can be obtained, and comparison made between in-bred strains. Moreover, there is also the prospect of knocking out the gene by homologous recombination technology, thus raising the prospect of creating a mouse model of mannosidosis.

Little was previously known of the isoenzymes of lysosomal α-mannosidases of mouse tissues, and so we describe here the types of isoenzymes found in a variety of tissues. Through cloning the full-length cDNA we have been able to show that both the A and the B isoenzymes can arise from a single transcript when expressed in COS cells. Judging by Northern blotting, the abundance of α-mannosidase mRNA, when compared with the abundance of glyceraldehyde-3-phosphate dehydrogenase, varies in different mouse tissues. Using this cDNA we have mapped, by an interspecific backcross, the α-mannosidase gene on mouse chromosome 8 [10].

EXPERIMENTAL

Materials

4-Methylumbelliferyl-α-D-mannopyranoside (4MUαMan), Mops, α-D-mannopyranoside, EDTA and neuraminidase from *Clostridium perfringens* type IV were from Sigma (St. Louis, MO, U.S.A.). Concanavalin-A–Sepharose, Sephacryl S-300 and molecular-mass standards were from Pharmacia (Uppsala,Sweden). DEAE-cellulose DE-52 was from Whatman (Maidstone, Kent, U.K.). A mouse macrophage cDNA library constructed in λgt11 was purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). We are grateful to Dr. Nebes for providing the cDNA clone for the human mannosidase.

[α-^{32}P]dCTP (3000 Ci/mol), [α-^{35}S]dATP (1000 Ci/mmol), Hybond C membranes, the multiprime DNA labelling system,

Abbreviations used: 4MUαMan, 4-methylumbelliferyl-α-D-mannopyranoside; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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The nucleotide sequence data reported has been deposited in the DDBJ/EMBL/GenBank Nucleotide Sequence Databases under the accession number U29947.

Sequenase sequencing kit, restriction endonucleases, DNA ligase and alkaline phosphatase were from Amersham International (Amersham, Bucks, U.K.). All other reagents were from BDH (Poole, Dorset, U.K.).

Assay of α-mannosidase

Enzyme activity was determined by using the fluorogenic substrate 4MUαMan. For assay, 50 μl of the sample was mixed with 100 μl of a 2 mM solution of substrate in 0.2 M sodium acetate buffer, pH 4.25. Then, after a period of incubation lasting between 30 min and 2 h, the reaction was stopped by adding 1.85 ml of 0.2 M glycine/NaOH buffer, pH 10.4. The fluorescence of the liberated 4-methylumbelliferone was measured on a Perkin–Elmer LS3 fluorimeter (excitation 360 nm, emission 446 nm).

Enzyme preparation

Extracts (10 %, w/v) were prepared by homogenizing tissues in 10 mM sodium phosphate buffer, pH 6.0, containing 0.1 M NaCl, followed by centrifugation at 10000 ***g*** for 30 min at 4 °C in a Sorvall RC-5B centrifuge. Enzyme activity was determined in the supernatant.

Ion-exchange chromatography

Samples of supernatants for ion-exchange chromatography were dialysed against 10 mM sodium phosphate buffer, pH 6.8, and loaded on to columns of DEAE-cellulose (3 ml) equilibrated with the same buffer. Unretained protein was eluted with the column buffer and then a linear gradient of NaCl (0–0.5 M) was applied. After the gradient, the column was eluted with 1.0 M NaCl in the same buffer. The protein concentration in eluates was monitored at 280 nm.

Concanavalin A–Sepharose chromatography

Concanavalin A–Sepharose affinity chromatography was performed using a column (4 cm length × 0.5 cm diameter) equilibrated with 20 mM Tris/HCl buffer, pH 7.4, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. Following DEAE-cellulose chromatography, pooled fractions were dialysed against the above buffer and then applied to the affinity column. A linear gradient containing α-methyl-D-mannoside (0–0.5 M) and 1.0 M NaCl in the starting buffer was used to elute the bound glycoproteins.

Enzyme characterization

pH optima were determined in 0.2 M sodium acetate and 0.1 M/0.2 M citric acid/sodium phosphate buffers in the pH range 3.0–7.5. Thermal stability of isoenzymes was determined by incubating samples (50 μl) in 0.2 M sodium acetate buffer at various temperatures ranging from 40 to 70 °C for 60 min. Samples were then cooled on ice for 2 h and assayed for enzyme activity at 37 °C. Molecular masses of the isoenzymes were determined by gel-filtration chromatography on Sephacryl S-300. Samples of each isoenzyme were loaded on to a column (60 cm length × 1.5 cm diameter) equilibrated with 20 mM sodium phosphate buffer, pH 6.8, containing 0.1 M NaCl.

