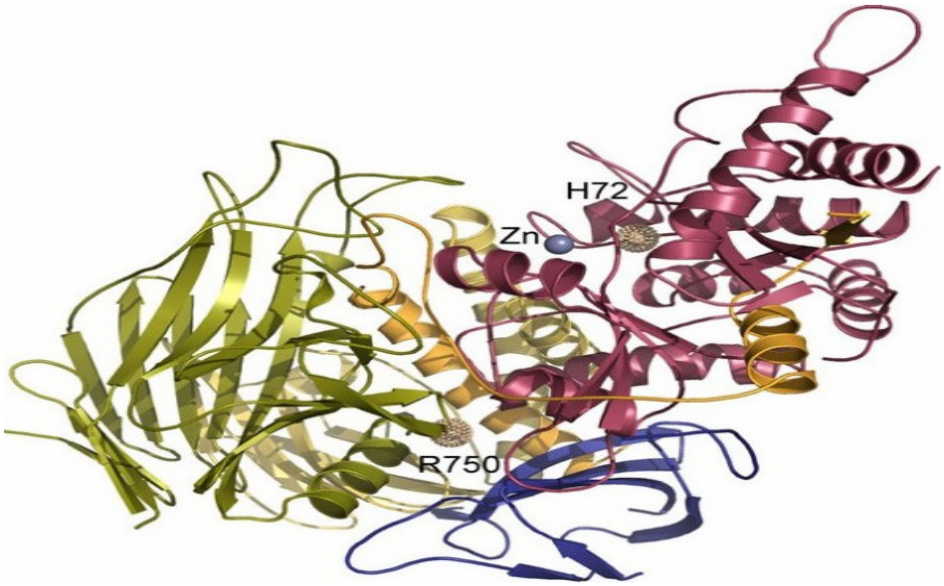
Functional characterization of alpha-mannosidosis associated sequence variants



By
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A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science in Molecular Biology

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December.2008

Table of contents

Acknowledgements

This work was performed at the Department of Medical genetic, university hospital of north-Norway, University of Tromsø, from august 2008 to December 20008.

First, I deeply acknowledge my supervisor, Hilde Monica F.Riise Stensland and co-supervisor, Øivind Nilssen for their fantastic support in guidance and proofreading through this study. My special thanks are to Vigdis Brox, Elisabeth Buvang and Mona Nystad for their great help. They introduced me with most of the techniques I used in this work which meana a lot to me.

I would like to thank Bjørn Hermansen and Karin Eilertsen for their support in administrative work.
Thanka to all members of the Medical Genetics research group for their contribution to the excellent scientific and social atmosphere I enjoyed during my work.

Finally, I would like to thank to my parents for always supporting me. Special greetings to my friends for creating a good social environment during my study time in Tromsø.

Abstract

Alpha-mannosidosis is a rare autosomal recessive lysosomal-storage disorder resulting from deficiency of lysosomal alpha-mannosidase activity. The disease shows a wide range of clinical phenotypes caused by intracellular accumulation of mannose-containing oligosaccharides, which ultimately may lead to mental retardation, hearing loss, skeletal changes and immune deficiency. Lysosomal alpha-mannosidase is a hydrolase that cleaves alpha-linked mannose residues from the non-reducing end of N-linked glycans of glycoproteins. So far, 40 alpha-mannosidosis associatied sequences have been reported, but no correlation of clinical and disease associated sequence variations has been detected so far. In previous work, seven of alpha-mannosidosis sequence variants were identified. Missense mutation p.H200L was previously reported as alpha-mannosidosis associated mutation (Sbaragli *et al*., 2005).

This study was undertaken as part of the functional characterization of mutant alpha-mannosidase. The mutations were introduced into an expression vector containing the wild-type LAMAN cDNA by *in vitro* mutagenesis, and the resulting proteins were expressed in COS-7 and BHK cells. Mutants p.C55F and p.R750W showed activity less than 8% of wild-type. The mutants were misfolded in the ER as inactive single-chain form and contained endo H sensitive N-glycans. The mutant p.P263L showed 8-10% of normal activity, whereas p.S318L, p.S453P, p.V457E and p.T745R expressed high residual activity (18-34%). They were processed into peptides abc and d which contained both high mannose and complex N-glycans, suggesting that a fraction of these mutants were transported to the lysosomes. The mutant p.T745R precursor contained only endo H sensitive glycans. It was proteolytically processed into the abc and d peptide but was not secreted into the medium. The mutant p.C55F was transport-arrested and accumulated in the endoplasmic reticulum (ER). In contrast, the mutant p.H200L, p.P263L, p.S.318L, p.S453F, p.V457E and p.T745R allowed a small fraction of LAMAN to be transported to the lysosomes.

