Purification of feline lysosomal α -mannosidase, determination of its cDNA sequence and identification of a mutation causing α -mannosidosis in Persian cats

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α -Mannosidosis is a lysosomal storage disorder that is caused by the deficiency of lysosomal α -mannosidase. Feline α -mannosidosis is a well-characterized animal model used for studying pathological and therapeutic aspects of lysosomal storage disorders. We here report the purification of feline liver lysosomal α -mannosidase and determination of its cDNA sequence. The active enzyme consisted of three polypeptides, with molecular masses of 72, 41 and 12 kDa, joined by non-covalent forces. The cDNA sequence of feline lysosomal α -mannosidase was determined from reverse transcriptase PCR products obtained from skin fibroblast mRNA. The deduced amino acid sequence contained the N-terminal sequences of the 72 and 41 kDa peptides. This indicated that the enzyme is synthesized as a single-chain precursor with a putative signal peptide of 50

amino acids followed by a polypeptide chain of 957 amino acids, which is cleaved into the three polypeptides of the mature enzyme. The deduced amino acid sequence was 81.1 and 83.2 % identical with the human and bovine lysosomal α -mannosidases sequences respectively. A 4 bp deletion was identified in an α -mannosidosis-affected Persian cat by DNA sequencing of reverse transcriptase PCR products. The deletion resulted in a frame shift from codon 583 and premature termination at codon 645. No lysosomal α -mannosidase activity could be detected in the liver of this cat. A domestic long-haired cat expressing a milder α -mannosidosis phenotype than the Persian cat had a lysosomal α -mannosidase activity of 2 % of normal. This domestic long-haired cat did not possess the 4 bp deletion, proving molecular heterogeneity for feline α -mannosidosis.

INTRODUCTION

α -Mannosidosis is an autosomal recessive disease resulting from the deficiency of lysosomal α -mannosidase (LAMAN) (EC 3.2.1.24). This lysosomal storage disorder has been identified in humans [1], cattle [2] and cats [3]. Typical clinical symptoms in humans include progressive mental retardation, recurrent infections, skeletal changes and hearing impairment [4,5]. Considerable clinical heterogeneity among affected individuals exists, and a continuum of phenotypes for human α -mannosidosis has been reported.

Human and bovine LAMANs are synthesized as single-chain precursors that are proteolysed into five polypeptides [6,7]. Starting with the peptide derived from the N-terminal part of the precursor, their molecular masses are within the range 35–39 kDa (a-peptide), 8–13 kDa (b-peptide), 20–22 kDa (c-peptide), 38–42 kDa (d-peptide) and 13–15 kDa (e-peptide). In human LAMAN there is an additional peptide of 70 kDa representing the unprocessed form of the abc-peptide [6]. The cDNAs encoding human [6,8,9] and bovine [7] LAMAN were recently cloned, and one human [6] and two bovine mutations [7] leading to α -mannosidosis have been reported.

α -Mannosidosis has been reported in domestic short-haired [3] and long-haired [10] kittens and in three widely dispersed families

of Persian cats as seen in Switzerland [11], the U.S.A. [12] and Belgium [13]. The disease in cats shows clinical, morphological and biochemical features closely resembling those of the human disease. All affected cats showed severe neurological disease, but clinical heterogeneity was apparent, as affected domestic long-haired cats had a milder and less progressive phenotype than affected Persian and short-haired domestic cats [10].

A colony of Persian cats has been established to study pathogenic mechanisms and therapeutic strategies for α -mannosidosis [11] (H. J. Baker and S. U. Walkley, unpublished work). A dramatic improvement in neurological symptoms and neural pathology was achieved by bone marrow transplantation of this animal model [14]. In contrast, very limited reduction in the neuronal storage was observed after bone marrow transplantation of GM2 gangliosidosis cats [15]. Based partly on the success of bone marrow transplantation for feline α -mannosidosis, gene therapy involving haematopoietic stem cells has been suggested as a therapeutic strategy for lysosomal storage disorders [16]. For such studies, feline α -mannosidosis will provide an attractive animal model, not only for the treatment of human α -mannosidosis, but also possibly for other neurological diseases. To facilitate these experiments we have characterized feline LAMAN and determined its cDNA sequence. We have also defined the mutation causing α -mannosidosis in a

Abbreviations used: LAMAN, lysosomal α -mannosidase (EC 3.2.1.24) (the symbol approved by the Human Genome Organization nomenclature committee for the human lysosomal α -mannosidase gene is MANB); RT-PCR, reverse transcriptase PCR; PNGase F, peptide-*N*⁴-(*N*-acetyl- β -glucosaminyl)asparagine amidase F (EC 3.5.1.52); endo H, endoglycosidase H (EC 3.2.1.96).