Treatment with neuraminidase

Samples of α-mannosidase B, from DEAE-cellulose chromatography, were incubated at 37 °C, in citric acid/sodium phosphate buffer, pH 6.0, for periods ranging from 4 to 12 h with 4 units of neuraminidase. After incubation, the reactions were cooled rapidly on ice, dialysed and loaded on to a DEAE-cellulose column.

Isolation, sequencing and expression of the cDNA encoding mouse lysosomal α-mannosidase

A total of 2×10^6 clones were screened using a 1.1 kb *Pst*I fragment of human α-mannosidase cDNA as a probe. Plaques giving positive hybridization signals were recovered and purified by repeated plating and screening. cDNA inserts from λgt11 were excised by digestion with *Eco*RI and subcloned into the pBluescript vector. Sequencing of the inserts in the plasmids was performed by using the dideoxy method [11], with either vector-specific oligonucleotides or oligonucleotides specific for the mouse mannosidase. Both strands of the cDNA were sequenced. COS-1 monkey kidney cells were maintained in α-minimal essential medium containing streptomycin (100 μg/ml) and penicillin (100 units/ml), and 10 % (v/v) fetal-bovine serum at 37 °C in CO_2/air (19:1). The 3.1 kb fragment containing the complete coding sequence of the mannosidase was subcloned into a mammalian expression vector PMT2 that was kindly provided by Dr. P. G. Pelicci (Policlinico Monteluce, Perugia, Italy). This construct (20 μg) was transfected into monkey COS-1 cells by using the calcium phosphate method [12] and after 72 h the cells were harvested and extracts assayed for α-mannosidase with the fluorogenic substrate at pH 4.25 as described above. α-Mannosidase isoenzymes in cell lysates of transfected and control cells were separated by DEAE-cellulose chromatography as described above.

Total RNA isolation and Northern blotting analysis

Total RNA isolation was performed on ten 3-month-old male Balb/c mice. The animals were killed and the tissues (liver, spleen, kidney, brain, testis and epididymis) quickly removed, frozen in liquid N_2 and stored at −80 °C.

Total RNA isolation and Northern blotting were performed as previously described [13]. Five separate pools of RNA were prepared for each tissue. The mouse α-mannosidase cDNA was used as a probe. The cDNA probe was labelled with [α-^{32}P]deoxycytidine 5′-triphosphate by random priming to specific activities of 5×10^8–1×10^9 d.p.m./μg. After hybridization with the mannosidase cDNA, the membranes were stripped [12] and reprobed with rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA kindly provided by Professor R. Donato (Dipartimento di Medicina Sperimentale e Scienze Biochimiche, University of Perugia). Hybridization signals were quantified using an InstantImager Electronic Autoradiography System (Packard Instruments, Meriden, CT, U.S.A.) and standardized to account for differences in RNA loading using the GAPDH signal. Readings within the linear range of the instrument are presented as means ± S.D.

RESULTS AND DISCUSSION

Isoenzymes of α-mannosidase in mouse tissues

Mammalian cells contain α-mannosidases of two types, both of which hydrolyse 4MUαMan [14]. The lysosomal enzyme has a pH optimum of 4.25, whereas those involved in processing N-linked oligosaccharides have a more neutral pH optimum. pH activity curves (results not shown) for α-mannosidase from different mouse tissues showed in each case a peak or shoulder in the curve corresponding to the pH optimum of the lysosomal