Abbreviations

aa	amino acids
amp	ampicillin
BHK	Baby hamster kidney
BSA	Bovine serum albumin
cDNA	complementary DNA
CHO	Chinese hamster ovary
COS-7	African green monkey kidney cells
DMEM	Dulbecco's modified Eagle's Medium
DNA	deoxyribonucleic acid
EDTA	Ethylene Diamine Tetraacetic Acid
ER	endoplasmic reticulum
ERT	Enzyme replacement therapy
FBS	Fetal bovine Serum
GlcNac	N-acetylglucosamine
Kb	kilo base
kDa	kilo Dalton
LAMAN	Lysosomal alpha mannosidase
LAMP 1	Lysosomal-associated membrane protein 1
LSD	Lysosomal Storage Disease
Man	Mannose
Man-6-P	Mannose-6-phosphate
MEM	Minimum essential medium
Met	Methionine
OD	optical density
PBS	Phosphate-buffered saline
PDI	Protein disulfide isomerase
PCR	Polymerase chain reaction
rpm	revolution per minutes
RT	Room temperature
TGN	Trans Golgi network
3-D	Three-dimensional

Introduction

Glycoproteins

Glycoproteins are proteins that have oligosaccharide chains covalently attached to their polypeptide side-chains (reviewed in Varki, 1993). The oligosaccharide chains are added to the proteins during the cotranslational or posttranslational modification process known as glycosylation. The glycoproteins are synthesized by the attachement of sugar chains to the amide nitrogen on the side chain of asparagine (N-glycosylation) or to the hydroxyl oxygen on the side chain of hydroxylysine, hydroxyproline, serine, or threonine (O-glycosylation)(reviewed in Helenius and Aebi, 2001). The N-linked glycans play important functions such as promoting protein folding, quality control, intracellular sorting and transport to the proteins final destination (Helenius and Aebi, 2001). Ultimately, the glycans are degraded in the lysosomes by a group of exoglycosidases acting at the nonreducing termini, and by endo-ß-N-acetylglycosaminidase and aspartylglycosaminidase at the reducing end (Aronson *et al*., 1989). Specific deficiences of these enzymes result in oligosaccharide accumulation that cause lysosomal storage diseases such as alpha-Mannosidosis, ß-Mannosidosis, Fucosidosis and Sialidosis (Winchester *et al*., 2000).

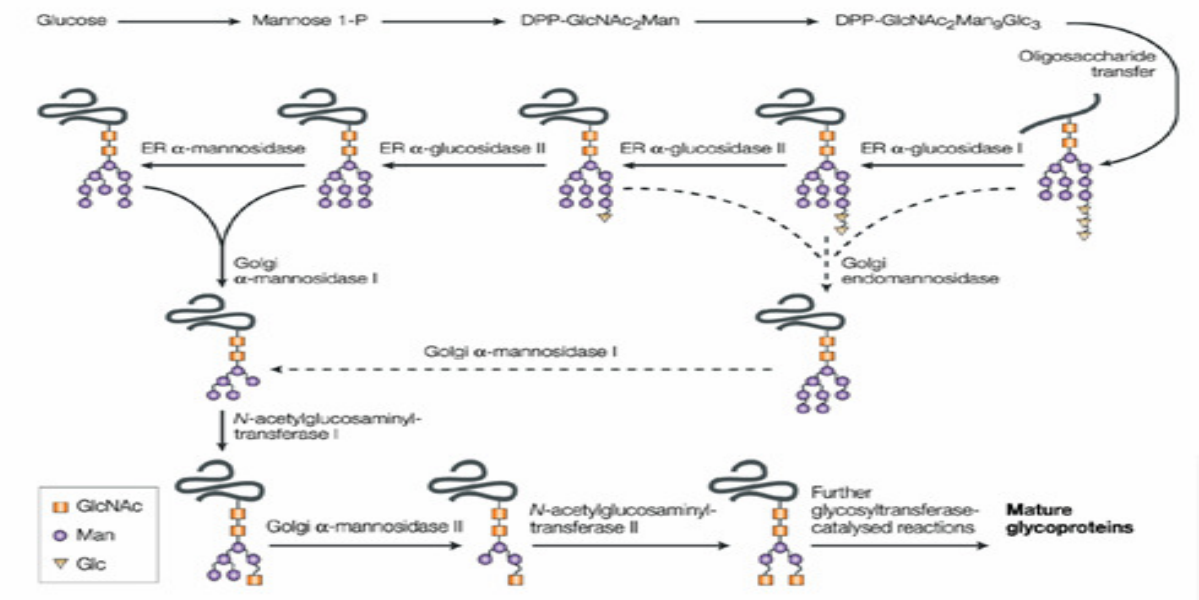


Figure 1 The biosynthesis of N-linked oligosaccharides. A block of 14 sugars is added to the newly synthesized polypeptide in the ER. The N-glycan is subjected to extensive modification as the glycoprotein move through ER and Golgi (Walsh *et al*., 2006).

