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Spectrum of Mutations in α-Mannosidosis

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Summary

α-Mannosidosis is an autosomal recessive disorder caused by deficiency of lysosomal α-mannosidase (LAMAN). The resulting intracellular accumulation of mannose-containing oligosaccharides leads to mental retardation, hearing impairment, skeletal changes, and immunodeficiency. Recently, we reported the first α-mannosidosis–causing mutation affecting two Palestinian siblings. In the present study 21 novel mutations and four polymorphic amino acid positions were identified by the screening of 43 patients, from 39 families, mainly of European origin. Disease-causing mutations were identified in 72% of the alleles and included eight splicing, six missense, and three nonsense mutations, as well as two small insertions and two small deletions. In addition, Southern blot analysis indicated rearrangements in some alleles. Most mutations were private or occurred in two or three families, except for a missense mutation resulting in an R750W substitution. This mutation was found in 13 patients, from different European countries, and accounted for 21% of the disease alleles. Although there were clinical variations among the patients, no significant LAMAN activity could be detected in any of the fibroblast cultures. In addition, no correlation between the types of mutations and the clinical manifestations was evident.

Introduction

α-Mannosidosis (MIM 248500) is a rare autosomal recessive disorder caused by deficient activity of lysosomal α-mannosidase (LAMAN; E.C.3.2.1.24). The major clinical manifestations of this lysosomal storage disorder are mental retardation, hearing impairment, various skeletal changes, and recurrent infections. The clinical variation is considerable, ranging from a severe form that includes profound mental retardation, hepatosplenomegaly, severe dysostosis multiplex, and early death to a mild form that includes moderate mental retardation, hearing impairment, milder dysostosis, and survival into adult life. The diagnosis of α-mannosidosis currently is based on clinical evaluation, detection of mannose-rich oligosaccharides in urine, and direct measurements of α-mannosidase activity in various cell types, such as leukocytes, fibroblasts, and amniocytes (Chester et al. 1982; Thomas and Beaudet 1995).

LAMAN is an exoglycosidase that cleaves α-linked mannose residues as part of the ordered, lysosomal degradation of N-linked oligosaccharides (Aronson and Kuranda 1989). The human enzyme is synthesized as a single-chain precursor that is processed into three main glycopeptides of 15, 42, and 70 kD (Nilssen et al. 1997). The gene encoding LAMAN (MANB) has been assigned to the centromeric region of chromosome 19 (Kaneda et al. 1987) and consists of 24 exons, spanning 21.5 kb (GenBank accession numbers U60885–U60899; Riise et al. 1997). The LAMAN transcript is ~3,500 nucleotides (nts) and contains an open reading frame encoding 1,011 amino acids (GenBank U60266; Nilssen et al. 1997). Thus far, the only human mutation reported is a missense mutation resulting in the His72Leu substitution, detected in two Palestinian siblings (HGMD accession number 119376; Nilssen et al. 1997). α-Mannosidosis also has been described in cattle (Hocking et al. 1972) and cats (Burditt et al. 1980). In these animals the association of specific mutations to distinct clinical phenotypes suggested a correlation between the genotype and phenotype (Berg et al. 1997*a,* 1997*b;* Tollersrud et al. 1997). Our study was undertaken to identify mutations responsible for α-mannosidosis and to investigate whether heterogeneity in the LAMAN gene underlies the clinical variability observed for this disorder.

Patients and Methods

Patients

Patients were diagnosed at the referring center, and fibroblast cultures, genomic DNA, and blood samples

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were provided by their physicians. Clinical information was available from 23 of the patients; however, since patients were evaluated by clinicians from several countries, clinical data were not collected in a standardized manner, and proper classification was not possible. There was no known consanguinity in any of the families.

DNA and RNA Isolation

Genomic DNA was isolated from skin fibroblasts or from whole blood, by use of an automated DNA extractor (341 Nucleic Acid Purification Systems, Applied Biosystems) or by a salting-out procedure (Miller et al. 1988). RNA was isolated from a 75-cm^2 culture flask of skin fibroblasts or from 1 ml whole blood, by use of the TRIzol Reagent System (Gibco BRL).

Southern Blot Analysis

Southern blot analysis was performed by use of standard procedures. Approximately 3–8 μg of genomic DNA was digested separately with *Eco*RI and *Pst*I (Gibco BRL), at 37°C overnight, and then was separated on a 1% agarose gel and transferred overnight to a GeneScreen Plus membrane (Dupont), by capillary action. A 3-kb fragment containing 98% of the LAMAN coding region was fluorescence labeled by use of the Gene Images random prime-labeling module (Amersham Life Sciences) and was hybridized to the membrane, at 65°C overnight. Bound probe was detected by the Gene Images CDP-star detection module (Amersham Life Sciences), followed by exposure to BioMax MR film (Kodak) for 5–72 h.

Reverse Transcription (RT) and PCR

Approximately 1 μg total fibroblast or lymphocyte RNA was reverse transcribed from an oligo-dT primer, at 45°C, by use of the Superscript II kit (Gibco BRL). One microliter of first-strand cDNA and 0.2 μM of primers mph5′F-RI and mph3′R-RI (table 1, PCR 1) were set up in a 25-μl PCR reaction, by use of the TaKaRa LA PCR kit (TaKaRa Shuzo). PCR conditions were as follows: 98°C for 20 s, 70°C for 4 min (7 cycles) and then 98°C for 20 s, 66°C for 4 min (32 cycles). The PCR product (3,098 bp), which contained the LAMAN coding sequence (3,030 bp), was diluted 1:50 in H_2O and was used as a template for nested PCRs (PCRs 2–7). The LAMAN exons and exon-intron borders were amplified from genomic DNA (PCRs 8–21). PCRs 18 and 19 did not give products and were diluted 1:50 prior to nested PCR (PCRs 18a, 18b, and 19a). For patients for whom no LAMAN-specific PCR products could be detected after nested PCR, the cDNA quality was evaluated by RT-PCR of the β-actin gene, using human β-actin–specific primers (Quick-Screen human cDNA–library panel, Clontech), or by RT-PCR of the human glycosylasparaginase (GA) gene, using human GA primer B (Park et al. 1991) in combination with GA reverse exon 5 primer (Nilssen et al. 1993). All amplifications were performed in a total volume of 25 μl, with 1 μl first-strand cDNA, 1 μl diluted PCR product or 50–500 ng genomic DNA, 0.8 mM dNTP, and 0.2 μM each primer, by use of DynaZyme DNA polymerase (Finnzymes) in 1 × DynaZyme buffer. Dimethyl sulfoxide was added to 5% in PCR 8. The PCR conditions were 94°C for 60 s and 66°C for 90 s (35 cycles), except for evaluation of cDNA quality, for which the conditions were 94°C for 60 s, 56°C for 60 s, and 72°C for 60 s.