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The nucleotide sequences have been submitted to the GenBank database and are available under the accession numbers AF010191 (cDNA sequence) and AF010192 (5'-end of the gene).

colony of Persian cats of Swiss origin and developed a DNA-based assay for the identification of carrier and affected animals. By using this assay we showed that feline α -mannosidosis is a genetically heterogeneous disease, as an affected domestic long-haired cat did not possess the mutation detected in Persian cats.

MATERIALS AND METHODS

Purification of feline LAMAN

Feline LAMAN was purified from about 220 g of homogenized cat liver using our published methods for purification of bovine LAMAN [7], except that the hydroxyapatite chromatography step was omitted, as feline LAMAN did not bind to this column. As a last purification step, the partially purified enzyme was subjected to SDS/PAGE on an 8–25% gradient of polyacrylamide using the Phast system (Pharmacia) without prior heat treatment. The gel was cut into several pieces, transferred to tubes containing PBS and, after overnight diffusion, enzyme activity was determined. α -Mannosidase activity was measured using the chromogenic substrate *p*-nitrophenyl α -D-mannopyranoside as previously described [7].

Western-blot analyses, N-terminal sequencing and deglycosylation studies

Western-blot analyses were carried out by standard methods using polyclonal antibodies against bovine LAMAN [7]. For N-terminal sequencing the LAMAN peptides were separated by SDS/PAGE after boiling for 2 min, stained with Coomassie Blue, electroblotted on to a PVDF membrane and subjected to micro-sequencing as described [17]. N-Terminal sequencing was carried out at the Biotechnology Centre, Oslo, Norway, by automated Edman degradation in an Applied Biosystems 477A Protein Sequencer. Deglycosylation studies using peptide-*N*⁴-(*N*-acetyl- β -glucosaminyl)asparagine amidase F (PNGaseF) and endoglycosidase H (endo H) (Boehringer-Mannheim) were performed as described for bovine LAMAN [7]. Deglycosylated peptides were analysed by Western blotting as described above.

Determination of LAMAN activity in livers of α -mannosidosis cats

Approx. 5 g of liver from two affected Persian cats, one affected domestic long-haired cat and one normal Persian cat was homogenized separately in 0.75 M acetic acid/0.15 M NaCl and fractionated using 40–60% (NH₄)₂SO₄. The precipitates were dissolved in a minimum volume of PBS and divided into two portions. One of the portions was subjected to Western blotting. The other was immunoprecipitated by standard procedures using antibodies against native feline LAMAN and *Staphylococcus aureus* (Sigma) as a co-precipitant. After three washes with PBS, α -mannosidase activity was measured directly in the resuspended pellets. Antibodies against feline LAMAN had been generated by subjecting the partially purified enzyme to SDS/PAGE without prior heat treatment (as seen in Figure 1, lane 2) and homogenizing the piece of gel containing α -mannosidase in 0.5 ml of PBS, mixing 1:1 with Freund's complete adjuvant before injecting into a Chinchilla rabbit. The rabbit was bled after two subsequent injections.

Isolation of nucleic acids

Genomic DNA was extracted from skin fibroblasts or homogenized liver by the Qiagen Genomic DNA Purification Kit. Total RNA was extracted from cultured feline skin fibroblasts using the TRIzol Reagent System (Gibco-BRL).

Table 1 Oligonucleotides used for PCR

mp, mph and mpf, primers designed from bovine, human and feline LAMAN cDNA sequences respectively. F indicates forward (sense) and R reverse primers. The positions of the 5'-nucleotide of the primers are relative to the A in the start codon. *Xba*I and *Eco*RI recognition sites introduced into primer mpf5ut2XF are underlined.

Primer	Position	Sequence
mpf5ut2XF	— 35	5'-GAATTCTAGAGGTGGCGGTGGAGGCA-3'
mp1F	91	5'-CCACCGCTCCCGCCTCTCT-3'
mph266R	109	5'-AGAGAGCGGGAGCGGTGG-3'
mpf2R	140	5'-CCAGGCGCCGCCAGCAGTAACA-3'
mpf301F	541	5'-GAAGACACCTTTGGCAAAGAT-3'
mpf263R	597	5'-GAAGGATCAATGTGCGAG-3'
mp303F	1002	5'-CAAGCTCATCCAGTTGGTCAATG-3'
mph26aR	1103	5'-GCCTTGTTCAGCTCCAGAGGTAA-3'
mpf28bF	1493	5'-TCAACGTGAGCGTCTGTCGGCT-3'
mpf5R	1642	5'-GCACGATGCGCATTCCGGT-3'
mp305F	1677	5'-TCAGGAGCTGCTTTTCTCAGCCT-3'
mp4R	1919	5'-TAGAAGGCTTGCGCAACAGGCAGC-3'
mp306F	1966	5'-GGTGCCTACATCTTCAGAC-3'
mpf309F	2662	5'-TCGGGGCTGGCGCGGAGCTG-3'
mpf3utR	3042	5'-CGCCTGCTCCAGCGTGTCTA-3'

