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HUMAN LYSOSOMAL ALPHA-MANNOSIDASE: ISOLATION AND NUCLEOTIDE SEQUENCE OF THE FULL-LENGTH cDNA[1]

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The amino acid sequence of the human lysosomal alpha-mannosidase precursor has been deduced by PCR mediated cloning and sequencing of the cDNA. The protein has 961 amino acids and a molecular weight of 107,644 Daltons. The amino acid sequence shows 38% identity to the Dictyostelium discoideum lysosomal alpha-mannosidase. The cDNA maps proximal to the centromere on chromosome 19q, the same locus as the MANB gene. Our results provide valuable information for the study of the lysosomal storage disease alpha-mannosidosis, an inherited disorder caused by mutations in the MANB gene which encodes the human lysosomal alpha-mannosidase.

Alpha-mannosidase (EC 3.2.1.24) is a lysosomal enzyme that is necessary for the catabolism of N-linked carbohydrates released during glycoprotein turnover. The soluble enzyme has been purified from various human sources and is a tetramer composed of two identical N-glycosylated 60 kDa subunits and two identical 27 kDa subunits with a pH optimum of 4.5 (1-3). A 110 kDa precursor is proteolytically processed to form the two subunits (4). The gene encoding this enzyme, MANB, has been mapped proximal to the centromere on the long arm of chromosome 19 by isoenzyme analysis of lysosomal alpha-mannosidase activity in human/rodent somatic cell hybrids (5).

Inherited lysosomal alpha-mannosidase deficiency results in the human lysosomal storage disease alpha-mannosidosis. Children with the severe form of this autosomal recessive disorder become mentally retarded, develop deafness, bone and joint deformities, muscle

[1]The nucleotide sequence reported in this paper has been submitted to the GenBank Database with accession number U05572.

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weakness, and die before the age of two (reviewed in refs. 6 and 7). There is no known treatment for mannosidosis. For gene therapy to become a potential form of treatment the MANB gene, which encodes lysosomal alpha-mannosidase, must be cloned and characterized. In this paper we present evidence that the cDNA we have cloned and sequenced encodes human lysosomal alpha-mannosidase.

Methods

Polymerase chain reaction (PCR)

PCR amplification of a 393 bp fragment of the MANB gene was accomplished using as a template 1 µgm of CsCl purified DNA from a HeLa cell cDNA library kindly provided by the laboratory of Dr. Leonard Guarante, MIT (8). The sequence of the 1,024 fold degenerate 5' primer used was 5'CCG GGA TCC C/AAT/C T/AG/CI CAT/C A/GT/AI GAT/C IC/TI GC/GI TGG 3'. The sequence of the 128 fold degenerate 3' primer was 5' CCG GGA TCC G/ATA/G ICC A/GAA IGT/G A/GTC IG/AG/T III CCA 3'. Nucleotide/ nucleotide indicates that both nucleotides were incorporated at a given position (eg. C/A means that both a C and an A were incorporated at position 10 in the 5' primer). BamHI sites were added to the 5' ends of both primers to facilitate subcloning of the PCR products. Deoxyinosine was incorporated to reduce primer redundancy at those positions where any nucleotide might be expected. Following a 5 min denaturation at 95°C samples were heated at each of the following temperatures for 30 secs: 95°C, 40 °C, and 72°C for forty cycles. The major PCR product estimated to be about 400 bp in size was subcloned into the BamHI site of the pBluescript SK- vector (Stratagene) resulting in the plasmid pHAM4. Both strands of the insert were sequenced using universal and reverse primers and [alpha-^{32}P]dATP in the Sequenase dideoxy sequencing protocol (United States Biochemicals) for double stranded templates.

