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Missense and Nonsense Mutations in the Lysosomal α-Mannosidase Gene (MANB) in Severe and Mild Forms of α-Mannosidosis

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Summary

α-Mannosidosis is an autosomal recessive lysosomal-storage disorder caused by a deficiency of lysosomal α-mannosidase activity. This disease shows a wide range of clinical phenotypes, from a severe, infantile form (type I), which is fatal at <3–8 years of age, to a less severe, late-onset form (type II), which ultimately may involve hearing loss, coarse face, mental retardation, and hepatosplenomegaly. To elucidate the molecular mechanism underlying this disease in both types of patients, we have used PCR, followed by either SSCP analysis or direct sequencing, to analyze the 24 exons and intron/exon boundaries of the α-mannosidase gene (MANB) from five patients. Two amino acid substitutions—H72L and R750W, in exons 2 and 18, respectively—and two nonsense mutations—Q639X and R760X, in exons 15 and 19, respectively—were identified in four type II patients. One amino acid substitution, P356R, was identified in exon 8 from a type I patient. This patient and three of the type II patients were homozygous for their mutations (H72L, P356R, R750W, and R760X) and one type II patient was heterozygous for the Q639X and R750W mutations. Transfection experiments of COS 7 cells, using the α-mannosidase cDNA containing one of the missense mutations—H72L, P356R, or R750W—revealed that each of these mutations dramatically reduces the enzymatic activity of α-mannosidase. These data demonstrate that widely heterogeneous missense or nonsense mutations of the MANB gene are the molecular basis underlying α-mannosidosis.

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Introduction

Lysosomal α-mannosidase (E.C.3.2.1.24) is a lysosomal hydrolase that cleaves α-linked mannose residues from the nonreducing end of N-linked glycoproteins. Human lysosomal α-mannosidase has been purified from various tissues, and two polypeptides have been identified to be either ~60 and 30 kD (Cheng et al. 1986; Emiliani et al. 1995) or 65 and 27 kD (Tsuji and Suzuki 1987). Recently, a single cDNA clone of this enzyme was isolated from retina/muscle cDNA libraries (Nebes and Schmidt 1994) and from spleen, fibroblast, and lung cDNA sources (Liao et al. 1996; Nilssen et al. 1997). Transfection experiments using this cDNA gave increased lysosomal α-mannosidase enzyme activity in murine fibroblasts (Wang et al. 1996) and in *Pichia pastoris* (Liao et al. 1996), confirming that the cloned cDNAs encode human lysosomal α-mannosidase. From the amino acid sequence of the protein purified from placenta, a single-chain precursor protein of lysosomal α-mannosidase was shown to be processed into three peptides, which have molecular weights of 70, 42, and 13 or 15 kD (Nilssen et al. 1997).

Deficiency of α-mannosidase results in the autosomal recessive lysosomal-storage disorder, α-mannosidosis (MIM 248500). α-Mannosidosis shows a wide range of clinical phenotypes, from a severe infantile phenotype (type I), which shows rapid mental deterioration, hypotonia, hepatosplenomegaly, severe dysostosis multiplex, and, often, death at <3–8 years of age, to a milder phenotype (type II), which is characterized by normal early development, mental retardation during childhood, and survival well into adulthood (Thomas and Beaudet 1995).

Using the α-mannosidase cDNA clone (Nebes and Schmidt 1994) as a probe, we isolated and analyzed genomic clones of the human MANB gene (Wakamatsu et al. 1997). Riise et al. (1997) have determined the genomic structure of the MANB gene by using a PCR-based strategy. This gene spans ~22 kb and consists of 24 exons. The 5′-flanking region of the gene shows a high G+C content and has two Sp1 sites and three AP-2 sites. The availability of a genomic clone made it pos-

sible to investigate the molecular defects associated with α-mannosidosis, with hopes of determining the molecular basis underlying both types of phenotypic variants. In this effort, we prepared specific oligonucleotides to amplify all 24 exons and flanking sequences, for use in a mutation analysis of the gene in five patients. We identified several mutations from five patients with α-mannosidosis, revealing not only that the disease is caused by mutation of the MANB gene but that a heterogeneous collection of nonsense and missense mutations provide the molecular basis underlying α-mannosidosis.

Subjects and Methods

Subjects

Patient 1 (OK1).—Patient 1 is a 47-year-old Japanese female born to a consanguineous marriage in which the parents were first cousins. She has suffered from recurring infections, such as bronchitis and otitis media, since her 1st year of life. Hearing disturbance and delayed psychomotor development were noticed at age 2 years, by her parents. When she was 9 years old, she entered a school for the deaf, where she did poorly. She gradually developed a gait disturbance and was admitted to our hospital at age of 36 years. A physical examination revealed mental retardation, with an IQ of 19; coarse face; retinal degeneration; sensorineural hearing loss; increased deep-tendon reflexes; spastic gait; and mild limb ataxia. There were vacuolated lymphocytes in her peripheral blood. Similar vacuoles were also present in her biopsied muscle cells and fibroblasts. The lysosomal α-mannosidase activity of the peripheral leukocytes was markedly decreased, to <1% of that in normal control subjects, whereas other lysosomal enzyme activities—such as α-galactosidase, β-galactosidase, and β-hexosaminidase—were all within the normal range. Thin-layer chromatography of the patient's urine showed an enhanced excretion of oligosaccharides. From these clinical and laboratory findings, she was diagnosed as having α-mannosidosis (table 1). Her younger sister (OK2) was age 42 years and had clinical history and features that were similar to those of patient 1; the results of the pathological examination of muscle from this sister have been reported elsewhere (Kawai et al. 1985).