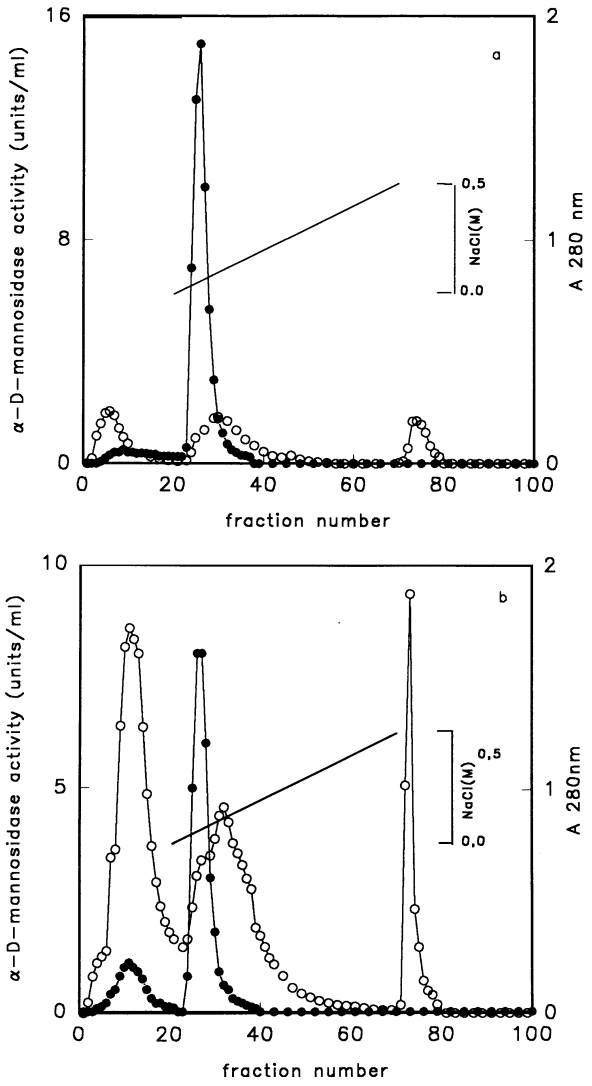

Figure 1 Separation of α-mannosidase from (a) epididymis and (b) testis by DEAE-cellulose chromatography

Fractions were assayed for activity towards 4MUαMan (●), and protein concentration was monitored at 280 nm (○). One unit of activity is the amount of enzyme that hydrolyses 1 nmol of substrate/min at 37 °C. For details see the Experimental section.

enzyme. The least interference from the neutral forms was found in epididymis and kidney, and a greater contribution to the activity, measured at pH 4.25, from the neutral forms was found in brain and liver. It was not possible therefore to obtain reliable estimates of the abundance of lysosomal α-mannosidase in all mouse tissues by a simple assay at pH 4.25. Elution profiles of α-mannosidase from mouse tissues on DEAE-cellulose revealed different, but reproducible, patterns of peaks when assayed with 4MUαMan (Figure 1). The separation of the total activity into a basic fraction (A) and a more acidic component (B) is similar to that seen with the human α-mannosidases A and B under identical conditions [14]. The profiles from mouse spleen and liver (results not shown) were similar to those of the testis (Figure 1b) and epididymis (Figure 1a) respectively. The profiles of mouse spleen and kidney (results not shown) were similar to that of testis. The profiles from testis, brain and spleen resembled each other in that a small proportion of activity was not retained by the column and the greater part of the activity was eluted in the salt gradient. This was reversed in the elution profile from kidney. α-Mannosidase A was almost completely lacking in extracts of epididymis and liver. We considered the possibility that the appearance of α-mannosidase in unretained fractions might have resulted from overloading of the column. Closely comparable results from each tissue were obtained over a range of total protein loaded, so we conclude that, as with its human counterpart, α-mannosidase A is an isoenzyme form distinct from form B. Both isoenzymes were retained by a column of concanavalin A–Sepharose and all the activity was recovered as a single peak after elution with α-methylmannoside. Thus both isoenzymes in the mouse tissues are glycoproteins. The B form was greatly diminished after neuraminidase treatment and a corresponding increase of an A-type enzyme was observed (results not shown). Thus the characteristic behaviour of B on DEAE-cellulose might be due to the presence of sialic acid residues. These results show that the mouse α-mannosidases A and B behave similarly to the human enzymes, which are thought to differ mainly in sialic acid content, and possibly to a small extent in conformation [15]. The molecular masses of mouse α-mannosidases A and B, determined by gel-filtration chromatography, were 290 ± 10 kDa and 270 ± 10 kDa respectively. Both isoenzymes showed an acidic pH optimum of 4.25 and were thermostable at 60 °C for 1 h. We conclude, therefore, on the basis of their behaviour on DEAE-cellulose and their biochemical properties, that mouse tissues have α-mannosidase isoenzymes similar to the B and A forms observed in human tissues [14].