Nonisotopic RNase-Cleavage Assay (NIRCA)

Prior to NIRCA, the LAMAN coding sequence was PCR amplified from cDNA, in four overlapping fragments, by use of primers containing the T7 (5′-TAATACGACTCACTATAGGG/A-3′) or SP6 (5′-ATTTAGGTGACACTATAGA/GA-3′) promoter sequences added 5′ to the LAMAN-specific sequence (table 1, PCRs 2–5). NIRCA was performed by use of the MisMatch Detect II kit (Ambion). Approximately 100 ng PCR product was transcribed by use of T7 and SP6 RNA polymerases in 10 μl at 37°C for 1 h, in separate tubes, and then 10 μl hybridization buffer was added. Seven microliters of transcription reactions of opposite polarity was mixed (patient-T7 to control-SP6, patient-SP6 to control-T7, and patient-T7 to patient-SP6), boiled for 3 min, and cooled slowly. A control-T7 to control-SP6 hybridization was used as a negative control of cleavage. Five microliters of hybridized transcript was subjected to RNase cleavage using 15 μl RNase solutions 1, 2, and 3 (diluted 1:100 in RNase digestion buffer), at 37°C for 45 min, in separate tubes. The cleavage products were analyzed on 2.5% agarose gels.

Sequencing and Restriction-Enzyme Analysis

PCR products were treated with exonuclease I and shrimp alkalic phosphatase, by use of the PCR-product Presequencing kit (Amersham Life Sciences), and were directly sequenced from the appropriate primers (see table 1 or Nilssen et al. 1997; Riise et al. 1997), by use of the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (United States Biochemical/Amersham Life Sciences). When PCR reactions produced multiple bands, each fragment was gel purified by use of the Qiaex Gel-Extraction kit (Qiagen) prior to sequencing. For restriction-enzyme analysis, PCR products were digested overnight with the appropriate enzymes (table 2) and were analyzed by gel electrophoresis.

Table 1

PCRs

PCR	Primer Pair[a]	Localization	Position[b]	Sequence (5′→3′)[c]	Expected Size (bp)
cDNA:					
1	mph5′F-RI	Exon 1	−23/4	aggacgaattCCCCAGGAGGAAGCTGCTGAGCCATGG	3,101
	mph3′R-RI	Exon 24	3057–3033	catgcgaattcGGAGGGCCCATCCCAGCAGACCTAA	
2	mph1F-T7	Exon 1	10–31	T7-TACGCGCGGGCTTCGGGGGTCT	989
	mph7R-SP6	Exon 7	958–941	SP6-AGTCCGAGCCCATGGTCA	
3	mph6F-T7	Exon 6	797–815	T7-ATCTGTGCTGGGATGTGCT	1,041
	mph14R-SP6	Exon 14	1797–1780	SP6-TCTTCTGGGGATGGGCTG	
4	mph14F-T7	Exon 14	1668–1685	T7-CAGCCAGGCGCACCCTCC	649
	mph19R-SP6	Exon 18/19	2276–2257	SP6-TAATCCCGCCTCCTCTCCAG	
5	mph18$_2$F-T7	Exon 18	2229–2242	T7-ACGCTTCTACACAGACAGCA	869
	mph3′R-SP6	Exon 24	3051–3042	SP6-CCCATCCCAGCAGACCTAAC	
6	mph28F	Exon 12	1442–1462	CTCGGCTCAGAGGCTTCAAAG	551
	mph306R	Exon 16	1992–1972	GGGTCTGAAGATGTAGGCACC	
7	mp306F	Exon 16	1972–1990	GGTGCCTACATCTTCAGAC	344
	mph306bR	Exon 19	2315–2295	GCCACGGGCTCCGTCTGGTTC	
Genomic DNA:					
8	mphf5F-T7	5′ Flank.	−372/−353	T7-ACGGACACCCTGGATTCCCA	650
	mphi1R-SP6	Intron 1	+79/+62	SP6-CAGACCCACCCACACCTC	
9	mphi1F-T7	Intron 1	−106/−90	T7-CGAGATCAGGCCTCTCT	695
	mphi3R-SP6	Intron 3	+94/+78	SP6-GCTTGCACGTGGCATGA	
10	mphi3F-T7	Intron 3	−39/−20	T7-CTCTGACCGCTGACCCTGAC	321
	mphi4R-SP6	Intron 4	+47/+28	SP6-GCCAGAGTGAGTGAAGAAGT	
11	mphi4F-T7	Intron 4	−42/−23	T7-CTGGGCACTAATTCACACTG	671
	mphi6R-SP6	Intron 6	+70/+50	SP6-TGCCTGTACCATGGAAAGAGC	
12	mphi6F-T7	Intron 6	−45/−27	T7-ATGGCCCAGGATCCTCTGG	264
	mphi7R-SP6	Intron 7	+62/+42	SP6-CCCAAGGCCCCCGGATGCAAG	
13	mphi7F-T7	Intron 7	−64/−43	T7-AGTGTGGGCCCCCAGGAAG	592
	mphi10R	Intron 10	+63/+41	CGGCCTAGGGGTGGTTTCCAACT	
14	mphi10F-T7	Intron 10	−39/−18	T7-GAGTCCCACAGAACCTCACTGGAC	221
	mphi11R-SP6	Intron 11	+33/+11	SP6-ACCCCCGTGTCTCCCAAGTCTCG	
15	mphi11F-T7	Intron 11	−153/−133	T7-TATAGTCAAGGGCAGCAGGGT	714
	mphi13R-SP6	Intron 13	+35/+16	SP6-GTCCCAGCGGGGGAATATTC	
16	mphi13F-T7	Intron 13	−36/−16	T7-TACTCTTGACTCAGTTTCCCT	292
	mphi14R-SP6	Intron 14	+30/+10	SP6-GCAGGAAAGGGGATTGAAATG	
17	mphi14F-T7	Intron 14	−116/−97	T7-TGGCTACAGAGTGAGACTCA	500
	mphi16R-SP6	Intron 16	+43/+21	SP6-CTTCCCCATTCCCAACTGCCCA	
18	mphi16$_2$F-T7	Intron 16	−30/−9	T7-CTTACACCCACCTCACCCGTTGT	456
	mphi18R	Intron 18	+95/+71	GTCTCCCAAACTCATGTAATCAGCA	
18a	mphi16$_2$F-T7	Intron 16	−30/−9	T7-CTTACACCCACCTCACCCGTTGT	343
	mp3R	Exon 18	2249–2227	CGGCCATTGCTGTCAGTGTAGAA	
18b	mph3.5F	Exon 17	2095–2114	CAGGTGGTTCGCCTGTACCC	357
	mphi18R	Intron 18	+95/+71	GTCTCCCAAACTCATGTAATCAGCA	
19	mph3F	Exon 18	2229–2249	CTACACAGACAGCAATGGCCG	~1,750
	mph2R	Exon 21	2535–2513	CAGCACCAGGTGGCGCCCTCGCA	
19a	mphi18F-T7	Intron 18	−93/−73	T7-TGACAGAGCGAGACTCCATC	457
	mphi20$_2$R-SP6	Intron 20	+31/+10	SP6-CTGGCCTCGGATGGGGCTCTGA	
20	mphi20F-T7	Intron 20	−49/−30	T7-CACCCACAAACCCACGACTC	357
	mphi21R-SP6	Intron 21	+40/+19	SP6-CTTCAATCCGGTCCTCTCTGCC	
21	mphi21F-T7	Intron 21	−42/−24	T7-CCCCGCCCCTCTCCAACTC	4,833
	mphi23R-SP6	Intron 23	+35/+16	SP6-CTCCACCCCTTCCCTACCCC	
22	mphi23F-T7	Intron 23	−72/−54	T7-GAGGAAGCATCGTGGGGTG	435
	mphf3′R-SP6	3′ Flank.	+109/+90	SP6-TTACAGGAGCAAGCCACCAC	