Patient 2 (cell line GM654).—The clinical characteristics of patient 2 have been described elsewhere (Autio et al. 1973; also see table 1). In brief, the patient is the only child of healthy nonconsanguineous parents. The parents did not notice anything abnormal during this child's 1st year of life, except for recurring infections. By age 17 mo, he was speaking only a few words, and impaired hearing was suspected. He was noticed to have coarse facial features, delayed psychomotor functions, and brisk tendon reflexes when examined at age 17 mo in the Children's Hospital. Approximately 80% of his peripheral blood leukocytes were vacuolized, and his α-mannosidase activity was decreased to only a few percent of that in normal control subjects. He often suffered from respiratory infections but was living well at home.

Patient 3 (cell line GM2051), patient 4 (cell line GM2817) and patient 5 (cell line GM4518).—The skin biopsies of patients 3, 4, and 5 were done at ages 7, 6, and 2 years, respectively. The clinical characteristics of these patients were not available, except for those of patient 5, who showed severe growth failure, with hypotonia, psychomotor retardation, and hepatosplenomegaly (table 1).

The cultured skin-fibroblast cell lines GM654, GM2051, GM2817, and GM4518 were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). These cell lines were maintained in α-MEM medium supplemented with 20% FCS at 37°C.

Enzyme Activity

Leukocytes and cultured fibroblasts were homogenized with 20 mM sodium phosphate buffer, pH 6.0, containing 0.5 % Triton X-100. The supernatants were assayed for α-mannosidase activity and protein concentration (Bradford 1976). Lysosomal α-mannosidase ac-

Table 1

Clinical and Biochemical Findings in Patient with α-Mannosidosis

Patient (Cell Line) [Sex; Age]	Origin	Clinical Finding(s)	Activity of α-Mannosidase[a] (% of normal)	Tissue Source
1 (OK1) [F; 47 years]	Japanese	Mental retardation, hearing loss, coarse facial features	<1	Leukocytes
2 (GM654) [M; 7 years]	Finnish	Mental retardation, hearing loss, coarse facial features	2	Fibroblasts
3 (GM2051) [F; 7 years]	Arabian	Unknown	<1	Fibroblasts
4 (GM2817) [F; 6 years]	Unknown	Unknown	2	Fibroblasts
5 (GM4518) [F; 2 years]	Unknown	Severe growth failure, hypotonia, psychomotor retardation, hepatosplenomegaly	<1	Fibroblasts

[a] For leukocytes, mean (± standard error of the mean) control activity was 2.98 (±0.29) nmol/min/mg protein ($n = 5$); for fibroblasts, mean (± standard error of the mean) control activity was 1.43 (±0.09) nmol/min/mg protein ($n = 5$).