Reverse transcription, PCR and DNA sequencing

About 2 μ g of total RNA was annealed to 500 ng of oligo(dT) primer and reverse-transcribed at 42 °C using Superscript II reverse transcriptase under the conditions described by the supplier (Gibco-BRL). A 1 μ l portion of this cDNA or 100 ng of genomic DNA was used as template for PCR. All PCRs were carried out in 25 μ l volumes containing 0.2 μ M each primer, 0.2 mM dNTPs, 1 unit of Takara EX-Taq (TaKaRa Shuzo Corp.) and 1 $\times$ Ex-Taq buffer (TaKaRa Shuzo Corp.). The sequences of the primers used in the PCRs are listed in Table 1. DNA sequencing based on the dideoxy chain termination method [18] was performed using commercially available kits purchased from Amersham Life Sciences. Primers for sequencing either were the same as for PCR (Table 1) or were those designed for characterization of the human and bovine LAMAN genes, and are listed in [7,19]. The sequences were computer-analysed using programs within the GCG program package [20].

Isolation of the 5'-end of the feline LAMAN gene

The 5'-end of the feline LAMAN gene was isolated using the PromoterFinder™ Construction kit (Clontech) according to the manufacturer's protocol. Briefly, 100 ng of genomic DNA from feline fibroblasts was subjected to restriction enzyme digestion followed by adapter-ligation. Adapter-ligated DNA was used as template in a primary PCR with the primer mpf2R and the adapter primer 1 (Clontech). The primary PCRs were diluted 1:50 and subjected to a nested PCR with primer mph266R and adapter primer 2 (Clontech). Initial cycling conditions were denaturation for 5 min at 95 °C followed by seven cycles of denaturation at 98 °C for 20 s and annealing/extension at 70 °C for 3 min. The annealing/extension temperature was then reduced to 66 °C in the 36 following cycles. An 850 bp fragment resulting from the nested PCR was cloned into a pTag vector (Ingenuis) and sequenced on both strands.

Determination of the feline LAMAN cDNA sequence

Primer mpf5ut2XF, located upstream of the first in-frame ATG of the corresponding human and bovine LAMAN cDNAs [6,7], was designed from the sequence of the 5' genomic fragment and

used in combination with primer mpf2R to amplify the 5'-part of the feline LAMAN-coding region from cDNA. The PCR conditions were 5 min at 95 °C, seven cycles of 98 °C for 20 s and 64 °C for 2 min, followed by 36 cycles of 98 °C for 20 s, 60 °C for 30 s and 72 °C for 1 min. To amplify the 3'-end of the LAMAN-coding region, PCR was performed on DNA extracted from a λ -ZAP[®] feline heart cDNA library (Stratagene) by using the library-specific M13 -40 sequencing primer (Pharmacia) and primer mp306F. The PCR conditions were 94 °C for 5 min, followed by 36 cycles of 62 °C for 1 min, 72 °C for 2 min and 94 °C for 1 min. Primer mpf3utR was designed from the sequence of the 3'-fragment and used together with mp1F to amplify the major part of the LAMAN-coding region (2900 bp) by PCR on the feline fibroblast cDNA. PCR conditions were 5 min at 95 °C, seven cycles of 98 °C for 20 s and 64 °C for 2 min, followed by 36 cycles of 98 °C for 20 s, 60 °C for 30 s and 72 °C for 1 min. The three overlapping PCR products covering the entire LAMAN coding region were sequenced on both strands and the DNA sequences assembled to give the full-length feline LAMAN cDNA sequence.

Identification of a feline α -mannosidosis mutation

Four overlapping PCR products covering the complete LAMAN cDNA of an affected Persian cat were produced using the primer combinations mpf5ut2XF/mpf2R, mp1F/mpf263R, mpf301F/mpf5R and mpf28bF/mpf3utR. PCR conditions were seven cycles of 98 °C at 20 s and 67 °C at 2 min, then 36 cycles of 98 °C at 20 s and 63 °C at 2 min. The PCR products were gel-purified (Qiagen Gel Purification Kit) and one strand of each PCR product was sequenced. To screen for the 4 bp deletion in genomic DNA, a fragment of approx. 1200 bp was amplified using the primers mp305F and mp4R. The PCR conditions were 34 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. Genomic sequencing revealed that, in addition to 243 bp of exonic sequence, the amplicon contained an intron of about 1 kb located between nucleotides 1824 and 1825 in the cDNA sequence. The deletion introduced a *Bsa*HI restriction site, and normal and mutant alleles were distinguished by agarose-gel electrophoresis after *Bsa*HI digestion of the 1200 bp amplicon.