Most glycoconjugates are degraded in the lysosomes, and a portion of the liberated monosaccharides are reused for glycoprotein synthesis (Freeze ,1999) . The breakdown of N-glycans from glycoproteins and glycopeptides is ordered and highly specific (Figure 2).

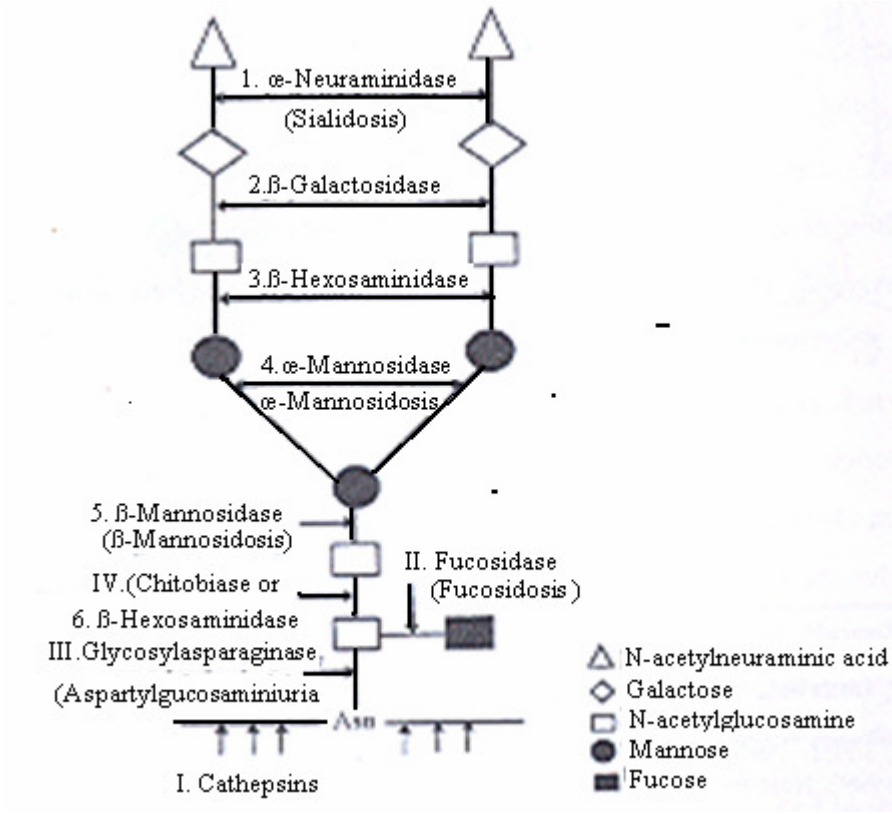


Figure 2 Lysosomal degradation of complex N-linked glycoproteins. The figure shows two sets of reactions (1-6 and I-IV) occuring idenpendently of each other. In reaction 1-6, exoglycosidases act stepwise in the removal of oligosaccharides from the non-reducing end. In reaction I-IV the protein and the protein-to-carbohydrate linkage region is hydrolysed (Aronson and Kuranda 1989).

Lysosomes and lysosomal enzymes

Lysosomes are intracellular organelles required for the final digestion of macromolecules such as proteins, lipids, carbohydrates and nucleic acids (Varki, 1999) The degradation of macromolecules is carried out by the action of at least 60 distinct hydrolytic enzymes, including proteases, nucleases, lipases, sulphatases, phosphatases and glycosidases (Mader, 2007). Most of these enzymes have been characterized and are active at low pH (de Duve *et al*., 1995). The lysosomal enzymes are synthesized on the ER membrane and follow the initial route of the secretion pathway (Figure 3).