Table 2

Primer Sequence Used for the MANB Gene

Analysis and Primer Name	Exon	Primer Sequence (5′→ 3′) Forward	Reverse	Annealing Temperature (°C)
PCR-SSCP:				
manE1S/A	1	ACCCCAGGAGGAAGCTGCTGA	TCACTCTGCCTCCTGTACGT	58
manE2S/A	2	GGGGGTTATTGACAAGGTAT	TGGGGATCCCAGGGACCAGT	56
manE3S/A	3	CTGCTGTTCCCTGAGAGCCT	CTGGGCTTCCTCTTTTCACT	60
manE4S/A	4	CTCTGACCGCTGACCCTGAC	GTGAAGAAGTGGGCCCAAGA	58
manE5S/A	5	GGTGAGAGGGCTGGGCACTAAT	GTGGCACCATGGCTGGCCCTGC	67
manE6S/A	6	GGTGATGGGCCACCCCTTGA	CATTGTATTGTATATACCGGACT	58
manE7S/A	7	GGATCCTCTGGCTTCAGGACT	CGGATGCAAGCGCACATGTGCA	57
manE8S/A	8	GCGGACTCCCGAGGGCTCACT	GGTCATGACCCACGGGGCATG	62
manE9S/A	9	TTGGCATGCCCCGTGGGTCAT	GTCGGGCCTCGGGGCTGCATG	67
manE10S/A	10	AGCCCCGAGGCCCGACAGGCT	TCCAACTTCAGCCTCAAACCT	62
manE11S/A	11	TGAGTCCCACAGAACCTCACT	CCTGTCTCCACCCCCGTGTCT	62
manE12S/A	12	CGTCCTGAACCCACCGGTCC	GCCCATCTGCCCTAGATCCA	58
manE13S/A	13	TGTGACCCATGCCCTCTCTG	GGAGTCCCAGCGGGGGAATA	58
manE14S/A	14	GGTACTCTTGACTCAGTTTC	ACAGGAGCAGGAAAGGGGAT	58
manE15S/A	15	TAACACACAACCCATCTGT	GCCCTAGCTCCATGCCGAGC	58
manE16S/A	16	CTAGGGCCCCTTACCTGACT	CATTCCCAACTGCCCACTC	62
manE17S/A	17	GCTCAGGTACAGACTGACAT	CCTCTGCCCTTGCTTCCACA	58
manE18S/A	18	AGAGGGGTTTATCCAAGGCT	CCACAGACCACCCCCTCAGT	58
manE19S/A	19	GGAGCCAGATCCCAAGCCTG	GGTGATTCCCTTTCTATCGA	58
manE20S/A	20	TGGGGTTGACTGCCCTCTAC	TGGGAGGACCCTGGCTCGGA	58
manE21S/nA	21	CAAGGACACCCACAAACCCACG	CCGGTGTCCGGCGGCTGCAG	62
manE21nS/A	21	TGGTGCTGCTGGACACAGCCCAG	AAACCCGGCTCCCTGGTAGACT	67
manE22S/A	22	CCGCCCCTCTCCAACTCAGC	TCTCTCCGATCTCCTTCTCA	58
manE23S/A	23	GCTTTGCCCCAACTCATCTG	TGCCGGCCCCAGGTAAGACT	58
manE24S/A	24	CACCTCCTCACTCCTCCTTC	ACACTCAGTCACAGAGCGAC	58
Restriction enzyme:				
manE2MuS/A	2	AACGTGCACCTGCTGCCTCACAAGC	TGGGGATCCCAGGGACCAGT	50
manE8S/A	8	GCGGACTCCCGAGGGCTCACT	GGTCATGACCCACGGGGCATG	62
manE15MuS/16A	15	AGCAACTCCTGCTGCCTGTTCTC	CATTCCCAACTGCCCACTC	62
manE18S/A	18	AGAGGGGTTTATCCAAGGCT	CCACAGACCACCCCCTCAGT	58
manE19MuS/A	19	TCACCCCCAACCCCAGGCGGGAATAT	GGTGATTCCCTTTCTATCGA	57
Mutagenesis:				
man72LS/A	1–5	GAAGCTGCTGAGCCATGGGCGCCTAC	AAGCCCATCTCGCGAAACAGCG	65
man750WS/A	16–21	CCAGTATAGGTGACAACGAAAGTGAC	TGCGCGGAGGAGCCCCGAGATTGTA	57
T3/man356RA1	1–8	AATTAACCCTCACTAAGGGG	GTAACAAGCGCGGGTGGAGTAG[a]	50
man356RS/A2	8–10	CTACTCCACCCGCGCTTGTTAC[a]	TGCACACCTGCAGGAAGTTGTA	50

[a] The mutation site is underlined.

tivity was determined by use of 2 mM 4-methylumbelliferyl (MU) α-mannopyranoside (Sigma) as substrate. The enzyme assay was terminated by the addition of 2 ml of 0.2 M glycine NaOH buffer, pH 10.6. One unit of enzyme activity was defined as the hydrolysis of 1 nmol of substrate for 1 min at 37°C. Product formation was determined by the increase in fluorescence, measured by a F3010 Fluorescence Spectrophotometer (Hitachi) with excitation wavelength at 365 nm and emission wavelength at 448 nm.

PCR Amplification, Screening by SSCP, and DNA Sequencing

Genomic DNA was isolated from peripheral blood or cultured skin fibroblasts, according to standard protocols. The primer pairs used for the amplification of each exon, exon/intron boundary, and part of the associated introns were determined according to the intron sequence near each splice junction (table 2). Fifty nanograms of genomic DNA from each patient and normal control subject was amplified by PCR in a total volume of 20 μl containing 0.2 μg of each primer, 20 μM of each dNTP, 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH8.4), 50 mM KCl, 1 unit of *Taq* DNA polymerase (Takara Shuzo), and 0.5 μl of α-[^{32}P]dCTP (5 μCi; 3,000 Ci/mmol). PCR samples were preheated at 94°C for 5 min. Thirty PCR cycles were performed; each cycle consisted of 40 s denaturation at 94°C, 40 s at optimal annealing temperature (table 2), and 1 min extension at 72°C. For SSCP analysis, PCR products were denatured by boiling,