[a] mph primers were designed from the human cDNA sequences (Nebes and Schmidt 1994; Nilssen et al. 1997), mp primers from the bovine cDNA sequence (Tollersrud et al. 1997), and mphi and mphf primers from the human genomic sequence (Riise et al. 1997). RI = *Eco*RI site added to the 5′ end of the primer. F = forward, and R = reverse.

[b] Position 1 refers to A in the first in-frame ATG (codon 1) in the cDNA sequence reported by Nilssen et al. (1997), but, in all patients and normal controls studied, an insertion of GCC (encoding Ala) was detected between nts 6 and 7. Since deletion of this GCC appears to be a rare polymorphism, we have changed the numbering of the cDNA and protein to include the insertion. For primer mph5′F-RI and for the intron and flanking primers, positive values indicate positions downstream of the previous exon, and negative values indicate positions upstream of the preceeding exon.

[c] Lowercase letters indicate 5′ overhangs containing *Eco*RI sites. Mismatches to the human cDNA sequence (Nilssen et al. 1997) or the genomic DNA sequence (Riise et al. 1997) are underlined.

Table 2

Detection of Mutations and Polymorphisms in the LAMAN Gene

Designation(s)	PCR Primers (Size [in bp])[a]	Method of Detection[b]	Expected Size of Bands (in bp)
Mutation(s):			
H72L	mphi1F/mphi3R (657)	RE, *Msl*I	Normal: 182+160+128+119+68 Mutant: 279+182+128+68
W77X	mphi1F-T7/mph30R (574)	RE, *Hin*fI	Normal: 7+31+410+126 Mutant: 7+31+160+250+126
IVS5-1G→C	mphi5F/mph262R (212)	RE, *Pst*I	Normal: 121+91 Mutant: 212
965delAT	mphi6F-T7/mphi7R-SP6 (264)	Sequencing	…
IVS7+2T→G	mphi6F-T7/mphi7R-SP6 (264)	RE, *Bsm*BI[c]	Normal: 170+94 Mutant: 264
T355P	mphi7F-T7/mphi10R (592)	Sequencing	…
P356R	mphi7F-T7/mphi10R (592)	RE, *Hha*I	Normal: 346+79+46+121 Mutant: 124+222+79+46+121
1076–1077insA	mphi7F-T7/mphi10R (592)	RE, *Hpa*I	Normal: 592 Mutant: 130+463
Y359X	mphi7F-T7/mphi10R (592)	RE, *Hpa*I	Normal: 592 Mutant: 130+462
1153–1154insCC	mph26bF/mph27R (98)	PAGE	Normal: 98 Mutant: 100
E402K	mphi7F-T7/mphi10R (592)	Sequencing	…
IVS11-3del25	mphi11F-T7/mphi12R-SP6 (409)	AGE	Normal: 409 Mutant: 384
IVS12+2T→G	mphi11F-T7/mphi12R-SP6 (409)	Sequencing	…
E563X	mphi13F/mphi14R (370)	RE, *Msp*I	Normal: 156+214 Mutant: 370
1815delA	mphi13F/mphi14R (370)	RE, *Mse*I	Normal: 285+85 Mutant: 369
IVS14+1G→C	mphi13F/mphi14R (370)	RE, *Dde*I	Normal: 21+69+44+36+200 Mutant: 21+69+44+36+133+67
IVS14-2A→G	mphi14F-T7/mph306R (382)	RE, *Msp*I	Normal: 134+248 Mutant: 125+9+248
W714R	mph3.5F/mphi18R (357)	RE, *Bsr*BI	Normal: 358 Mutant: 45+312
IVS17+1G→A, IVS17+1G→T	mphi16F/mphi18$_2$R (573)	RE, *Hph*I	Normal: 24+52+33+156+198+110 Mutant: 24+52+33+354+110
R750W	mph3.5F/mphi18R (357)	RE, *Hae*III	Normal: 57+184+70+46 Mutant: 57+254+46
L809P	mph306bF/mphi20$_2$R-SP6 (317)	RE, *Msp*I	Normal: 46+271 Mutant: 46+209+62
Polymorphism:			
L/V278	mphi5F/mph262R (212)	RE, *Msp*I	L278: 59+153 V278: 59+129+24
T/I312	mphi6F-T7/mphi7R-SP6 (264)	RE, *Tsp*RI[c]	T312: 93+96+75 I312: 189+75
R/Q337	mphi6F-T7/mphi7R-SP6 (264)	RE, *Msp*I	R337: 68+96+67+33 Q337: 68+163+33
N/S413	mph26bF/mphi10R (335)	RE, *Bbv*I	N413: 153+182 S413: 153+57+125
IVS10+34A/C	mph26bF/mphi10R (335)	RE, *Aci*I[c]	IVS10+34A: 22+205+108 IVS10+34C: 22+205+78+30
IVS18+63G/A	mphi16F/mphi18$_2$R (573)	RE, *Hph*I	IVS18+63G: 24+52+33+156+198+110 IVS18+63A: 24+52+33+156+198+51+59