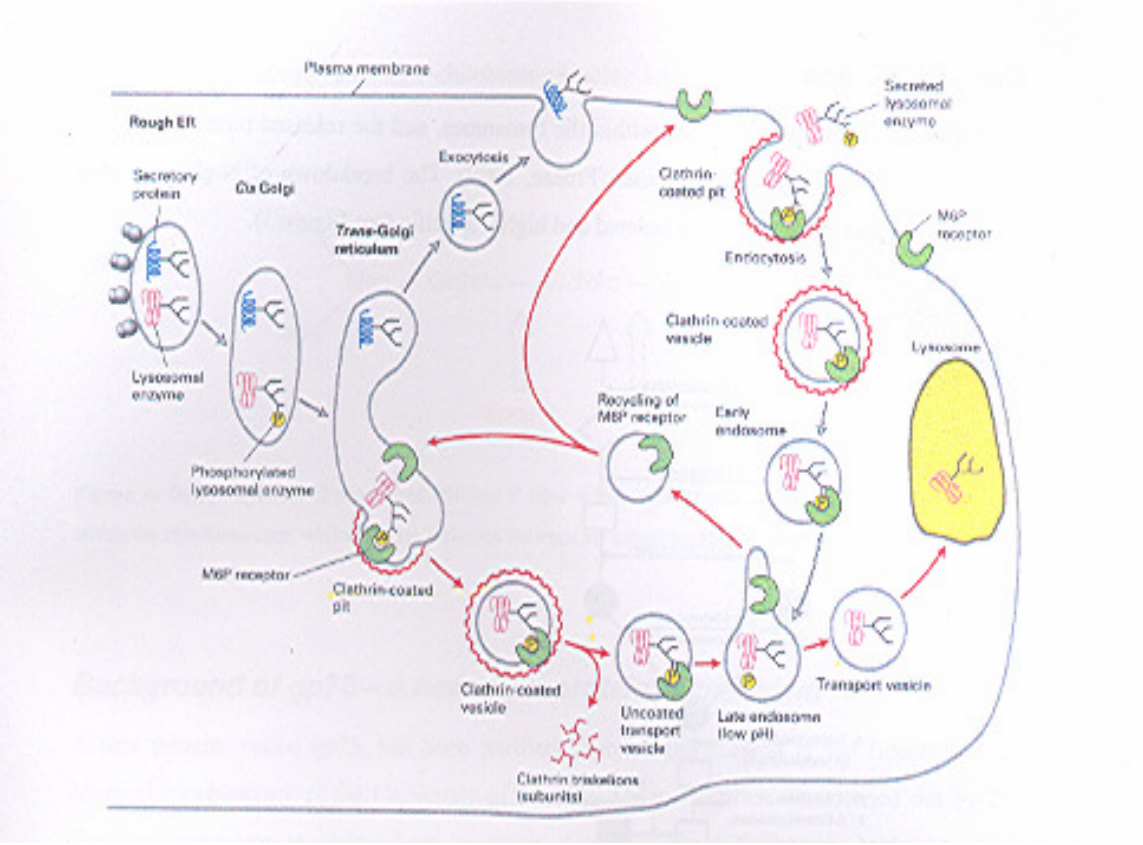


Figure 3 intracellular targeting of lysosomal (pink) and secretory (blue) proteins. The lysosomal protein follows the M-6-P pathway, while the secretory protein does not acquire the M-6-P-marker and is thus secreted (from Lodish *et al*., 1999).

The synthesis is initiated by the translation of a signal sequence, which is recognised by a signal recognition particle (SRP). Interaction with the SRP results in the translocation of the polypeptide into the lumen of the ER. In the ER, the protein is N-glycosylated by the oligosaccharide-transferase complex which transfers the oligosaccharide precursor to the consensus sequence Asn-X-Ser/Thr (where X is any amino acid except proline)

of a nascent chain polypeptide (Silberstein and Gilmore 1996). Following a series of trimming reactions of the N-glycans, the lysosomal proteins are properly folded and transported to the cis-Golgi for addition of the mannose-6-phosphate marker (M-6-P). This process involves two enzymatic steps (Waheed *et al*., 1981). In the first step, the enzyme UDP-N-acetylglucosamine 1- phosphotransferase recognizes and transfers a alpha-*N*-acetylglucosamine 1-phosphate residue to the 6-position of a mannose on a high-mannose oligosaccharide as the protein is transported from the ER to the Golgi apparatus (Sousa and Parodi, 1995). In the second step, the N-acetylglucosamine residue is trimmed from the oligossacharide chains by a glucosidase (N-acetylglucosamine 1-phosphodiester-*N*-acetylglucosaminidase) exposing it for recognition and uptake by the mannose 6-phospate receptor (Varki *et al*., 1983). After reaching the trans-golgi network (TGN), the phosphorylated mannose residues bind to the receptor, which direct the packing into transport vesicles. The lysosomal protein is recognised by two independent M-6-P receptors with different specificity that direct their transfer to lysosomes. After binding of the enzymes to the receptors, the complexes are incorporated into clathrin-coated vesicles that bud off from the trans-Golgi network and fuse with acidic prelysosomal compartments termed endosomes. The low pH dissociates the lysosomal enzyme from the M-6-P receptor, and phosphatases remove the phosphate from the lysosomal enzyme to prevent rebinding to the receptor. The receptors are then returned to the Golgi complex for reultilization, while the lysosomal enzymes enter the lysosomes through fusion between endosomes and lysosomes (see Schmid *et al*., 1997).