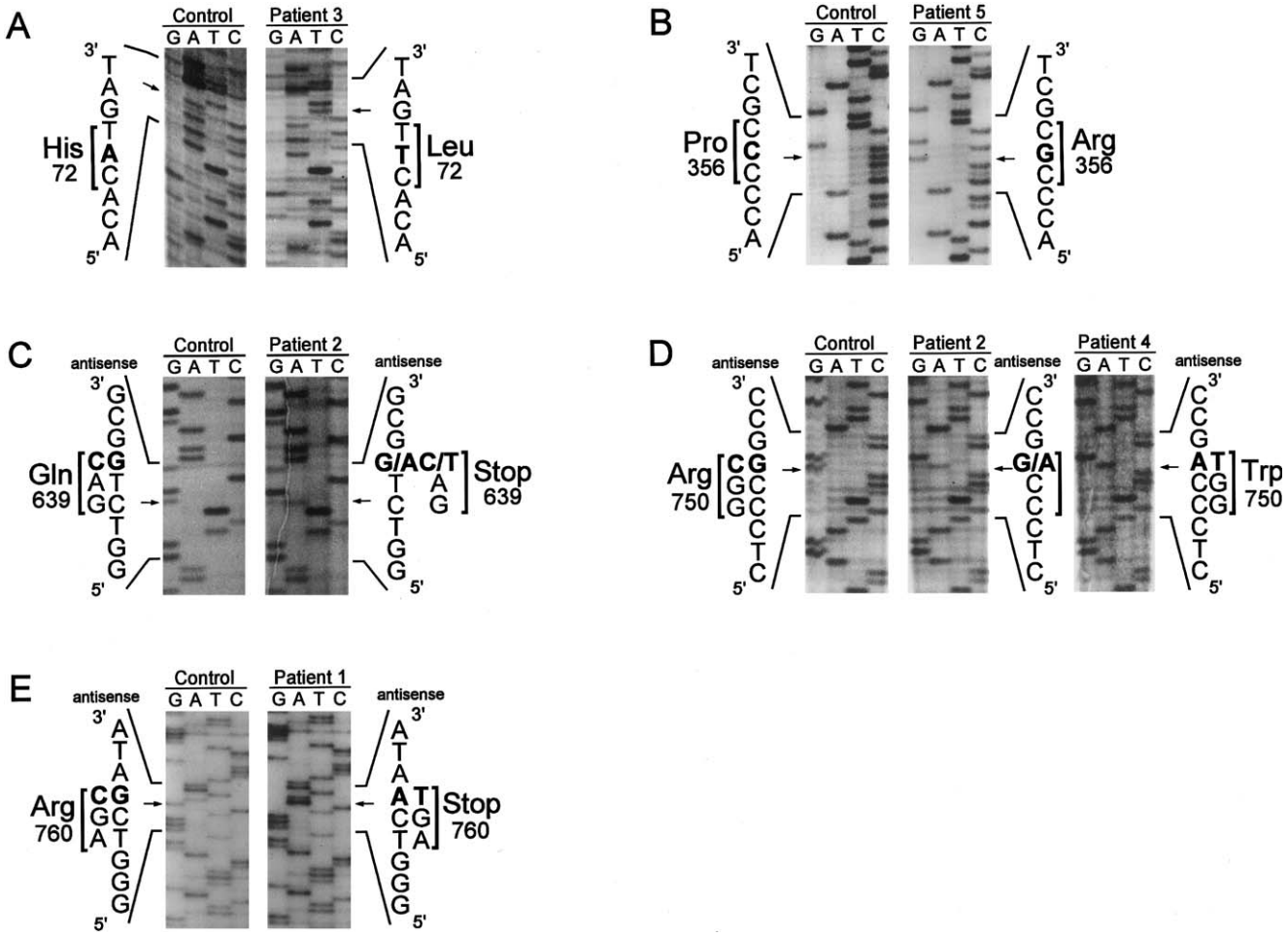

Figure 1 Partial nucleotide sequences of amplified MANB genomic DNA, confirming single-base substitutions in exons 2 (*A*), 8 (*B*), 15 (*C*), 18 (*D*), and 19 (*E*). Arrows indicate the positions of the nucleotide substitutions.

immediate cooling on ice, and separation on 6 % nondenaturing polyacrylamide gels containing 10% glycerol, 1 × Tris borate EDTA, for 12 h at room temperature. The gels were dried and exposed to X-ray film (X-OMAT; Kodak).

Identification of Mutations

On SSCP analysis, patients' samples that, compared with those from normal control subjects, exhibited either band shifts or additional bands were used for direct sequencing. Genomic DNA from patients and normal control subjects were amplified by PCR, with the same primer pairs that were used in the SSCP analysis. The resultant PCR products were isolated from 8% polyacrylamide gels, were purified, and were directly sequenced by Sequenase (UBS), with α-[^{35}S]dATP used as tracer (Winship 1989). In the case of patient 1 (OK1), the SSCP pattern failed to reveal band shifts for any of the PCR products, so direct sequencing of all exons and flanking sequences was performed.

To identify the mutations, PCR-dependent restriction-enzyme diagnostic tests were established for each case. Fifty nanograms of genomic DNA was used in each of the following PCR amplifications. Genomic DNA containing exon 2 and part of intron 2 of the MANB gene from either patient 3 or normal control subjects was amplified by specific primers (sense, manE2MuS; and antisense, manE2A; see table 2), to detect creation of a *Hin*dIII site in the mutant DNA. Genomic DNA containing intron 15, exon 16, and part of exon 15, and part of intron 16 of the MANB gene from patient 2 and form normal control subjects was amplified by specific primers (sense, manE15MuS; and antisense, manE15A; table 2) to detect creation of an *Xba*I site in the mutant DNA. Genomic DNA containing exon 19, part of intron 18, and part of intron 19 of the MANB gene from pa-

Table 3

Mutations Identified in the MANB Gene

Patient (Cell Line)	Codon	Nucleotide Change	Coding Effect	Consequence	Homozygosity Status[a]	Region
1 (OK1)	760	CGA→TGA	Nonsense	R760X	+	Exon 19
2 (GM654)	639	CAG→TAG	Nonsense	Q639X	–	Exon 15
	750	CGG→TGG	Missense	R750W	–	Exon 18
3 (GM2051)	72	CAT→CTT	Missense	H72L	+	Exon 2
4 (GM2817)	750	CGG→TGG	Missense	R750W	+	Exon 18
5 (GM4518)	356	CCC→CGC	Missense	P356R	+	Exon 8