Isolation and characterization of a cDNA encoding mouse α-mannosidase

In order to study the expression of mouse α-mannosidase at molecular level and to confirm that both isoenzymes can be encoded by the same gene, we have isolated and sequenced a cDNA encoding mouse lysosomal α-mannosidase. Six positive plaques were identified after screening a mouse cDNA library using the human cDNA as probe. One of these (3.1 kb) was a full-length cDNA, containing the complete coding sequence, an open reading frame of 3036 bp, and short 5′- and 3′-untranslated sequences of 7 bp and 77 bp respectively. There is a single polyadenylation signal (ATTAAA) 14 bp upstream from the poly(A)$^+$ tail (DDBJ/EMBL/GenBank Nucleotide Sequence Databases, accession number U29947). This polyadenylation occurs in only approx. 10% of mRNAs [16] and it is notable that the same polyadenylation signal is conserved in the human α-mannosidase cDNA sequence.

Alignment of the mouse and human cDNA sequences using the University of Wisconsin Genetic Computer Group GAP alignment program showed 75% similarity. It should be noted that the human α-mannosidase sequence we have used for comparison is the most recent version (DDBJ/EMBL/GenBank Release 96.0, accession number U05572) and not the original sequence reported in the paper by Nebes and Schmidt [8]. Comparison of the deduced amino acid sequences of the mouse and human α-mannosidase showed that they had 75% identity and 83% similarity (Figure 2). The human protein is two amino acids shorter than the mouse. There is N-terminal divergence between human and mouse sequences. These regions contain the signal sequences that are lost during processing and therefore have no functional significance in the mature protein. There are eleven potential glycosylation sites in the mouse sequence, the same number as in the human sequence but only six (N309, N366, N496, N645, N692 and N766) of these are conserved

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Figure 2 Alignment of the deduced amino acid sequence of mouse α-mannosidase with the sequence of human α-mannosidase

The upper sequence is mouse and the lower human.

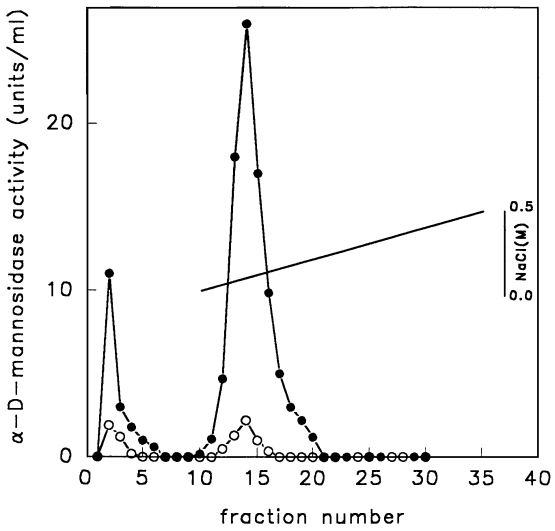

Figure 3 Separation of α-mannosidases expressed by transfected COS cells

Lysates of COS cells transfected with mouse α-mannosidase cDNA cloned into the expression vector PMT2 (●) and lysates from control cells (○) were analysed by chromatography on columns of DEAE-cellulose as described in the Experimental section. Fractions were assayed for activity towards 4MUαMan. One unit is the amount of enzyme that hydrolyses 1 nmol of substrate/h at 37 °C.

between the two species. The greater variability observed in the mannosidase isoenzyme profiles of mouse tissues, than in those of man, might be due to the utilization of different glycosylation sites. Rat endoplasmic reticulum, yeast vacuolar, human and mouse lysosomal and the mouse Golgi complex α-mannosidases all have a region of sequence similarity that might include all or part of the catalytic domain [17]. The mouse lysosomal α-mannosidase is also highly conserved in this region and extends between H (69) and H (199) (Figure 2). The high similarity of the amino acid sequences and the immunological cross-reactivity observed among α-mannosidases from different mammalian species [18] suggest a strong structural similarity between mouse and human α-mannosidases. In the mouse and human sequences there are two possible initiating ATG codons. Analysis of the human sequence preceding these ATGs showed that both match with the translation initiation consensus sequence described by Kozak [19]. In the mouse it is only the sequence preceding the first ATG that corresponds closely to the consensus sequence. Thus it is likely that translation is initiated from the first ATG in human and mouse lysosomal α-mannosidases mRNAs.