Secretion of lysosomal enzymes

Many cell types secrete lysosomal enzymes. The enzymes are secreted either as higher molecular weight precursors or as proteolytically processed mature forms (Jessyp *et al*., 1985). In mammalian cells, secretion of the lysosomal enzymes may be result of the absence of specific targeting signals (Reitman *et al.*, 1981), or defective or absence of receptors (Robbins and Myerowitz *et al.,* 1981). In this case the lysosomal enzymes never home to the lysosomes and are instead secreted directly from the Golgi (Figure 3). Secretion of lysosomal proteins in high-producer cells , may be caused by saturation of the sorting system in the trans Golgi network (Ling *et al*., 1993) .

Lysosomal alpha-mannosidase

Lysosomal alpha-mannosidase (MAN2B1; EC 3.2.1.24) is a member of the Glycosyl Hydrolase family 38 (Henrissat and Bairoch, 1996). It is an acidic exoglycosidase, which cleaves alpha-linked mannose residues from the nonreducing end of *N*-linked glycoproteins (al Daher *et al*., 1992; DeGasperi et *al*., 1991) during the ordered degradation of N-linked oligosaccharides . In addition to the cleavage of the natural substrates, the enzyme is also specific for the synthetic substrates, p-nitrophenyl- alpha-D-mannopyranoside and 4-methylumbelliferyl alpha-D-mannopyranoside (DeGaseri *et al*., 1991). The three-dimensional structure of two GH38 enzymes, Golgi alpha-mannosidase II from *Drosophila melanogaster* (Elsen *et al.*, 2000) and LAMAN from bovine kidney have been solved (Heikinheimo *et al.*, 2003).

The human LAMAN has been purified from various tissues. The human placenta LAMAN is synthesized as a polypeptide of 1011 amino acids (Nilssen *et al*., 1997). The enzyme is post-translationally modified by N-glycosylation, disulphide bridge formation, proteolysis and zinc binding (Nilssen *et a.l*, 1997). The enzyme is synthesized as a precursor that is active in various cell types (Thomas and Beudet , 2001) and processed into three glycopeptides of 70, 42 and 15 kDa (Nilssen *et al*, 1997), the 70 kDa peptide abc is further processes in bovine kidney (Tollerud *et al.,* 1997), but this peptide is only partially processed in the human enzyme (Nilssen *et al*., 1997)

The human and bovine LAMAN contain 11 and 8 potential N-glycosylation sites, respectively. Most N-glycosylation sites of the human LAMAN enzyme are occupied by high mannose and complex type N-linked oligosaccharides (Nilsen *et al.,* 1997). In addition, the bovine LAMAN also contains hybrid-type glycans (Tollersrud *et al*., 1997; Faid *et al.*, 2006). The p.N497 glycosylation site is evolutionary conserved among LAMANs, and it may be important for the stability of the enzyme. Both the secreted and the lysosomal forms contain endoglucosidase H complex type glycans suggesting a common pathway through the trans Golgi network (Hansen *et al*., 2004).

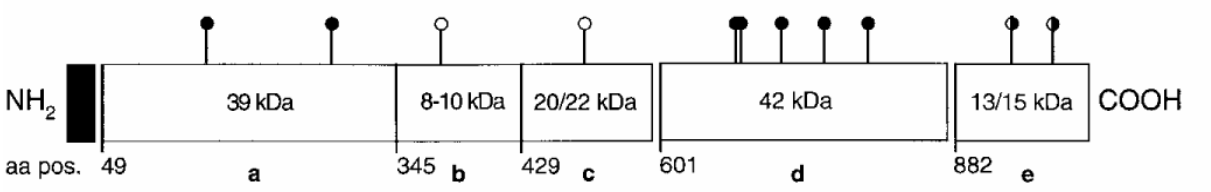


Figure 4. **Human placenta LAMAN**. The LAMAN peptides are labelled with a, b, c, d and e . The black filled box shows the signal peptide. The amino acid numbers under the diagram indicate the cleavage site positions. The 11 N-glycosylation sites are shown as "lollipops" above the diagram. Open circles indicate high mannose type oligosscharides whereas filled circles indicated complex type oligossacharides. The type of oligossacharides linked to the e-peptide is still unknown (Nilssen *et al*., 1997).

The gene encoding lysosomal alpha-mannosidase (Genbank accession no. U60266.1) has been localized close to the centromere of chromosome 19 (19p13.2) (Gonzalez *et al*., 1999). The gene spans approximately 22 kb and consists of 24 exons. The LAMAN transcript is about 3,5 kb and contains an open reading frame encoding 1011 amino acids with two potential initiation sites for translation (Nilssen *et al*., 1997). The 5' flanking region of the gene does not contain TATA or CAAT boxes, but several GC rich regions and putative biding sites for the transcription factor Sp1 have been identified. Multiple transcription start sites have been identified at posistion -191,-196 and -309 and at position -28 and -20 (Riise *et al*., 1997). The exons 2-14 encode polypeptide "abc", whereas peptide "d" is mainly encoded by exons 15-21, and the smallest polypeptide " e" is encoded by exons 22-24.