Library screening

The PCR fragment described above was used to screen 150,000 plaques from a human retina cDNA library using standard techniques (9). Phage were plated at a density of 50,000 plaques/ 15 cm plate and transferred to Nytran filters (Schleicher and Schull). The pHAM4 insert was labelled with alpha [^{32}P]dATP by random priming and hybridized to the filters my the method of Amasino (10). The single positive clone identified was purified to homogeneity and the pBluescript (Stratagene) phagemid insert of the Lambda Zap based clone was excised in vivo. The 2.15 kb of cDNA in the phagemid, pHAM20, was sequenced in both directions using a series of oligonucleotide primers corresponding to previously sequenced regions of the insert. The pHAM20 insert was used to rescreen 500,000 lambda plaques from the retina cDNA library. Four additional positive clones were obtained and phagemids were excised in vivo. One of the new positive clones extended the 3' end of the cDNA by 220 bp and was used to screen 500,000 plaques from a human muscle cDNA library (Strategene). A positive clone with a 1.8 kb insert, pHAM18, was obtained and sequencing revealed that it overlapped the 3' end of the 2.15 kb insert by 950 bp and extended the 3' end of the cDNA by 765 bp. To form the full length cDNA, plasmid pHAM31, a BssHII/EcoRV fragment from pHAM18 was ligated into the pHAM20 plasmid cut with BssHII and SmaI .

Mapping

DNA from the NIGMS human/rodent somatic cell hybrid mapping panel #1 was screened for lysosomal alpha-mannosidase specific sequences by PCR (11,12). We tested various primer pairs by PCR to find a set that would amplify a region of human genomic DNA that did not contain an intron (ie. cDNA and genomic DNA would be the same size) and that would allow us to distinguish the human DNA from hamster or mouse DNA found in the hybrids. Using a primer pair that met these requirements, we amplified a 153 bp fragment from our cDNA and human genomic DNA and screened DNA from the collection of 18 human/rodent somatic cell hybrids. Human chromosomes found in fewer than 5% of cells were considered to be below the limit of detection. PCR reaction buffers are identical to those described above except for the $MgCl_2$ concentration which was increased to 3mM, the DNA templates (100 ngms of DNA from each cell line was used per reaction), and the PCR primers which were 5'TTC CTG TGT GGC ATT TGT CTG CTG GTG3' and 5'ATCAAG AAT GAC ATC CAG CAC GCC GGT GTG 3' (100 pmol per reaction). Following a 5'

denaturation step at 95°C, samples were heated for 30 seconds at each of the following temperatures: 95°C, 55°C, and 72°C for 40 cycles. PCR reaction products were analyzed on 5% Tris/Borate/EDTA polyacrylamide gels. DNA from the chromosome 19q somatic cell hybrid panel (ref. 5 provided by Dr. Michael J. Siciliano, University of Texas) was screened as described above.

Results and Discussion

The initial step in our cloning strategy was to amplify a small fragment of cDNA by PCR using degenerate primers. The primers were designed using a region of amino acid sequence similarity deduced from four cloned alpha-mannosidase genes. The amino acid sequences used to design the primers are indicated in Fig.1. and listed in Methods. The major PCR product of 393 bp was amplified from a HeLa cell cDNA library (8), subcloned, and sequenced. The PCR fragment encoded an open reading frame that was similar in sequence to cloned alpha-mannosidases. Sequence alignments are shown in Fig 1.

The region of sequence similarity between the rat endoplasmic reticulum and the yeast vacuolar alpha-mannosidases has been noted in the literature and it was suggested that this region might include all or part of the catalytic domains of the enzymes (17). The finding that the human and slime mold lysosomal alpha-mannosidase and the mouse Golgi complex alpha-mannosidase sequences are all very similar in this region supports this idea. A carboxyl group is thought to be important for the catalytic activity of alpha-mannosidases and three invariant aspartic acid residues are found within this highly conserved domain. Additionally, a transition state analog that inhibits a broad spectrum of alpha-mannosdidases has recently been synthesized adding support to the idea that alpha-mannosidases have similar active sites.