RESULTS

Purification and characterization of feline LAMAN

LAMAN was purified from cat livers to a maximum specific activity of 16 units/mg by following a previously described protocol [7]. Three isoelectric forms of acid α -mannosidase with pI values of about 6.0, 5.6 and 5.3 were separated by DEAE anion-exchange chromatography. These isoforms had identical immunological properties as determined by Western blot using antibodies against denatured bovine LAMAN (results not shown). The pH optimum was 4.2 and the enzyme activity remained stable after 15 min at 60 °C (pH 7.4), which is similar to LAMANS from other mammals [6,7].

Three major bands were detected on SDS/PAGE in the absence of prior heat denaturation (Figure 1, lane 2). By cutting the gel into several pieces and eluting it overnight in PBS, we were able to localize the acid α -mannosidase activity to the uppermost band, which had hardly migrated into the gel, indicating that this band contained the native LAMAN complex. No α -mannosidase activity was associated with the other bands in this lane, indicating that they represented contaminating polypeptides. On heat denaturation, the activity-containing band disappeared and was replaced by three other peptides with apparent masses of 72, 41 and 12 kDa (Figure 1, lane 1). As these

Figure 1 Feline liver LAMAN

SDS/PAGE of partially purified LAMAN stained with Coomassie Blue (lanes 1 and 2). The band containing LAMAN activity hardly migrated into the gel and is indicated by an arrow. The estimated molecular masses of the peptides are shown to the left together with the N-terminal sequences of the abc-peptide (72 kDa) and the d-peptide (41 kDa). X in these sequences indicates amino acid residues that could not be identified. The two other major bands indicated with asterisks represent contaminating peptides (see the text). Western blot (lanes 3–5) showing the abc-peptide and d-peptide immunostained with antisera generated against the denatured bovine LAMAN. Before SDS/PAGE, feline LAMAN was heat-denatured (100 °C for 2 min) in the presence of denaturing agent and deglycosylated as indicated in the diagram. Migrations of molecular-mass standards are indicated on the right: α -lactalbumin (14.4 kDa), soya-bean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa) and phosphorylase b (94 kDa).

three peptides originated from the active LAMAN, they probably constituted non-covalently linked peptides within the LAMAN complex. N-Terminal sequencing of the 72 and the 41 kDa peptides revealed similarity to the N-terminal sequences of bovine LAMAN peptides referred to as the abc-peptide and d-peptide respectively [7]. The identities of the two peptides were confirmed by Western-blot analyses using antibodies to the denatured bovine LAMAN (Figure 1). Furthermore, specific antibodies to the bovine abc-peptide and the d-peptide cross-reacted with the 72 and 41 kDa peptide respectively (results not shown), supporting the idea that the 72 kDa peptide constituted the feline abc-peptide and the 41 kDa peptide was the feline d-peptide. The e-peptides of 13–15 kDa found in human and bovine LAMANS originate from the C-terminal part of the single-chain LAMAN precursor in these species [6,7]. The feline 12 kDa peptide associated with the LAMAN complex had about the same molecular mass as the human and bovine e-peptides, suggesting that this could be the feline e-peptide.

Endo H treatment reduced the molecular mass of the 72 kDa peptide (abc-peptide) and the 41 kDa peptide (d-peptide) to 70 and 39 kDa respectively. Further reduction to 58 and 29 kDa was observed after PNGase F treatment (Figure 1), indicating that each of the two peptides contained one high-mannose and three to four complex-type sugar chains. Feline LAMAN was sialylated, as the enzyme migrated faster on native SDS/PAGE after sialidase treatment (not shown).

Feline LAMAN cDNA sequence

By using a PCR-based strategy we amplified and sequenced the complete coding region of feline LAMAN cDNA (Figure 2, top). The cDNA sequence of 3158 nucleotides was determined from two overlapping RT-PCR products obtained from feline skin

[illegible]

Top, amino acid residues corresponding to the N-terminal sequences of the abc-peptide and d-peptide are underlined. Note that the residues not matching the N-terminal sequences are double underlined. Potential N-glycosylation sites are boxed and putative poly(A) sites are shown in bold letters. Arrows indicate cleavage sites of the precursor confirmed by the N-terminal sequences, whereas arrowheads show the position of potential cleavage sites as predicted from human and bovine LAMANs. The 4 bp deleted in affected Persian cats are overscored. The sequences are numbered relative to the putative start codon. Bottom, the organization of the abc-, d- and e-peptides in the LAMAN precursor. The signal peptide and the amino acids between residues 600 and 632 are probably cleaved off and are therefore shaded. Potential N-glycosylation sites are indicated with .