[a] PCR conditions were 95°C for 1 min, 64°C for 1 min, and 72°C for 1 min (for 35 cycles). Primer sequences are listed in table 1, in the report by Nilssen et al. (1997), or in the report by Riise et al. (1997), except for primers mphi12R-SP6 (SP6-CTTCGCAGCCCA-CGTAATTTCACT, position +108/+85 in intron 12), mphi16F (AATTATGTGGCGGTGACTGAAGGT, position −134/−111 in intron 16), and mphi18$_2$R (GGCTCCCCCGCCCTTCACTCT, position +109/+129 in intron 18). F = forward, and R = reverse.

[b] RE = restriction-enzyme digestion, and AGE = agarose-gel electrophoresis.

[c] Sequencing was performed because of ambiguous results after restriction-enzyme digestion.

Immunoprecipitation

Polyclonal antiserum was obtained by immunization of male Chinchilla rabbits with native human placenta LAMAN (Nilssen et al. 1997) and was IgG enriched by use of standard protocols. Fibroblasts from one 75-cm^2 culture flask were lysed in PBS containing 1% Triton-X100, incubated at 4°C for 1 h, and centrifuged. The supernatant was assayed for α-mannosidase activity, in 0.1 M sodium acetate, pH 4.5, and 4 mM 4-methylumbelliferyl α-D-mannopyranoside (Sigma), at room temperature for 2–7 h. The enzyme activity was correlated to the total amount of protein in the sample, which was measured by the D_C-protein assay kit (Bio-Rad). LAMAN was immunoprecipitated from the supernatant, by use of standard protocols, and the immunoprecipitate was assayed for α-mannosidase activity. The enzyme activity was correlated to the amount of protein prior to immunoprecipitation. All assays were performed in duplicate.

Results

Outline of Strategy for Mutation Analysis

By screening for the mutation resulting in the H72L substitution, described by Nilssen et al. (1997), we excluded this mutation in the 43 patients. Southern blot analysis using LAMAN cDNA as a probe was performed to search for large gene aberrations. Extra bands were observed in five patients. RNA and cDNA were prepared from the 32 patients from whom fresh blood or fibroblasts were available, and the LAMAN coding region was PCR amplified in four overlapping fragments. RT-PCR products were obtained from 24 of these patients. Of the eight patients who failed to give LAMAN RT-PCR products, four expressed normal levels of β-actin and GA mRNA, as determined from agarose-gel electrophoresis of RT-PCR products, indicating lack of LAMAN mRNA production. The remaining four patients showed no or very low amounts of control RT-PCR products, indicating that the quality of the cDNA was poor. LAMAN RT-PCR products of reduced sizes were observed in six patients. These fragments were directly sequenced, and the underlying splicing mutations were determined from genomic DNA. Fragments of sizes indistinguishable from the control fragments were subjected to mutation analysis using enzymatic cleavage of mismatched heteroduplexes (NIRCA). RT-PCR products corresponding to the cleaved fragments were sequenced, and the mutations also were verified by sequencing of genomic DNA. For some of the patients with at least one unidentified mutation after the cDNA analysis (patients 10, 14, 16, and 30) or from whom cDNA was not available (patients 2, 5, and 22), all 24 exons and the exon-intron borders were PCR amplified from genomic DNA and directly sequenced. Using this combined strategy, we identified 16 new mutations. All patients were screened for these mutations, by restriction-enzyme analysis or DNA sequencing. Five additional mutations were found during this screening. The 21 mutations (tables 3 and 4 and fig. 1) were present in 36 of the 43 patients (table 5). Genomic DNA was available from the family members of 12 patients (5, 7, 13, 14, 15A, 15B, 19, 20A, 20B, 21A, 21B, and 22), and, in these families, the parents were found to carry the mutations.

LAMAN Mutations

The mutations detected in this study are listed in tables 3 and 4. Splicing mutations (table 3) were predicted when the RT-PCR analysis revealed lack of an entire exon or parts of an exon. The cryptic exonic splice sites that were activated conformed to the consensus GT-AG rule (Padgett et al. 1986), except for the site activated as a result of IVS5-1G→C, in which case the activated acceptor site in exon 6 was TG, not the consensus AG dinucleotide. Nonconsensus splice sites are rare and usually are involved in alternative splicing (Jackson 1991). The IVS7+2T→G mutation resulted in the production of two different transcripts, devoid of 9 and 12 nts. A cryptic GT donor site at position 1015 in exon 7 was used for both transcripts. There were no genomic sequence alterations in the intron 7 acceptor site in this patient, and the inclusion of an extra codon in the largest transcript appeared to result from use of an alternative acceptor site 3 nts upstream of the commonly used site of intron 7. Splicing heterogeneity is also supported by the presence of this extra codon at the same position in the cDNA sequence reported by Nebes and Schmidt (1994). The alternative splicing is probably a rare event, since the codon was absent from all control cDNAs tested.