[a] A plus sign (+) denotes presence of homozygosity, and a minus sign (−) denotes absence of homozygosity.

tient 1 and normal control subjects was amplified by specific primers (sense, manE19MuS; and antisense, manE19A; table 2) to detect creation of an *Ssp*I site in the mutant DNA. The remaining mutations, including both the shared mutation in patients 2 and 4 and the mutation in patient 5, abolish an *Msp*I site and created a *Hha*I site, in exon 18 and exon 8, respectively. These were evaluated, in patient and control samples, by PCR of genomic DNA by specific primers (table 2). Amplification was performed for 30 cycles; each cycle consisted of denaturation for 40 s at 94°C, annealing for 40 s at optimal annealing temperature (table 2), and extension for 1 min at 72°C. The PCR products were digested with *Hin*dIII, *Xba*I, *Ssp*I, *Msp*I, or *Hha*I, were electrophoresed on 8% polyacrylamide gels, and were stained with ethidium bromide.

Preparation of Expression Vectors for Normal and Mutant Human Lysosomal α-Mannosidase

For each of the constructs, first-strand cDNA was synthesized, by reverse transcription of 10 μg of total RNA from the patient's fibroblasts (cell lines GM2051 and GM2817), by 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) and 0.2 μg of specific antisense primers, for 50 min at 42°C (table 2).

1. Construction of the α-mannosidase expression vector, pCMV-Man.—The 3.1-kb *Not*I fragment of full-length human MANB cDNA in plasmid SK(+) (Nebes and Schmidt 1994) was subcloned into the *Not*I site of pCMV, an expression vector driven by the human cytomegalovirus promoter/enhancer (pCMV-Man).

2. Introduction of exon 2 mutation (H72L).—The 3.1-kb *Not*I fragment of the MANB cDNA was inserted into the *Not*I site of plasmid KS(+) (KS-Man). A part of the MANB cDNA extending from exon 1 to part of exon 5 was amplified from cell line GM2051, by PCR (table 2). The amplification was performed for 30 cycles; each cycle consisted of 1 min denaturation at 94°C, 1 min annealing at 65°C, and 2 min extension at 72°C. The PCR product was digested with *Xho*I and *Stu*I and was inserted into the *Xho*I/*Stu*I site of cleaved KS-Man (KS-Man^{72L}). Finally, the 3.1-kb *Not*I fragment of pCMV-Man was replaced by the 3.1-kb *Not*I fragment of KS-Man^{72L} (pCMV-Man^{72L}).

3. Introduction of exon 18 mutation (R750W).—A portion of the MANB cDNA extending from part of exon 16 to part of exon 21 was amplified from cell line GM2817 by PCR (table 2). Amplification was performed for 30 cycles; each cycle consisted of 1 min denaturation at 94°C, 1 min annealing at 57°C, and 2 min extension at 72°C. The PCR product was digested with *Eco*81I and was inserted into the *Eco*81I-cleaved pCMV-Man (pCMV-Man^{750W}).

4. Introduction of exon 8 mutation (P356R).—Two parts of the MANB cDNA, extending from exon 1 to part of exon 8 and from exon 8 to part of exon 10, were amplified by specific primers (T3/man356RA1 and man356RS/A2 respectively; see table 2) from plasmid KS-Man, by *Pfu* DNA polymerase (Stratagene). In each PCR incubation, one primer has the codon 356 (CCC→CGC) mutation (see table 2, where the mutation site is underlined). Amplification was performed for 20 cycles; each cycle consisted of 1 min denaturation at 94°C, 1 min annealing at 50°C, and 3 min extension at 72°C. The PCR products were electrophoresed on a 1.5 % agarose gel, were isolated, and were purified with QIAEX II (QIAGEN). The purified PCR products were mixed and amplified by *Pfu* DNA polymerase, with T3/man356RA2 primers. Amplification was performed for 20 cycles, under the same conditions. The PCR product was digested with *Nae*I and *Not*I and was inserted into the *Nae*I/*Not*I site of cleaved 5.7-kb pCMV-Man (pCMV-Man^{356R}). The structures of these expression vectors were confirmed by restriction mapping, as well as by nucleotide-sequence analysis.

Cell Culture, DNA Transfection, and α-Mannosidase Activity

COS 7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS. The cells were transfected with 10 μg of test plasmid and 0.5 μg of the pCMVβ control plasmid, which is an expression vector containing the *Escherichia coli* β-galactosidase gene. Transfection was accom-