The residues extending from position 51 to position 69 of the deduced amino acid sequence matched the N-terminal sequences of the abc-peptide (72 kDa), whereas residues extending from 631 to 645 matched the N-terminal sequence of the d-peptide (41 kDa). The residues preceding the N-terminus of the abc-peptide contained a putative signal peptidase-cleavage site according to the $-3 -1$ rule of von Heijne [21] resulting in cleavage between residues 50 and 51. This corresponds to the position of the predicted signal peptidase-cleavage site in human and bovine

Figure 3 Alternative splicing of codon 344

PAGE of PCR products (*) amplified from normal feline cDNA by using the primers mp303F and mph26aR. Amplicon with codon 344 is 102 bp, whereas amplicons lacking codon 344 are 99 bp. A sequencing ladder for size determination is shown to the left. On the right is a suggested model for splicing of the pre-mRNA (middle), resulting in transcripts with (above) or without (below) codon 344. Codon 344 is underlined. Each transcript is represented by two bands, of which the upper band probably results from the addition of a terminal base by *Taq* polymerase [22].

LAMAN [6,7]. The molecular mass of the abc-peptide (residues 51–630) as predicted from the deduced amino acid sequence is 65.7 kDa. However, the observed molecular mass of the abc-peptide as determined by SDS/PAGE is 58 kDa after deglycosylation. The cleavage between the human abc-peptide and d-peptide occurs between the amino acids corresponding to residues 599 and 600 of the feline LAMAN. If this cleavage is conserved in feline LAMAN, then the predicted molecular mass of the abc-peptide is 62.2 kDa. Since this is in better agreement with the observed molecular mass for the abc-peptide, we propose that this cleavage also takes place for feline LAMAN, and as a result of this, the residues between 599 and 631 are probably cleaved off as a propeptide. The position of the cleavage between the d- and e-peptide in the human enzyme corresponds to position 881 in feline LAMAN. If this cleavage is conserved in feline LAMAN, the molecular mass of the d-peptide, as predicted from the cDNA sequence, is 28.5 kDa. Since the observed molecular mass of the unglycosylated d-peptide is 28 kDa (Figure 1, lane 5), it is likely that feline LAMAN is also cleaved at about this position. Such a cleavage results in the generation of a 14 kDa peptide, as deduced from the cDNA sequence. In the human and bovine LAMANS, a peptide of 13–15 kDa (e-peptide) corresponding to this part of the LAMAN precursor has been identified [6,7]. Thus the 12 kDa peptide associated with the feline LAMAN complex (Figure 1) is probably similar to the bovine and human e-peptides. A proposed model for the organization of the peptides within the LAMAN precursor is shown in the bottom panel of Figure 2.

Three polymorphic positions were identified in the cDNA from the normal cat. The cat was heterozygous for A/G at nucleotide positions 750 and 2590. Both 750A and 750G result in alanine. However, 2590A results in threonine, whereas 2590G results in alanine. The third polymorphic position observed in the normal cat was found at codon 344. Extension of the sequencing reactions through codon 344 resulted in double sequence starting from this codon. As judged from the sequencing gel, this heterogeneity apparently resulted from deletion of codon 344 from a portion of the transcripts. This was supported by PAGE of RT-PCR products covering codon 344 showing two products differing in size by 3 bp (Figure 3). Surprisingly, genomic sequencing revealed that this heterogeneity was absent from the genomic DNA of the same cat. Codon 344 resides in an intron/exon boundary containing three CAG repeats. Probably, these CAG repeats in the exon/intron boundary cause alternative splicing resulting in two isoforms of the LAMAN transcript, one containing codon 344 and one lacking this codon (Figure 3). The relative intensity of the RT-PCR products indicated that the two

alternative transcripts occurred with approximately the same frequency. In the bovine LAMAN cDNA, a codon corresponding to 344 is present. Two of the reported human LAMAN cDNA sequences [6,8] lack the codon corresponding to 344, whereas the third sequence [9] lacks the codons corresponding to both codon 344 and 345, suggesting that alternative splicing might also occur in the human LAMAN gene. However, the heterogeneous positions in feline LAMAN cDNA probably represent polymorphisms that do not influence LAMAN activity, since they were detected in cDNA from a normal cat.

The coding region of feline LAMAN cDNA showed 84.4 and 84.5% identity with human and bovine cDNAs respectively. The deduced amino acid sequence was 81.1 and 83.2% identical with human and bovine enzyme. Eleven potential N-glycosylation sites were identified in the amino acid sequence; eight of these sites (at positions 134, 369, 499, 643, 649, 690, 764 and 927) are also present at the corresponding positions of human and bovine LAMANS.