In addition, IVS11-3del25 resulted in the production of two types of transcripts, the largest of which was a result of activation of a cryptic acceptor site in exon 12. Agarose-gel electrophoresis of RT-PCR products covering exon 12 showed that this transcript was less abundant than the transcript that lacked the entire exon. IVS17+1G→A and IVS17+1G→T also resulted in production of two types of transcripts. As determined from gel electrophoresis, these transcripts were present in equal amounts.

We identified six new missense mutations (table 4). None of the 140 Norwegian control alleles contained the T355P, P356R, E402K, W714R, or R750W substitutions, whereas the L809P replacement was found on 1 of 196 Norwegian normal alleles. The T355P, P356R, E402K, and W714R variants were private. The mutation leading to R750W was detected on 4 of 12 Finnish dis-

Table 3

Splice Mutations in the LAMAN Gene

Mutation Designation	Sequence Alteration	Effect on Transcript	Effect on Protein
IVS5-1G→C	AG→AC in acceptor site of intron 5	Deletion of the first 15 nts of exon 6 (764–778)	Deletion of Val256–Gly260
IVS7+2T→G	GT→GG in donor site of intron 7	(I) Deletion of the last 12 nts of exon 7 (1015–1026); (II) Deletion of the last 12 nts of exon 7 (1015–1026) plus inclusion of 3 nts of intron 7	(I) Deletion of Val339–Gln342; (II) Deletion of Val339–Ala341
IVS11-3del25	Deletion of 25 bp preceeding −2 in acceptor site of intron 11	(I) Deletion of exon 12 (1420–1527); (II) Deletion of the first 66 nts of exon 12 (1420–1485)	(I) Deletion of Val474–Arg509; (II) Deletion of Val474–Gln495
IVS12+2T→G	GT→GG in donor site of intron 12	Deletion of exon 12 (1420–1527)	Deletion of Val474–Arg509
IVS14+1G→C	GT→CT in donor site of intron 14	Deletion of exon 14 (1645–1830)	Deletion of Val549–Glu610
IVS14-2A→G	AG→GG in acceptor site of intron 14	Deletion of the first 31 nts of exon 15 (1831–1861)	Frameshift after Glu610, termination at codon 612
IVS17+1G→A	GT→AT in donor site of intron 17	(I) Deletion of exon 17 (2047–2165); (II) Deletion of the last 24 nts of exon 17 (2142–2165)	(I) Frameshift after Lys682, termination at codon 694; (II) Trp714Cys plus deletion of Ser715–Gly722
IVS17+1G→T	GT→TT in donor site of intron 17	Same as for IVS17+1G→A	Same as for IVS17+1G→A

NOTE.—We have used the mutation nomenclature suggested by Antonarakis (1998). For an explanation of the numbering of nucleotides and codons, see footnote b in table 1. "(I)" and "(II)" refer to alternative forms of transcripts resulting from the same splicing mutation.

ease alleles but was excluded as a polymorphism in Finns, by the screening of 80 control alleles from this population. The same mutation was found on five of eight Polish disease alleles, as well as in single cases originating from Germany, Italy, Sweden, The Netherlands, and Turkey. Also, a cell line deposited in the National Institute of General Medical Sciences (NIGMS) cell repository contained this mutation, but information concerning the patient's country of origin was not available. In total, the R750W variant was present on 18 (21%) of the 86 disease alleles. The L809P substitution was present on one Norwegian and three Swedish alleles.

Southern blot analysis revealed the presence of extra bands in five patients (1, 3, 8, 14, and 25), when compared with 80 control alleles. Patients 1 and 25 showed abnormal restriction patterns for both *Eco*RI and *Pst*I. Patients 3, 8, and 14 showed abnormal patterns only for one enzyme (*Eco*RI or *Pst*I). Patient 14 was heterozygous for a mutation that disrupted a *Pst*I site (IVS5-1G→C), which probably explains the abnormal restriction pattern. The molecular basis of the other abnormal bands was not determined.

Six allelic variants were identified in this study (table 4). T/I312 and R/Q337 have been described elsewhere (Nilssen et al. 1997; Riise et al. 1997). The two intronic polymorphisms did not seem to affect the splicing process. These six variants were classified as polymorphisms not associated with α-mannosidosis, since they were present in homozygous states in several controls.

LAMAN Activity in α-Mannosidosis Fibroblasts

α-Mannosidase activity was assayed in fibroblasts from 22 of the patients and was found to be 5%–10% of the activity in controls (data not shown). However, since other α-mannosidases, such as Golgi α-mannosidase II (Moremen and Robbins 1991), also are active at a low pH, the specific LAMAN activity was determined after immunoprecipitation with antibodies against human placenta LAMAN. In all patients, the level of cross-reacting α-mannosidase activity was <1.3% of that in controls (not shown), but there was no consistent variation between the patients.