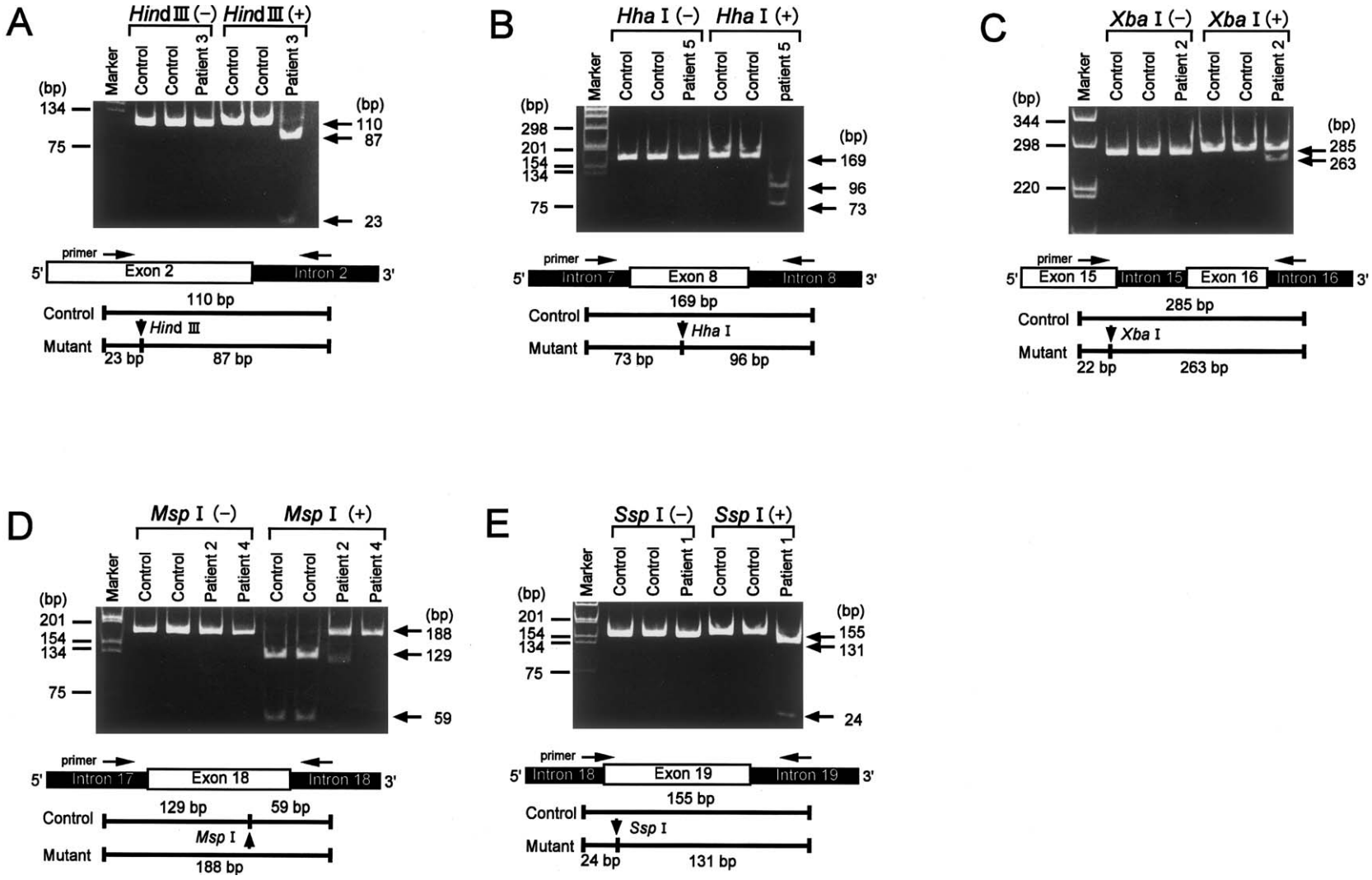

Figure 2 Confirmation of mutations, by PCR-dependent restriction-enzyme diagnosis. Genomic DNA from the patients and from two unrelated normal individuals was amplified by specific primers (table 2). The schematics below each panel show both the position of the restriction-enzyme site and the size of the fragments produced after restriction-enzyme digestion of the PCR product from normal control DNA and from the mutant DNA. At the top of each panel the migration of the restricted fragments can be seen in an 8% polyacrylamide gel stained with ethidium bromide. *A*, Patient 3. *B*, Patient 5. *C*, Patient 2. *D*, Patients 2 and 4. *E*, Patient 1.