Isolation and sequencing of the 5'-end of the feline LAMAN gene

The sequence of the genomic fragment isolated by PCR showed similarity to exon 1 of the corresponding bovine and human genes (Figure 4). The DNA sequence preceding the putative start codon had a high GC content, 75% from nucleotide –240 to –1, and contained two GC boxes (GGGCGG), which are putative binding sites for the SP-1 transcription factor. One CCAAT box [23] but no typical TATA boxes [23] or binding sites for AP-2 (GCCN₃CCG) [24] could be identified. However, several putative binding sites (GGA^{A/T}) for transcription factors within the ETS family known to stimulate transcription from TATA-less promoters were present [25]. Thus the 5'-flanking region of the LAMAN gene has features in common with housekeeping-gene promoters, which are characterized by a high GC content immediately upstream of the start codon and often have multiple binding sites for SP-1 [26].

Determination of LAMAN activity in affected cats and identification of a mutation leading to α -mannosidosis in Persian cats

The enzyme activity was measured in livers from cats affected with α -mannosidosis. In immunoprecipitates from the two Persian cats there was no detectable activity, whereas in the domestic long-haired cat the activity was 2% of normal. This was similar to previous reports of 0.003% in the Persian cats [11]

-746
GTAGAGACAGGGAAGTGACCCCTGTC

-720
CTCTGGCAATTTAGCACATGGGCTCTGCTGACCTGAGGAATGTGGGCTTTGATTTTCTTA

-660
TCAGACAAACTGGGTAATATCAGCCAACTGGATGGAATGACAGGGACTGGGAAAAGCATG

-600
AAAGGCTGAGACCTCCCCACTGGTCACTCCCTCACCTGAGTACTGTGGGTATTTCTTTA

-540
ACAACTAGGCTGGATACCTCCCAACCTCAGCACTCTCAGGGCAGTTCCCGTATCCGTAAT

-480
CCTCGTAGGACAGAACAGGACCGCCATCAACCCCTGCTCCTAGCCAAGCTAGGTCCCTCC

-420
AAGACCCAGCCTTAAAGGTCAATGGGACACCCTAGGATCCTGTGCACACCCTCCACCTC

-360
CTAGCCAAAATTACACCCATGGGAAAATCTGAGACCGATGCATCCACGGGGACAAAGGGG

-300
CACTCCGAGGGCCACTCGGAGGTAGGGAGACACTAAAGGCTCTTCTGGCCAAACCAGCACC

-240
CTCGGGGGGGGGGGACACGCCCTAACGCCTCCCTACACTCCTAGCGTCCTCGGGGGTAGC

-180
CAGAAACAGCCTCAGCCCCAGTGAGGCGCCGGGCCCCCTGCCGCACTTTACGTCCGCCCCCG

-120
CCAGCAACCAATTGGCCAGGTGGGGTCTCTGGGGCGGGGGCGCTTGCCCCGCCTTTGCCGGG

-60
CCCCGGGAACCCCCCGGAGGCGGCCGAGGTGGCGGTGGAGGCAGCGGAGGCTGCCGGAGCC

+1
ATGGGGGCGGACGCGCGGCCTTTGGGGGTCCGCGCTGGCGGTGGCGGCCGGGGCGCGGCG

+60
AGGCCCGGGACGAGCTCCCGCGCGCTCCCGCCACCGCTCCCGCCTCTCT

Figure 4 DNA sequence of the 5'-flanking region of feline LAMAN gene

Identical nucleotides in the human, bovine and feline LAMAN genes are shown in bold. GC (GGGCGG/CCGCC) and CCAT (CCAAT/ATTGG) boxes are boxed. The A of the start codon is referred to as +1.

and 2.5% in the domestic long-haired cats [10]. No LAMAN peptides could be detected in the liver extracts from the two affected Persian cats by Western blotting. However, a very faint signal was detected in the liver of the domestic long-haired cat (results not shown).

To determine the molecular defect underlying feline α -mannosidosis, the complete LAMAN cDNA sequence of an affected Persian cat was determined and compared with the normal sequence. Two differences from the normal cDNA sequence were detected. The first alteration was a silent C-to-T transition of nucleotide 2925. The second difference was a deletion of four nucleotides (CCAG) extending from position 1749 to 1752 (Figure 5, top). The mutation, designated 1748del4, leads to a frame-shift from codon 583 and consequent termination 62 codons further downstream.