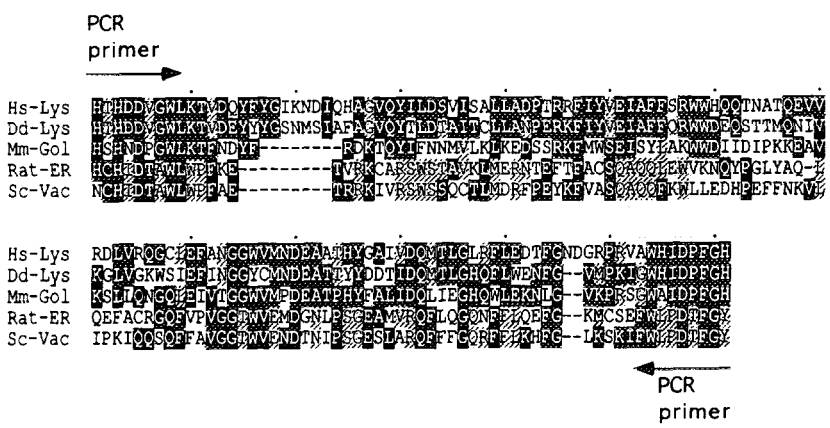

Fig. 1. Alignment of alpha-mannosidase sequences.
The initial MANB cDNA fragment was amplified from a cDNA library by PCR using degenerate primers. Fragments of the four alpha- mannosidase amino acid sequences used to design the PCR primers are shown aligned with the translated amplified sequence (Hs-Lys: Homo sapiens, lysosomal). Arrows indicate the regions encoded by the degenerate primers. The primer sequences are shown in Methods. The deduced amino acid sequences used were from alpha-mannosidases found in the Dictyostelium discoideum lysosome (Dd-Lys, Ref. 15), the mouse Golgi complex (Mm-Gol, Ref. 16), the rat endoplasmic reticulum (Rat-ER, Ref. 17), and the yeast, Saccharomyces cerevisiae vacuole (Sc-Vac, Ref. 18). Amino acids aligned in three or more sequences are highlighted on black backgrounds. Amino acids aligned in two sequences are shown on hatched backgrounds.

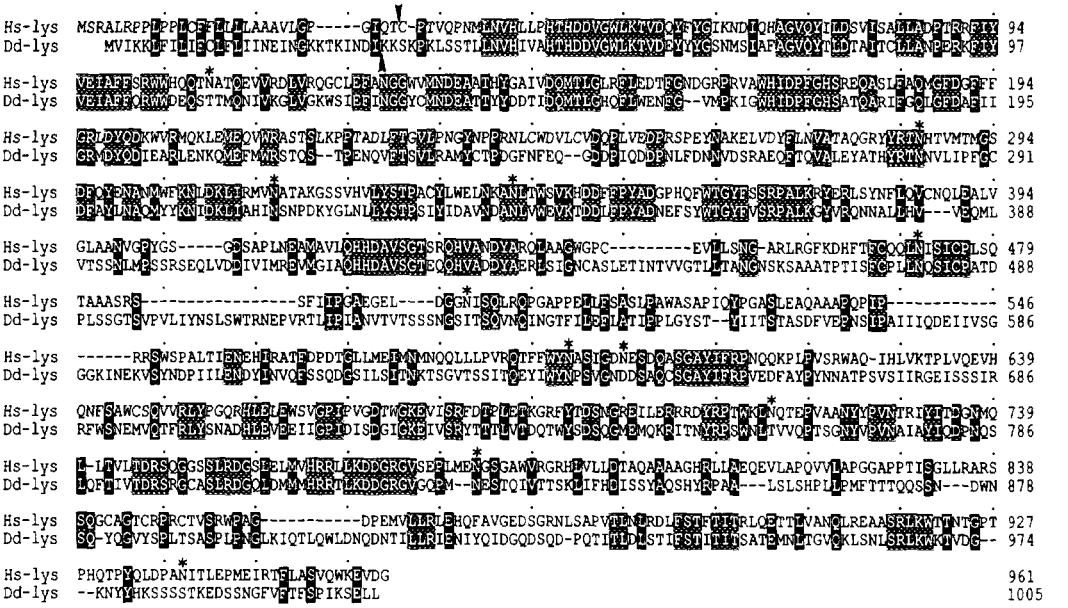

Fig.2. Comparison of the human and Dictostellium discoideum lysosomal alpha-mannosidase proteins.