Lysosomal storage disorders

Lysosomal storage disorders (LSD) are inherited metabolic disorders that result from defects in lysosomal function (see table 1).There are more than 45 different diseases caused by defect of lysosomal degradation of macromolecules (Neufeld, 1991; Gieselmann, 1995). Gaucher disease was the first of these disorders to be described, in 1882, followed by Fabry disease in 1898 (Winchester *et al*., 2000). Most of these disorders are autosomal recessively inherited, however a few are X-linked recessively inherited, such as Fabry disease and Hunter syndrome. The general feature of these diseases is the accumulation of undigested products that result in disruption of the

normal function of lysosomes, and eventually leading to cell death (Beutler *et al*., 2001). Accumulation of the undigested products are mainly caused by deficiency of the hydrolytic enzymes. The LSDs show a wide spectrum of clinical phenotypes. The severity of a given LSD depends partially on the type of storage material product and on which cells or tissues that accumulate the storage material. However, genetic background and other factors also influence the disease outcome.

Table 1 Examples of major groups of Lysosomal storage diseases.

Disease (s)	Enzyme defect	Accumulated material
Sphingolipidosis GM 1	Beta-galactosidase	GM_1 ganglioside
Tay-Sachs disease GM_2	Hexosaminidase A	GM_2 gangliosides
Sandhoff's disease GM_2	Hexosaminidase A	GM_2 gangliosidosis
Krabbe's disease	Galactosyl ceramid beta-	Galatocerebrosides
Nieman-pick disease, type	Sphiingomyelinase	Sphingomyelin
Gaucher's disease	beta-glucocerebrosidase	Glucosylceramide
Fabry's disease	alpha-Galactosidase A	Glucosyceriamide
Glycoprotein storage	alpha-Fucosidase	Trihexosylceramide
diseases	**alpha-Mannosidase**	Glycopeptides

Alpha-mannosidosis

Alpha-mannosidosis (MIM 248500) is an rare autosomal recessive lysosomal-storage disorder caused by the deficiency of LAMAN (Thomas and Beaudet 1995). The disease has been characterized in man (Ockermann, 1967), cattle (Hocking *et al*.1972; Healy *et al*., 1990), cats (Burditt *et al*., 1980; Cumming *et al*., 1988) and guinea pigs (Crawley *et a*l.,1999). Carrier parents have a risk of having an affected child of 25 percent. A prevalence is of about 1:500000. Typical clinical symptoms are coarse facial features, mental retardation, variable hepatosplenomegaly, hearing loss, and dysostois muliplex (Thomas and Beaudet, 1995). Alpha-mannosidosis shows a wide range of clinical phenotypes with varying degrees of severity. The variation in clinical

phenotype is not only caused by lack of enzyme activity, it may be due to both environmental factors and genetic factors. Lacking of enzyme activity found in patients who have different clinical phenotypes (Berg *et al*., 1997). The clinical diagnosis of alpha-mannosidosis is based on detection of mannose-rich oligosaccharides, and direct measurements of alpha-mannosidase activity in various cell types, such as leukocytes, fibroblasts, and amniocytes (Chester *et al*., 1982; Thomas and Beaudet,1995).

So far, more than 110 alpha-mannosidosis-associated sequence variants have been detected in over 170 unrelated patients (Riise Stensland *et al.*, manuscript in preparation). Forty alpha-mannosidosis-associated sequence variants have been reported in litteratures (Nilssen *et al*., 1997; Gotoda *et al*., 1998; Berg *et al*., 1999; Becarri *et al*., 2003; Olmez *et al*., 2004; Urushihara *et al*., 2004; Sbaragli *et al*., 2005; Castelnovo *et al*., 2007; Lyons *et al*., 2007; Pittis *et al*., 2007;). Most of alpha-mannosidosis-associated sequence variants occur in one or a few families, but the specific sequence variant mutation c.2248C>T (p.R750W) appears to account for a large fraction of alpha-mannosidosis disease alleles. The mutation was identified in 13 patients from different European countries (Berg *et al*., 1999). There is no clear correlation between clinical phenotype and disease-associated sequence variants, and disease-causing variants are responsible for the inactivation of LAMAN by misfolding and mislocation of the LAMAN enzyme or by active site distortion (Hansen *et al*., 2004). Mutant LAMAN enzyme may be mislocalized to nonlysosomal compartments, even though containing residual activity upon testing at the appropiate pH (Nilssen *et al*., 1997). Therefore, to establish the relationship between genotype and phenotype suggested, the enzyme activity should be measured on fractionated lysosomes.