Expression of mouse α-mannosidase cDNA in COS cells

The full-length cDNA was subcloned into a mammalian expression vector and expressed in COS cells. Lysates from transfected cells showed a 20-fold increase in α-mannosidase specific activity over control cells, whereas there was no significant change in the specific activity of another lysosomal enzyme, hexosaminidase, in the transfected cells. Expression of the cDNA clone in this way confirms that it codes for an α-mannosidase with an acidic pH optimum. When lysates of COS cells expressing the α-mannosidase cDNA were analysed by DEAE-cellulose chromatography (Figure 3) two peaks of activity were separated: one not retained by the column and the other eluted in the salt

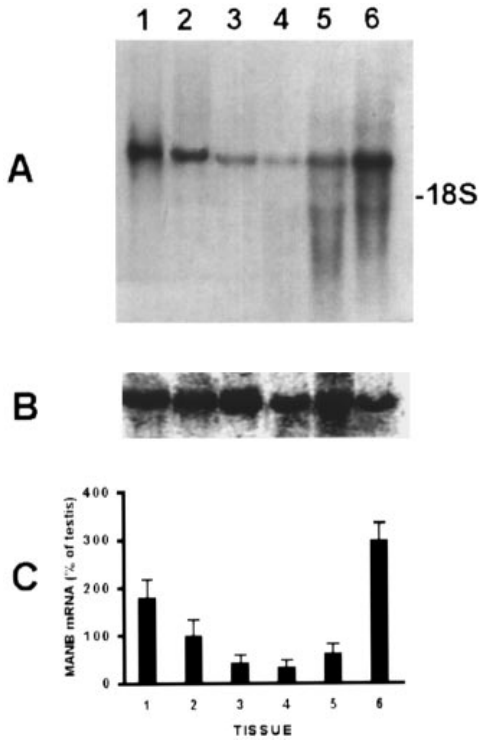

Figure 4 Analysis of α-mannosidase mRNA by Northern blotting

Total RNA (20 μg) was denatured, run on a 1% (w/v) agarose gel containing 2.2 M formaldehyde and blotted on to nitrocellulose by capillary transfer. RNA was extracted from: lane 1, spleen; lane 2, testis; lane 3, brain; lane 4, liver; lane 5, kidney; lane 6, epididymis. (**A**) Membrane probed with the α-mannosidase cDNA. Location of 18S (rRNA) is indicated. (**B**) The filter was stripped and rehybridized with a GAPDH cDNA probe. A quantitative representation of the tissue distribution of α-mannosidase mRNA is shown in (**C**). RNA was isolated from five separate pools of tissue. Radioactivity in the 3.0 kb α-mannosidase mRNA band was recorded for each tissue on a InstantImager Electronic Autoradiography System and standardized with radioactivity in the GAPDH mRNA recorded in the same way. Bars depict means ± S.D of five separate Northern blotting experiments and were expressed relative to the signal of testis set at 100%.

gradient. The activity of both peaks was appreciably higher than those of control cells (Figure 3). This result shows that both α-mannosidase isoenzymes can be expressed as products of a single mouse gene, as they can in man, and it should therefore be feasible to create a mouse model of mannosidosis by targeting the α-mannosidase gene on chromosome 8 by homologous recombination technology. There is no known treatment for mannosidosis, and the creation of a mouse model is necessary to study the pathophysiology of the disease as well as to develop therapeutic strategies.

Expression of α-mannosidase mRNA in mouse tissues

Northern blotting analysis demonstrated the presence of 3.0 kb α-mannosidase transcripts in all tissues that were examined (Figure 4). Tissues with greatest α-mannosidase mRNA abundance were epididymis, spleen and testis. Relative to GAPDH mRNA, mouse α-mannosidase mRNAs showed a 10-fold range of abundance from epididymis, the tissue with the highest level, to liver with the lowest. In addition to the major transcript, smaller minor transcripts were detected in kidney and epididymis. The nature of these transcripts, smaller than the size of the coding region of any known lysosomal α-mannosidase [20], is presently unknown. A transcript of 3.6 kb is expressed in human spleen, thymus and leukocytes [20], but a transcript of this size was not detected in the mouse tissues we tested.

It is notable that the highest mRNA levels and activity of α-mannosidase are in the epididymis, an organ that has high activities of many glycosidases. In a previous paper we have shown that the specific activity of β-*N*-acetylhexosaminidase, as well as the expression of the β-subunit of this enzyme, were higher in epididymis than in other mouse tissues [13]. It has been known for a long time that in rodents the specific activity of these epididymal glycosidases increases during sexual maturation, and decreases after surgical removal of the testis [1]. This effect can be partially reversed by treatment with testosterone, a fact suggesting that the expression of epididymal α-mannosidase and β-*N*-acetylhexosaminidase are under hormonal regulation.

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