Clinical Classification of Patients

Clinical classification was based on symptoms described by Chester et al. (1982). There was considerable variation in the severity of skeletal abnormalities and mental retardation. One patient (17) with a splicing mutation died at 26 mo of age from respiratory failure and may represent the most severe form of α-mannosidosis (Aylsworth et al. 1976; Patton et al. 1982; Thomas and Beaudet 1995). Other patients (1, 6, 12–16, 18–22, 24–28, 31, and 32) were less severely affected, but their symptoms still were not as mild as those of the patients

Table 4

Mutations and Polymorphisms in the LAMAN Gene

Mutation Designation	Sequence Alteration	Effect on Coding Sequence
Missense:		
H72L[a]	A→T at 215 in exon 2	His72Leu susbstitution
T355P	A→C at 1063 in exon 8	Thr355Pro susbstitution
P356R	C→G at 1067 in exon 8	Pro356Arg substitution
E402K	G→A at 1204 in exon 9	Glu402Lys substitution
W714R	T→C at 2140 in exon 17	Trp714Arg substitution
R750W	C→T at 2248 in exon 18	Arg750Trp substitution
L809P	T→C at 2426 in exon 20	Leu809Pro substitution
Insertion or deletion:		
1076–1077insA	Insertion of A between 1076 and 1077 in exon 8	Direct termination at codon 359
1153–1154insCC	Insertion of CC between 1153 and 1154 in exon 9	Frameshift after Pro384, termination at codon 476
965delAT	Deletion of AT at 965–966 in exon 7	Direct termination at codon 322
1815delA	Deletion of A at 1815 in exon 14	Frameshift after Leu605, termination at codon 623
Nonsense:		
W77X	G→A at 231 in exon 2	Termination at codon 77
Y359X	C→A at 1077 in exon 8	Termination at codon 359
E563X	G→T at 1687 in exon 14	Termination at codon 563
Polymorphism:		
L/V278	C or G at 832 in exon 6	Leu or Val at codon 278
T/I312[a]	C or T at 935 in exon 7	Thr or Ile at codon 312
R/Q337[a]	G or A at 1010 in exon 7	Arg or Gln at codon 337
N/S413	A or G at 1238 in exon 10	Asn or Ser at codon 413
IVS10+34A/C	A or C at +34 in intron 10	No effect
IVS18+63G/A	G or A at +63 in intron 18	No effect

NOTE.—We have used the mutation nomenclature suggested by Antonarakis (1998). For an explanation of the numbering of nucleotides and codons, see footnote b in table 1.

[a] Reported previously by Nilssen et al. (1997) and Riise et al. (1997).

reported by Warner et al. (1984) and Bennet et al. (1995). Moderate clinical manifestations in the majority of the patients was consistent with a previous study based on >60 cases (Chester et al. 1982).

Discussion

This study presents the first spectrum of mutations in α-mannosidosis. Twenty-one new mutations were characterized and included eight splicing mutations, six missense mutations, three nonsense mutations, two small insertions, and two small deletions (tables 3 and 4 and fig. 1). Putative disease-causing mutations were identified on 62 (72%) of the 86 disease alleles studied. In addition, four exonic and two intronic nonpathogenic polymorphisms were identified.

Technical Aspects of the Mutation Detection

cDNA analyses, involving agarose-gel electrophoresis and NIRCA, revealed mutations only on 16 alleles from the 24 patients (33%) who were shown to express LAMAN mRNA. One explanation for this low degree of detection is that cDNA analysis does not detect mutations that reduce the production or stability of mRNA. This was probably the case for seven patients (1, 3, 6, 10, 11, 13, and 17) who, by comparison of the cDNA and genomic sequences, were found to express mRNA from one allele only. Notably, six of the mutations (IVS7+2T→G [leading to the deletion of 12 nts from the transcript], 1063A→C, 1204G→A, 1238A→G, 2248C→T, and 2426T→C) identified by sequencing of genomic DNA were not detected by NIRCA, although sequencing later showed that these mutations were present in the cDNA. This demonstrates the limited sensitivity of this method of mutation detection. The cDNA analyses were also complicated by the presence of alternatively spliced transcripts for which no underlying mutations could be detected. The aberrant transcript that occurred most frequently contained intron 13 and lacked exon 8 and thus was identical to a transcript reported by Liao et al. (1996) that did not encode a functional LAMAN.

Seven patients were analyzed by the sequencing of all exons and exon-intron borders. Mutations were identified in all these patients, except for the mutation on the paternal allele of patient 14. RT-PCR showed that this patient expressed only the maternal allele carrying the IVS5-1G→C mutation, which disrupts a *Pst*I site. Southern blot analysis revealed the presence of an additional band after *Pst*I digestion. This pattern also was present in the mother and is probably a result of the IVS5-1G→C mutation. It is possible that the mutation

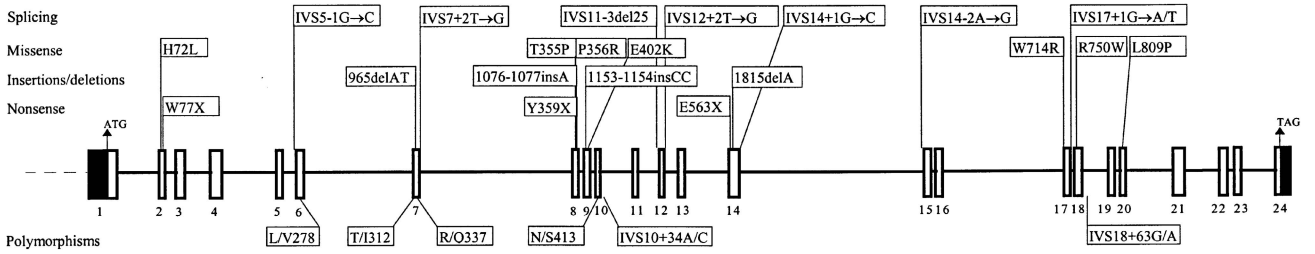

Figure 1 Localization of the mutations and polymorphisms in the LAMAN gene. Exons are represented by boxes; blackened areas indicate UTRs.

on the paternal allele is outside the analyzed regions or that unidentified intronic polymorphisms lead to allele-specific amplification of the maternal allele. Abnormal restriction patterns also were seen in patients 1, 3, and 25, in whom only one of the mutations had been found by the screening of genomic DNA. These patients also showed the normal bands, indicating heterozygosity for the mutation causing the abnormal restriction pattern. Additional bands also were present in patient 8, who appeared to be homozygous for a 2-bp deletion in exon 7. Determination of the molecular basis for these abnormal bands is in progress. Eighteen patients appeared to be homozygous for specific mutations, but DNA was available only from the parents of 6 of these patients. We therefore cannot exclude the possibility that the 12 remaining patients (2, 4, 8, 9, 18, 23, 27, 28, and 30–33) carry additional mutations causing allele-specific amplification or genomic rearrangements not detectable by Southern blot analysis.