There is no effective therapy for alpha-mannosidosis, but when the purified active enzyme was added to the medium of mannosidase-deficient fibroblasts, the accumulation of lysosomal storage products was corrected (Abraham *et al*, 1985). ERT have been performed on alpha-mannosidosis mouse, the result showed to decrease in mannose-containing oligosaccharides in tissues, including brain (Roces *et al*., 2004). Therefore, it is possible that ERT could be used to treat alpha-mannosidosis in humans in the future. The lysosomal vacuoles in alpha mannosidosis guinea pigs decreased markedly in liver, kidney, spleen, pancreas and trigeminal ganglion neurons (Crawley *et al*., 2006), but in a guinea-pig model, no histologic changes were seen in the brain. ERT has not been tested on human. Bone-marrow transplantations (BMTs) have been

performed in alpha-mannosidosis with varying outcomes. Early BMT attempts were unsuccessful in one child (Will *et al*., 1987) and successful in another (Wall *et al*., 1998). The donors are not available for the majority of alpha-mannosidosis patients. BMT had been performed in feline alpha-mannosidosis, where BMT has led to replacement of alpha-mannosidosis activity in cells of the central nervous system (Walkey *et al*., 1994).

Aims of this study

In previous work (Nguyen, 2008 and Stensland *et al*., manuscript in preparation), the alpha-mannosidosis associated sequence variants, p.C55F, p.H200L, p.P263L, p.S318L, p.S453F, p.S457E and p.T745R in the LAMAN gene were identified. The mutant, p.H200L had been suggested to be disease-associated based on activity measurements and modelling into the three dimensional structure, but the consequences on intracellular processing and localization were not investigated.

This study was undertaken to characterize these sequence variants at the biochemical and cellular level to study their effect on the LAMAN protein and to investigate if they are disease-causing mutations.

Specific goals were:

- Enzyme activity analysis of mutant LAMANs
- characterization the intracellular processing and localization of mutant LAMANs.

Subjects and Methods

Subjects

In a previous work (Nguyen 2008), putative alpha-mannosidosis associated sequence variants were detected in the patient from three unrelated families from Grea Britain , Italy and Chile. Four other putative disease-associated sequence variants were detected in four unrelated patients from Turkey, Great Britain and Germany, respectively (Riise Stensland *et al.*, manuscript in preparation) (See table 2). All patients were diagnosed with alpha-mannosidosis by enzyme assays/thin layer chromatography at the referring center , and DNA and/or blood samples from patients and family members were provided by their physicians. The study was a part of the HUE-MAN project and approved by the Regional Ethics Committee, University of Tromsø, Norway.

Table 2 Patients with alpha-mannosidosis and LAMAN mutations investigated in this study . Mutations in black color were detected in my project work (Nguyen 2008), mutations in green color were identified in previous work (Riise Stensland *et al*., unpublished). "Sequence variants were named as recommended (Den Dunnen and Antonarakis, 2000) as well as from the online version from the Human Genome Variation Society. (www.hgvs.org/mutnomen/). Nucleotide positions are as in the human MAN2B1 reference sequence (GenBank accession no U60266) where position +1 corresponds to A in the ATG translation initiation codon."

cDNA level		**Protein level**	**Exon**	**patient**	**Origin**
Alle 1	alle 2	Mutation name			
[c.1371T>A]	[c.2248C>T]	[p.V457E]+[p.R750W]	11 , 18	1a-b	Great Britain
[c.165G>T]	[c.599A>T]	[p.C55F]+[p.H200L]	2, 4	2a-b	Italy
[c.2248C>T]	[c.2248C>T]	p.R750W]+[p.R750W]	18	3	Chile
[c.788C>T]	[c.2355G>A]	[p.P263L]+[splicing mutation]	6	4	Turkey
c.909+731del6272	[c.953C>T]	(IVS6+731del6272) +[p.S318L]	7	5	Great Britain
[c.1358C>T]	[c.1358C>T]	[p.S43F]+ [p.S453F]	11	6a-b	Germany
[c.2234C>G]	[c.2234C>G]	[p.T745R]+ [p.T745R]	18	7	Turkey

Site-directed mutagenesis

The cDNA encoding the human LAMAN contained in the expression vector pcDNA 3.1 (Hansen *et al*, 2004) served as template for site-directed mutagenesis using the QuickChangeTM Site-Directed Mutagenesis Kit (Stratagene), and the primers were used are listed in the table A (see additional materials). The amplification was performed for 19 cycles. Each cycle consisted of 50 seconds denaturation at 95^{o}C, 50 seconds annealing at 60 ^{o}C, and 7 minutes extension at 68 ^{o}C. After PCR amplifcation, the reactions were placed on ice for 2 minutes to cool the reaction to ≤37°C. 1 µl of the *Dpn* I restriction enzyme (10 U/µl) added directly to each reactions and gently thoroughly mixed centrifuged for 1 minute and then immediately incubate at 37°C for 1 hour to digest the parental (i.e., the nonmutated) supercoiled dsDNA.