A rapid test involving PCR amplification and *Bsa*HI digestion was developed to screen for the mutation in genomic DNA (Figure 5, bottom). This test confirmed that the affected cat was homozygous and that two obligate carriers from the same colony were heterozygous for the deletion. The mutation was absent from the DNA of an affected domestic long-haired cat expressing a milder α -mannosidosis phenotype.

DISCUSSION

We have shown that feline LAMAN consisted of three polypeptides that originate from a single-chain precursor. The three peptides were joined by non-covalent forces, and, on the

basis of their similarity to human and bovine LAMAN peptides, they were designated the abc-peptide (72 kDa), d-peptide (41 kDa) and e-peptide (12 kDa). In contrast with human and bovine LAMANS [6,7], no internal cleavage of the feline abc-peptide could be detected. The feline cDNA contained an open reading frame coding for 1007 amino acids with 81.1% and 83.2% identity with human and bovine LAMAN amino acid sequences respectively. The amino acid sequence contained 11 potential N-glycosylation sites. Deglycosylation by endo H and PNGase F revealed that most of these sites were occupied, predominantly by complex-type sugar chains. A similar glycosylation pattern has been described for human and bovine LAMAN [6,7].

The 5'-flanking region of the feline LAMAN gene showed regions with similarities to the human and bovine genes and shared common features with housekeeping-gene promoters. Nilssen et al. [6] showed that the human LAMAN gene was expressed in all tissues examined, supporting the notion that it is a housekeeping gene. No consensus poly(A) signal could be identified at the 3'-end of the cDNA. However, the presence of various non-consensus poly(A) signals has been reported for several lysosomal enzymes [6,27–29].

The 1748del4 mutation identified in a colony of Persian cats causes a frame shift at codon 583 of the LAMAN coding sequence and leads to premature termination at codon 645. Since the three previously reported α -mannosidosis mutations are missense mutations [6,7], the 1748del4 mutation is the first reported α -mannosidosis mutation that results in a truncated

Figure 5 Detection of the 1748del4 mutation in α -mannosidosis cats

Top, DNA sequence showing the 1748del4 in the cDNA of an affected Persian cat. Bottom, separation on a 2% agarose gel after *Bsa*HI digestion of amplicons from DNA of a normal cat (lane 2), two obligate carrier Persian cats (lanes 3 and 4), an affected Persian cat (lane 5) and an affected domestic long-haired cat (lane 6). To the right are the estimated sizes of the digests. Lane 1 shows undigested PCR product and L the 100 bp ladder from Gibco-BRL.

LAMAN. The presence of a truncated LAMAN in affected Persian cats was consistent with the finding of no LAMAN activity in immunoprecipitates from liver extracts of the affected cats. The Persian cats examined in this work were derived from the family reported by Vandeveld et al. [11]. A milder α -mannosidosis phenotype and a larger residual activity of 3–5% was reported in two other family lines of Persian cats [12,13], indicating that different mutations could exist in these family lines. An affected domestic long-haired cat from the family first described in [10] did not possess the 1748del4 mutation, thus proving molecular heterogeneity for feline α -mannosidosis. Detection of about 2% of normal LAMAN activity in this cat confirmed that a different mutation had caused the disease. Affected domestic long-haired cats were found to express a milder form of α -mannosidosis with a later onset, and they were spared the skeletal deformities, ocular abnormalities and hepatomegaly described in affected Persian cats [10].

The larger residual activity in affected domestic long-haired cats is consistent with their milder phenotype compared with

affected Persian cats. A correlation between the degree of enzyme impairment and severity of the disease has been reported in human α -mannosidosis [30] and in some of the other lysosomal storage disorders [31]. However, for bovine α -mannosidosis there appears to be no correlation between residual LAMAN activity and severity [7]; affected Galloway calves are either aborted or stillborn, and yet liver LAMAN activity is about 2% of normal, and, although the disease is milder in affected Angus calves, the enzyme is expressed at only 0.15% of normal. This shows that it might be difficult to establish a correlation between residual α -mannosidase activity and degree of severity for α -mannosidosis. It seems, however, that for both feline α -mannosidosis (this study) and bovine α -mannosidosis [7] there is a correlation between genotype and clinical phenotype, as the different mutations are associated with different degrees of severity.

Characterization and cloning of feline LAMAN will facilitate clinical trials of gene- and enzyme-replacement therapy using the cat as an animal model. The detection of the 1748del4 mutation enabled us to design a DNA-based test, involving PCR and restriction enzyme digestion, for rapid and reliable determination of the α -mannosidosis genotype of the animals within the colony of Persian cats. This will be of great value for maintaining the feline α -mannosidosis colony and for setting up future therapeutic trials on this animal model.