The deduced amino acid sequences (single letter code) of the human (Hs-lys) and slime mold Dd-lys) lysosomal alpha-mannosidase proteins were aligned using the Maligned computer program. Identical amino acids are highlighted on black backgrounds. Amino acids are numbered on the right. The nucleotide sequence has been submitted to the GenBank Data Bank with accession number U05572. Potential signal sequence cleavage sites are indicated by arrowheads and potential N-linked glycosylation sites by asterisks.

The 393 bp PCR fragment was used as a probe to screen a human retinal cDNA library. Positives from the retinal cDNA library were used to screen a human muscle cDNA library and a full length cDNA was constructed from two of the positive clones as described in Methods.

The cDNA encodes an open reading frame of 961 amino acids with a predicted molecular weight of 107,644 Da (See Fig.2). The first 30 amino acids have characteristics typical of a signal sequence--a charged N-terminus, a central hydrophobic core, and a pattern of amino acids that could form a signal sequence cleavage site (13). Other than this putative signal sequence, there are no hydrophobic regions that are long enough to span a membrane, in keeping with the soluble nature of the lysosomal alpha-mannosidase enzyme. The 60 kDa subunit of the human lysosomal alpha-mannosidase is known to be N-glycosylated. Eleven potential N-linked glycosylation sites are found in the sequence at amino acid positions 109, 285, 316, 342, 471, 501, 594, 600, 715, 783, and 939. The 3' noncoding sequence has a consensus polyadenylation signal 14 bp upstream from the start of a 23 nucleotide poly(A) sequence. When expressed in E. coli a 110 kDa protein is induced from the cDNA (data not shown). The alpha-mannosidase precursor is estimated to be 110 kDa by polyacrylamide gel electrophoresis (4). Taken together the above data indicate that the cDNA could express the full-length alpha-mannosidase precursor.

Searches of GenBank showed that the deduced human lysosomal alpha-mannosidase amino acid sequence was most similar over the entire sequence to the Dictyostelium discoideum lysosomal alpha-mannosidase sequence. An alignment of the two sequences, which are 38% identical is shown in Fig 2. To date no other mammalian lysosomal alpha-mannosidase genes have been cloned. Most of the gaps in the alignment that are greater than 5 amino acids occur between amino acids 440 and 550. This region aligns with a domain that is proteolytically cleaved from the slime mold lysosomal alpha-mannosidase precursor protein. Pro-regions, for example in the subtilisin family of serine proteases, are quite varied in sequence and in length although the regions that become the active enzyme are quite similar in sequence (14 and refs. therein).

The cDNA was mapped to chromosome 19 by analysis of two somatic cell hybrid mapping panels. DNA from the NIGMS human/rodent somatic cell hybrid mapping panel #1 was screened for lysosomal alpha-mannosidase specific sequences by PCR (11,12). Our results are shown in Figure 3. Our analysis maps the putative MANB cDNA to chromosome