Characteristics of the Mutations

The deletions, insertions, and nonsense mutations all resulted in premature termination of translation, which indicates that these mutations were pathogenic. The splicing mutations were probably also disease causing, even though the reading frame remained intact in some of the misspliced transcripts. Six sequence variants (L/V278, T/I312, R/Q337, N/S413, IVS10+34A/C, and IVS18+63G/A) were found in homozygous states in normal controls, proving that these polymorphisms do not cause α-mannosidosis. In contrast, the mutations leading to T355P, P356R, E402K, W714R, and R750W were not found on 140 control alleles, whereas 1 of 196 control alleles carried the mutation leading to the L809P substitution. The absence or very low frequency of carriers in a normal population does not prove that these six missense mutations are disease causing, but several observations indicate that they are pathogenic. First, T355P, R750W, and L809P were identified in patients for whom all exons and exon-intron borders were sequenced, which indicates that there were no other disease-causing mutations on these alleles. Second, all six amino acid substitutions resulted in profound changes in the side-chain properties of the affected residues. Third, T355 is conserved among all class 2 α-mannosidases, whereas the five other amino acid residues are conserved among the four mammalian LAMANs cloned to date (i.e., human [Nilssen et al. 1997], cattle [Tollersrud et al. 1997], cat [Berg et al. 1997*b*], and mouse [Beccari et al. 1997]). E402K was found on the same allele as the splicing mutation IVS7+2T→G. The splicing mutation probably has a more profound effect on the gene product than does the E402K substitution, since the splicing mutation leads to deletion of three or four amino acids. Expression studies are required, to determine which of these two mutations causes loss of activity. It also is possible that both mutations are required for inactivation of the gene product.

The high degree of mutational heterogeneity of α-mannosidosis, with 22 mutations identified thus far, is comparable to that of many other genetic disorders. Sixteen of the 33 unrelated patients included in this study appeared to be homozygous. This indicates that α-mannosidosis often is associated with consanguinity, although we could not confirm this in any of the families. Most mutations were private, but seven mutations occurred in more than one family. The 1153–1154insCC and IVS14-2A→G mutations were found in two Norwegian families, whereas Y359X and IVS17G→T were present in two German and two Italian families, respectively. Both IVS14+1G→C and L809P were present in three families each, all originating from northern Europe. The mutation leading to R750W was found in patients from several countries and accounted for 21% of the disease alleles. Haplotype analysis is required, to address whether the mutations occurring in more than one family are identical by descent or are recurrent mutations. Unfortunately, we were unable to perform a proper haplotype analysis, since we lacked DNA samples from the family members of many patients. By testing the five R750W homozygotes (patients 5, 18, 23, 31, and 33) for the L/V278, R/Q337, N/S413, IVS10+34A/

Table 5

Mutations in α-Mannosidosis Patients

Patient	Ethnicity	Mutation on Allele 1	Mutation on Allele 2	Source(s)
1[a]	Dutch	W77X	ND	O. P. van Diggelen, Rotterdam
2[a,b]	Dutch	1076–1077insA	1076–1077insA	R. Wevers, Nijmegen, The Netherlands; Jansen et al. (1987)
3[a,b]	Dutch	R750W	ND	O. P. van Diggelen, Rotterdam
4[a]	Finnish	IVS14+1G→C	IVS14+1G→C	M. Renlund, Helsinki
5[a]	Finnish	R750W[c]	R750W[c]	M. Renlund, Helsinki
6[a,b]	Finnish	R750W	ND	GM00654;[d] Autio et al. (1973)
7[a]	Finnish	R750W[c]	ND	M. Renlund, Helsinki
8[a]	French (Réunion)	935delAT	935delAT	E. Vamos, Brussels; Farriaux et al. (1975)
9[a,b]	French (Réunion)	IVS17+1G→A	IVS17+1G→A	C. Largillière, Lille, France; Farriaux et al. (1975)
10[a,b]	German	Y359X	IVS11-3del25	M. Beck, Mainz, Germany
11[a,b]	German	Y359X	IVS7+2T→G, E402K	M. Beck, Mainz, Germany; Spranger et al. (1976)
12[b]	German	1815delA	IVS14+1G→C	M. Beck, Mainz, Germany; Gehler et al. (1975)
13	German	R750W[c]	ND	M. Beck, Mainz, Germany
14[a,b]	Greek	IVS5-1G→C[c]	ND	H. Michelakakis, Athens
15A	Italian	IVS12+2T→G[c]	IVS12+2T→G[c]	R. Gitzelmann and N. U. Bosshard, Zurich
15B	Italian	IVS12+2T→G[c]	IVS12+2 T→G[c]	R. Gitzelmann and N. U. Bosshard, Zurich
16	Italian	T355P	IVS17+1G→T	R. Gatti, Genoa
17[a]	Italian	IVS17+1G→T	ND	R. Gatti, Genoa; Cerruti Mainardi et al. (1982)
18[a]	Italian	R750W	R750W	B. Bertagnolio, Milan
19[a,b]	Norwegian	1153–1154insCC[c]	1153–1154insCC[c]	D. Malm and L. Tranebjærg, Tromsø, Norway; Malm et al. (1995)
20A[a,b]	Norwegian	1153–1154insCC[c]	IVS14-2A→G[c]	D. Malm and L. Tranebjærg, Tromsø, Norway; Malm et al. (1995)
20B[b]	Norwegian	1153–1154insCC[c]	IVS14-2A→G[c]	D. Malm and L. Tranebjærg, Tromsø, Norway; Malm et al. (1995)
21A[b]	Norwegian	IVS14-2A→G[c]	IVS14-2A→G[c]	D. Malm and L. Tranebjærg, Tromsø, Norway
21B[b]	Norwegian	IVS14-2A→G[c]	IVS14-2A→G[c]	D. Malm and L. Tranebjærg, Tromsø, Norway
22[a]	Norwegian	IVS14+1G→C[c]	L809P[c]	D. Malm and L. Tranebjærg, Tromsø, Norway
23[a,b]	Polish	R750W	R750W	O. P. van Diggelen, Rotterdam
24[a,b]	Polish	R750W	ND	B. Czartoryska, Warsaw
25[a]	Polish	R750W	ND	B. Czartoryska, Warsaw
26[a]	Polish	R750W	ND	B. Czartoryska, Warsaw
27[a,b]	Spanish	W714R	W714R	E. Vamos, Brussels; Loeb et al. (1969)
28[b]	Spanish	E563X	E563X	A. Chabàs and M. J. Coll, Barcelona
29[b]	Swedish	R750W	L809P	J.-E. Månsson, Mølndal, Sweden
30[a,b]	Swedish	L809P	L809P	J.-E. Månsson, Mølndal, Sweden
31[a,b]	Turkish	R750W	R750W	O. P. van Diggelen and J. G. N. de Jong, Rotterdam; Jansen et al. (1987)
32[a,b]	Caucasian	P356R	P356R	GM04518[d]
33[a,b]	Caucasian	R750W	R750W	GM02817[d]