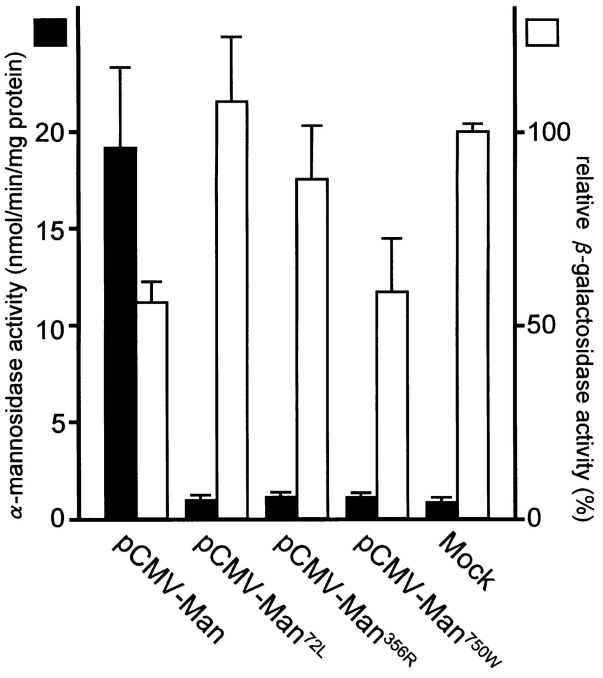

Figure 3 α-Mannosidase activities in COS 7 cells transfected with α-mannosidase cDNAs. COS 7 cells were cotransfected with 0.5 μg of pCMVβ control plasmid and 10 μg of test plasmid containing either normal mannosidase cDNA (pCMV-Man) or mutant cDNA (pCMV-Man^{72L}, pCMV-Man^{356R}, and pCMV-Man^{750W}), or they were mock transfected (i.e., were transfected without test plasmid [Mock]). α-Mannosidase activity was measured, with 4-MU α-D-mannopyranoside used as a substrate. The columns indicate the mean of α-mannosidase (*blackened cols.*) and *E. coli* β-galactosidase (*unblackened cols.*) activities, and vertical bars indicate standard error of the mean ($n = 3$).

plished by the calcium phosphate coprecipitation method (Wigler et al. 1978). Ten hours later, the medium was replaced with the fresh medium, and, after an additional 48-h culture, the COS 7 cells were harvested and homogenized with 20 mM sodium phosphate buffer, pH 6.0, containing 0.5 % Triton X-100. The supernatants were assayed for α-mannosidase activity, β-galactosidase activity (An et al. 1982), and protein concentration (Bradford 1976).

Results

Identification of Mutations in the MANB Gene

On Southern blot analysis of *Eco*RI-digested genomic DNA, with the full-length lysosomal α-mannosidase cDNA (kindly provided by V. L. Nebes) used as a probe, the DNA samples from the five patients showed patterns identical to those from control subjects, indicating that the patients had no large deletions of the gene (data not shown). We next screened for mutations in the patients' samples by using SSCP analysis, after all 24 exons and flanking intron sequences were amplified by primers specific for the MANB gene (table 2). Mobilities that were abnormal in comparison with those of control samples were identified in exon 2 from patient 3 (cell line GM2051), in exon 8 from patient 5 (cell line GM4518), in exon 15 from patient 2 (cell line GM654), and in exon 18 from both patient 2 (cell line GM654) and patient 4 (cell line GM2817) (data not shown). On the basis of direct sequence analyses of mutant PCR products from four patients' samples, three homozygous point mutations were identified in three patients: in patient 3, a His(H)-to-Leu(L) substitution at codon 72 (CAT→CTT) (fig. 1*A*); in patient 5, a Pro(P)-to-Arg(R) substitution at codon 356 (CCC→CGC) (fig. 1*B*); and, in patient 4, an Arg(R)-to-Trp(W) substitution at codon 750 (CGG→TGG) (fig. 1*D*). Moreover, two heterozygous mutations, a Gln(Q)-to-stop(X) substitution at codon 639 (CAG→TAG) (fig. 1*C*) and an Arg(R)-to-Trp(W) substitution at codon 750 (CGG→TGG) were identified in patient 2 (fig. 1*D*). No abnormal mobilities were identified, by SSCP analysis, in the PCR products from patient 1 (OK1). We therefore turned to direct sequencing of all 25 PCR products in this patient, in a search for probable mutations. Only one homozygous point mutation, an Arg(R)-to-stop(X) substitution at codon 760 (CGA→TGA), was identified (fig. 1*E*). The codon numbering used here is that of Wakamatsu et al. (1997). The differences from the sequences of the MANB gene reported by Nilssen et al. (1997) are due to an additional three nucleotides—GCC, inserted after the first ATGGGC nucleotide in the cDNA—which we also identified in genomic DNA as well as in the cDNA. The differences in the codon numbers of the mutations identified in our previous report (Gotoda et al. 1996) are due to the differences in the sequences reported by Nebes and Schmidt (1994) and to numbering from the first ATG (Wakamatsu et al. 1997). The identified mutations are summarized in table 3.

Confirmation of Mutations, by PCR-Dependent Restriction-Enzyme Diagnosis

To confirm the five mutations identified in patient leukocytes and cell lines, we established diagnostic tests specific to each mutation that were based on restriction-enzyme cleavage of PCR products by primers flanking the mutation site (table 2). The assays confirmed that patients 1 and 3–5 appeared to be homozygous for their identified mutations and that patient 2 was heterozygous for the mutations Q639X and R750W (fig. 2). The sister of patient 1 also was homozygous for the R760X mutation (data not shown). Both alleles are confirmed as R760X, since these patients' parents are first cousins. We used these same diagnostic testes to screen for each

mutation in a set of 48 normal control subjects; and none of the mutations was identified (data not shown), confirming that these mutations do not represent frequent polymorphisms.

α-Mannosidase Activity in COS 7 Cells

To establish whether the mutations H72L, P356R, and R750W affect α-mannosidase activity, we introduced each into the pCMV-Man vector, and the plasmids were transiently expressed in COS 7 cells. Expression of the pCMV-Man vector in COS 7 cells produced α-mannosidase activity that was ~20 times higher than that in mock-transfected (i.e., transfected with pCMVβ plasmid only) COS 7 cells. However, α-mannosidase activity in the mutant plasmids containing H72L, P356R, or R750W showed no increase over background levels (fig. 3).