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REFERENCES

- Öckerman, P. A. (1967) *Lancet* **ii**, 239–241
- Hocking, J. D., Jolly, R. D. and Batt, R. D. (1972) *Biochem. J.* **128**, 69–78
- Burditt, L. J., Chotali, K., Hirani, S., Nugent, P. G., Winchester, B. G. and Blakemore, W. F. (1980) *Biochem. J.* **189**, 467–473
- Chester, M. A., Lundblad, A., Öckerman, P. A. and Autio, S. (1982) In *Genetic Errors of Glycoprotein Metabolism* (Durand, P. and O'Brien, J., eds.), pp. 89–122, Edi-Ermes, Milano and Springer Verlag, Berlin
- Thomas, G. H. and Beaudet, A. L. (1995) In *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. A. and Valle, D., eds.), pp. 2529–2561, McGraw-Hill, New York
- Nilssen, Ø., Berg, T., Riise, H. M. F., Ramachandran, U., Evjen, G., Hansen, G., Malm, D., Tranebjærg, L. and Tollersrud, O. K. (1997) *Hum. Mol. Genet.* **6**, 717–726
- Tollersrud, O. K., Berg, T., Healy, P. J., Evjen, G., Ramachandran, U. and Nilssen, Ø. (1997) *Eur. J. Biochem.* **246**, 410–419
- Nebes, V. L. and Schmidt, M. C. (1994) *Biochem. Biophys. Res. Commun.* **200**, 239–245
- Liao, Y. F., Lal, A. and Moremen, K. W. (1996) *J. Biol. Chem.* **271**, 28348–28358
- Cummings, J. F., Wood, P. A., de Lahunta, A., Walkley, S. U. and Le Boeuf, L. (1988) *J. Vet. Intern. Med.* **2**, 163–170
- Vandeveld, M., Fankhauser, R., Bichsel, P., Wiesmann, U. and Herschowitz, N. (1982) *Acta Neuropathol. (Berl.)* **58**, 64–68
- Jezyk, P. F., Haskins, M. E. and Newman, L. R. (1986) *J. Am. Vet. Med. Assoc.* **189**, 1483–1485
- Maenhout, T., Kint, J. A., Dacremont, G., Ducatelle, R., Leroy, J. G. and Hoorens, J. K. (1988) *Vet. Rec.* **122**, 351–354
- Walkley, S. U., Thrall, M. A., Dobrenis, K., Huang, M., March, P. A., Siegel, D. A. and Wurzelmann, S. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2970–2974
- Walkley, S., Thall, M., Dobrenis, K., March, P. and Wurzelmann, S. (1994) *Brain Pathol.* **4**, 376
- Barranger, J. A. (1996) *Gene Ther.* **3**, 379–380
- Tollersrud, O. K. and Aronson, Jr., N. N. (1989) *Biochem. J.* **260**, 101–108
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467

- 19 Riise, H. M. F., Berg, T., Nilssen, Ø., Romeo, G., Tollersrud, O. K. and Ceccherini, I. (1997) *Genomics* **42**, 200–207
- 20 Devereux, J., Haeberli, P. and Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395
- 21 von Heijne, G. (1984) *J. Mol. Biol.* **173**, 243–251
- 22 Clark, J. M. (1988) *Nucleic Acids Res.* **16**, 9677–9686
- 23 Bucher, P. (1990) *J. Mol. Biol.* **212**, 563–578
- 24 Williams, T. and Tjian, R. (1991) *Genes Dev.* **5**, 670–682
- 25 Kageyama, R., Merlino, G. T. and Pastan, I. (1989) *J. Biol. Chem.* **264**, 15508–15514
- 26 Dynan, W. S. (1986) *Trends Genet.* **2**, 196–197
- 27 Stein, C., Gieselmann, V., Kreysing, J., Schmidt, B., Pohlmann, R., Waheed, A., Meyer, H. E., O'Brien, J. S. and von Figura, K. (1989) *J. Biol. Chem.* **264**, 1252–1259
- 28 Chen, H., Leipprandt, J. R., Traviss, C. E., Sopher, B. L., Jones, M. Z., Cavanagh, K. T. and Friderici, K. H. (1995) *J. Biol. Chem.* **270**, 3841–3848
- 29 Stoltzfus, L. J., Sosa Pineda, B., Moskowitz, S. M., Menon, K. P., Diott, B., Hooper, L., Teplow, D. B., Shull, R. M. and Neufeld, E. F. (1992) *J. Biol. Chem.* **267**, 6570–6575
- 30 Bennet, J. K., Dembure, P. P. and Elsas, L. J. (1995) *Am. J. Med. Genet.* **55**, 21–26
- 31 Gieselmann, V. (1995) *Biochim. Biophys. Acta* **1270**, 103–136

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