NOTE.—ND = not determined. In the patient designations, "A" and "B" indicate that the the patients are siblings.

[a] Southern blot analysis performed; underlining indicates that abnormal results were obtained.

[b] LAMAN activity determined.

[c] Parents analyzed.

[d] Deposited in the NIGMS Human Genetic Mutant Cell Repository.

C, and IVS18+63G/A polymorphisms, we found that 750W appeared on two different haplotypes. The Finnish, Italian, and Polish homozygotes, as well as the GM02817 cell line, shared the same haplotype (L278, R337, S413, IVS10+34C, and IVS18+63G), whereas the Turkish patient differed at one position (N413). The presence of 750W on two different haplotype backgrounds suggests that there may have been two independent mutational events. This is supported by the dispersed geographic distribution of the mutation and by

its location in a CpG dinucleotide, which is a hot spot for mutations (Cooper and Youssoufian 1988).

Relationship between the Mutations and the Biochemical and Clinical Features

The LAMAN activity in immunoprecipitates from patient fibroblasts was <1.3% of normal. Because of fluctuations among parallel samples, we were unable to obtain precise values for the enzyme activity in individual patients. However, the range of values (0.3%–1.3%) remained constant, which indicates that residual activities were below the detection limit of the method. The results from previous studies show both absence (Burditt et al. 1978; Pohlmann et al. 1983) and presence (Mersmann and Buddecke 1977; Poenaru et al. 1980) of crossreacting activities in patients. This could reflect different mutations resulting in different levels of residual LAMAN activity. Alternatively, these variations could be due to differences in the specificity of the antibodies (Thomas and Beaudet 1995). Bach et al. (1978) suggested that the relatively mild phenotype of two Palestinian siblings was due to a high level of residual α-mannosidase activity, as measured by cellogel electrophoresis followed by an enzyme assay. However, we could not detect any significant crossreacting α-mannosidase activity even in these patients.

Except for an Italian patient (17) who had died at age 2 years, all patients presented moderate clinical features. Two siblings (patients 21A and 21B) presented with relatively mild mental retardation and skeletal changes, and one of them is still, at age 32 years, employed at a "sheltered workplace." These siblings were homozygous for a frameshift splicing mutation that most likely abolished LAMAN activity. The more severely affected patient (17) was heterozygous for a splicing mutation. Although the mutation on the other allele remained unidentified for this patient, our results indicate that no specific class of mutations is associated with moderately or more severely affected patients. Since no patients with mild phenotypes were available for this study, we cannot exclude the possibility that particular mutations are restricted to such patients.

Clinical variation within sibships has been reported for α-mannosidosis (Mitchell et al. 1981; Michelakakis et al. 1992), as well as for related disorders such as fucosidosis (Thomas and Beaudet 1995). Both environmental and other genetic factors may contribute to variations in clinical expression. Since α-mannosidosis patients, especially those at a young age, suffer from recurrent infections, environmental factors may contribute to severe infections that lead to early death and thus, by definition, presentation of a "severe" clinical picture. Other α-mannosidases may contribute to salvage pathways for the degradation of glycoproteins. Indeed, the pattern of oligosaccharides in the urine of α-mannosidosis patients indicates that at least partial degradation of α-mannosidic linkages occurs in the absence of LAMAN (Chester et al. 1982; Warner et al. 1984; Michalski 1996). Interestingly, strong evidence of a role for cytosolic α-mannosidase in the intracellular degradation of oligosaccharides was provided recently (Grard et al. 1996). It therefore is possible that allelic heterogeneity of this or other extra-lysosomal α-mannosidases could influence the clinical expression of α-mannosidosis. However, Warner et al. (1984) observed similar patterns and levels of mannose-containing oligosaccharides in the urine of a very mildly affected and two more severely affected patients, which indicates that factors other than α-mannosidase activity contribute to the clinical heterogeneity.

In conclusion, we found considerable mutational heterogeneity in α-mannosidosis, with a higher prevalence of certain mutations in some restricted geographic locations. Our study forms the basis of DNA-based diagnostics for α-mannosidosis. However, such diagnostic studies cannot be used to predict clinical expression, since environmental and epigenetic factors appear to be the main causes for the clinical variation observed in α-mannosidosis.

Note added in proof.—Recently, Gotodo et al. (1998) reported mutations in five α-mannosidosis patients, including P355R in one patient and R750W in two patients. These three patients are identical to patients 32, 6, and 33, respectively, in our study (see table 5). Gotodo et al. also identified a nonsense mutation (Q639X) in the patient corresponding to patient 6 in our study, while their patient with the H72L substitution is identical to the Palestinian patient reported by Nilssen et al. (1997).

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov (for cDNA [U60266] and genomic DNA [U60885–U60899])
Human Genomic Mutation Database (HGMD), http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html (for mutation H72L [119376])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for α-mannosidosis [248500])

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