Concordance Data

Cell line	Response	Human Chromosome 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
pHAM20 (cDNA)	+																								
NAIMR91 (human)	+	100																							
NA0037A (mouse)	-	0																							
NAI0908 (CHO)	-	0																							
NA09925	+	74	24	0	74	76	60	82	78	0	0	4	68	6	86	78	14	98	96	46	84	0	76	0	0
NA09926	+	69	75	75	65	2	88	85	69	0	68	0	2	77	73	93	2	81	75	84	96	2	4	2	0
NA09927	+	69	83	75	77	0	93	79	73	0	82	0	0	77	79	90	0	81	73	87	89	0	0	0	0
NA09928	+	0	84	58	0	48	32	0	66	0	2	0	0	4	76	92	0	98	0	28	0	70	82	0	78
NA09929	-	0	0	61	59	0	43	2	49	0	0	33	49	0	59	2	0	96	0	2	31	0	0	2	0
NA09930A	-	0	34	62	4	12	0	26	4	0	0	6	22	56	82	12	0	86	78	0	22	82	76	6	8
NA09931	-	0	0	0	0	26	0	78	0	0	46	0	64	0	100	0	0	100	0	0	78	90	0	0	14
NA09932	-	0	0	0	68	86	46	0	80	0	2	28	26	0	0	0	0	96	0	2	0	92	0	0	0
NA09933	+	50	0	84	16	54	76	92	54	0	6	0	50	84	78	92	0	88	70	80	32	94	88	0	32
NA09934	-	0	50	0	0	83	79	4	87	0	0	77	87	0	2	89	0	90	89	0	91	89	2	0	0
NA09935A	-	0	0	52	10	28	12	0	0	0	8	0	22	74	72	0	0	93	59	0	9	91	71	0	0
NA09936	-	0	0	0	18	0	46	70	10	0	16	34	0	2	88	2	0	100	0	44	24	0	18	0	0
NA09937	-	0	0	54	38	0	62	54	70	0	4	0	42	0	70	60	0	96	66	0	0	0	0	0	0
NA09938	-	0	0	2	88	60	88	86	4	0	0	36	92	0	80	4	0	92	0	4	80	76	60	0	2
NA09940	-	0	0	46	0	0	0	84	62	0	0	0	0	0	0	62	0	100	0	0	0	0	0	0	0
NA10324	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	90	0
NA10567	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	98	0	0	0	0	0	0	0	0
NA10611	-	0	0	0	0	0	0	0	0	69	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
# discordant (+/- or -/+)		1	3	6	7	8	7	7	6	6	5	11	11	3	6	4	5	10	5	1	8	9	6	7	5
# concordant (+/+ or -/-)		17	15	12	11	10	11	11	12	12	13	7	7	15	12	14	13	8	13	17	10	9	12	11	13
% Concordance		94	80	50	36	20	36	36	50	50	62	0	0	80	50	71	62	0	62	94	20	0	50	36	62

Fig. 3. PCR mapping data.
PCR screening of the NIGMS human/rodent somatic cell hybrid mapping panel DNAs was used to detect the presence of the MANB gene in the cell lines. The mapping panel cell lines are indicated in the first column. The presence or absence of PCR products is indicated in the second column along with a photograph of the PCR products run on a polyacrylamide gel and stained with ethidium bromide. The percentages of somatic cell hybrids retaining a particular human chromosome is shown in the remaining columns. If fewer than 5% of cells retained a given chromosome, those hybrids were considered to be below the limit of detection and treated as lacking that chromosome. The number of mismatches of the MANB gene and the presence of a particular chromosome is shown in the discordant row. The number of matches of the MANB to a particular chromosome is shown in the concordant row. The data are tabulated and the percent concordance is calculated as previously described (19).

19 or chromosome 1 with a concordance of 94%. To resolve this ambiguity we obtained a chromosome 19 hybrid mapping panel (5) that divides the q arm of chromosome 19 into 22 ordered subregions and analyzed it by PCR using the same methods as described above. A 153 bp PCR fragment was amplified from the cDNA and from the two hybrids that are positive for MANB by isoenzyme analysis (data not shown). The MANB gene has been mapped proximal to the centromere on 19q using this panel of somatic cell hybrids. These data are consistent with the cDNA mapping to the MANB locus.

This paper presents no direct evidence that the cDNA described here encodes the human lysosomal alpha-mannosidase. To provide direct evidence, N-terminal amino acid sequence from the purified alpha-mannosidase protein subunits would need to be obtained and shown to match regions of the deduced amino acid sequence from the cDNA. Alternatively, mutations that cause alpha-mannosidosis could be shown to map to this sequence. However, the indirect evidence presented here is consistent with the cDNA encoding the human lysosomal alpha-mannosidase. The deduced amino acid sequence is similar to other alpha-mannosidases and most closely resembles the only known lysosomal alpha-mannosiadase sequence. The hydrophobicity of the deduced amino acid sequence suggests that the cDNA encodes a soluble protein with a signal sequence which are properties of the human lysosomal enzyme. The cDNA when expressed in bacteria makes a 110 kDa protein, the size expected for the precursor. Most compelling is the map location of the cDNA which corresponds to the MANB locus. The data presented here could make the mapping of alpha-mannosidosis mutations possible and provide a necessary reagent for eventual gene therapy for this disease.

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