Discussion

In the present study, we describe five mutations (H72L, P356R, Q639X, R750W, and R760X) of the human MANB gene in patients with α-mannosidosis. Given the wide spectrum of clinical heterogeneity in patients with this disease, which in part could be due to the presence of residual enzyme activity and altered kinetics of the enzyme, Desnick et al. (1976) hypothesized that this disease most likely is caused by missense mutations. To elucidate the specific molecular defects associated with the MANB gene, we examined mutations in five patients with α-mannosidosis. Patient 1 (OK1) and patient 2 (cell line GM654) have mild clinical phenotypes. Both patients showed psychomotor retardation after the 1st year of life and developed recurring infections, hearing loss, impaired speech, and coarse facial features. They survived to age >7 years (patient 2) and age >47 years (patient 1). Two others, patient 3 (cell line GM2051) and patient 4 (cell line GM2817), also most likely have a mild form of α-mannosidosis, because they have also survived to age ⩾10 years. In contrast, patient 5 (cell line GM4518) showed severe growth failure with hypotonia after birth and appeared to have a severe form of the disease. Taken together, patients 1–4 probably have type II α-mannosidosis, and patient 5 probably has type I α-mannosidosis. The four distinct mutations identified in the patients confirm a probable genetic basis for the heterogeneity in this disease (Gotoda et al. 1996), although the nonsense mutations were unexpected in the patients with type II α-mannosidosis.

Transfection studies using α-mannosidase cDNAs containing the missense mutations H72L, P356R, or R750W, which were identified in both types of patients, did not show an increase in α-mannosidase activity in COS 7 cells. This indicates that these mutations are likely to be at least three distinct genetic causes of α-mannosidosis. The R750W mutation probably affects local charge and secondary structure of the protein, with consequent loss of enzymatic activity. In contrast, the H72L mutation, which has been identified in the mild form of the disease (Nilssen et al. 1997), is a conservative change that does not affect the charge of the enzyme. This amino acid, however, is probably critical to the function of the enzyme, since it is conserved among the lysosomal α-mannosidases of man, cattle, and *Dictyostelium discoideum*, as well as among the class 2 α-mannosidases of *Drosophila* and human Golgi and rat endoplasmic reticulum (Nilssen et al. 1997). It is possible that the H-to-L substitution affects the catalytic site or substrate-recognition site on the enzyme.

The P356R mutation was identified in a severe form of the disease. The affected residue, Pro356, and surrounding amino acids are all conserved between α-mannosidase in man and that in *D. discoideum* (Schatzle et al. 1992). Use of computer programs that calculate probable secondary structure on the basis of amino acid sequences suggests that this region contains hydrophobic amino acids predicted to form a β-sheet structure (Schatzle et al. 1992). The positive charge on the arginine—and subsequent disruption of the putative bend resulting from this mutation—would dramatically change the secondary structure of the enzyme.

α-Mannosidosis is a clinically heterogeneous disorder, but enzyme-activity assays using a single concentration of substrate do not easily account for the range in clinical severity described in many patients (Noll et al. 1989). Indeed, all five patients in the present study had a similar deficiency of lysosomal α-mannosidase, at 2 mM 4-MU-α-D-mannopyranoside (table 1). Recently, Bennet et al. (1995) compared the biochemical analyses of α-mannosidase in patients with each clinical type of α-mannosidosis. Kinetic analysis showed that α-mannosidase from type I patients had an ~400-fold reduction in substrate affinity, whereas those with type I had a substrate affinity reduced by only 40-fold. Bennet et al. concluded that the markedly decreased substrate affinity of the enzyme correlated with the severity in phenotypic expression. In this context, the P356R mutation would be expected to strongly change the secondary structure of the enzyme, thereby markedly reducing its affinity for the substrate and resulting in a severe form of the disease. In contrast, the R750W mutation would more likely have less of an effect on substrate affinity, resulting in a milder form of the disease. However, the fact that both types of patients have been documented as being in the same family (Spranger et al. 1976; Mitchell et al. 1981) suggests that the phenotype may be influenced by factors other than just the primary gene defect; environmental agents or unidentified modifier genes may contribute to the heterogeneous clinical presentation. Nevertheless, a

more extensive accumulation of different mutations from patients with mild to severe forms of the disease should help in the elucidation of any genotypic/phenotypic relationships.

The homozygous nonsense mutation, R760X, was identified in exon 19 from a patient with a milder form of α-mannosidosis. This is in stark contrast with what is seen in other lysosomal-storage diseases, such as Tay-Sachs disease and Sandhoff disease, where homozygous nonsense mutations are observed mostly in patients with the severe, infantile form of the disease (Gravel et al. 1995). The nonsense mutation in the α-mannosidase gene is located in exon 19, at a point ~75% along the length of the protein. This nonsense mutation likely encodes an unstable mRNA, such that a relatively stable but defective protein is produced that can show partial activity toward α-mannose residues in vivo. It also suggests that the C-terminus may be of limited importance for enzyme synthesis or lysosomal targeting. In the effort to further clarify the functional relevance of the C-terminus of α-mannosidase, the R760X mutation or a similar deletion near the C-terminus may provide important clues relative to the activity of the defective enzyme and its kinetics.

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Electronic-Database Information

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for α-mannosidosis [MIM 24850])

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