Transformation and culturing of bacterial cells

XL-10 Gold Ultra supercompetent cells (Stratagene) were transformed with the mutagenesis-mix as recomanded by the manual instruction. The 45 µl of supercompetent cells were added to 2µl of mutagenesis-mix and incubated on ice for 30 minutes, heat-shock for 45 seconds at 42 ^{o}C and immediately placed on ice. Cells were added 500 µl of SOC medium and incubated at 37 ^{o}C for 1 hour with shaking, plated on LA-plates containing 100 µg/ml ampicillin, and incubated at 37 ^{o}C overnight.

Five colonies from each plate were dispersed in 4 ml LB medium containing 100 µg/ml ampicillin to make over-night cultures and streaked separately on new amp plates to check for false positive colonies.

Plasmid purification

The plasmids from the 4 ml cultures or 60 ml overnight cultures were purified using Mini/Midi-preps DNA Kit (QIAGEN) according to manual instruction. The plasmid DNA was eluted and dissolved in sterile water. The plasmid concentrations were measured by spectrometry (Thermo Scientific NanoDrop^{TM} Spectrophotometers 1000).

DNA sequencing

The presence of mutation was verified by DNA sequencing (Bigdye 3.1 ABI). The LAMAN insert was sequenced for each mutant to ensure that no other mutation had been introduced by the PCR-based mutagenesis procedure. The sequencing reaction was run using the condition given in table 3. The primers 263R, 30F, 262F, 304bF, 305bF and 306bF were used to sequence the insert (see Riise *et al*., 1997).

Table 3 Cycling parameters for sequencing reactions

Cycle number	Parameter	Temperatur (^{o}C)	time
25	Denaturation	95	0 seconds
	Annealing	50	5 seconds
	Extension	60	4 minutes
	Hold	4	

Transfection of COS-7 , BHK-21 and CHO-K1 cells

COS-7, BHK-21 and CHO-K1 cells (CRL-1651; American Type Culture Collection) were cultured and maintained in DMEM (Dulbecco's modified Eagle's ,medium) (Gibco, Invitrogen), whereas BHK cells were maintained in MEM (minium essential Eagle's medium) (Gibco, Invitrogen) supplemented with 10 % FBS (foetal bovine serum) and antibiotics (penicillin and streptomycin) at 37 ^{o}C in 5% CO_2 incubator. For transfection, the cells were seeded in 6-wells plates at a density of 3.0 x 10^6 cells with 2 ml medium per well 1 day before transfection. The next day, cells were transfected with 2 µg of respective plasmid DNA diluted into 250 µl OPTI-MEM I Reduced Serum Medium (Invitrogen Life Technologies). The plasmid DNAs were added to a mix containing LipofectAMINE^{TM} 2000 (Invitrogen) diluted in reagent 250 µl OPTI-MEM reagent and incubated at RT for 20 minutes . After incubation, the medium was replaced by the fresh medium and added the plasmid-mix to each well. Cells were incubated for 12, 24, 36 and 48 hours. Each construct was transfected in duplicate for at least two independent experiments.

Enzyme activity analysis and protein concentration measurement.

The media from each well was removed for further analysis, were washed 2x with PBS and lysed in 250 µl MPER- extract reagent (Thermo Scientific) and protease inhibitor mixture (Roche Biochemicals). The mediasand cell lysates were centrifuged at 13000 rpm for 5 minutes, and the supernatants were transferred to new tubes. Enzymic activity was measured in the cell lysate and media by incubation with 4 mM *p*-nitrophenyl alpha-D-mannoyranoside, (pH 4.7)(Sigma) for 1 hour at 37 ^{o}C. The reaction was stopped by the addition of an equal volume of stopp-solution contained 13 mM glycine, 67 mM NaCl, and 83 mM Na_2CO_3 (pH 10.7). The absorbance was recorded at 405 nm; 1 unit of enzymic activity was defined as the amount of enzyme that liberated 1 µlmol of *p*-nitrophenol/min. The protein concentration was measured by the D_c-protein assay kit (Bio-Rad) as described by the supplier. The absorbances were recorded at 750nm (Microplate Reader with Softmaxr, biocompare). The enzyme activity and protein concentration measurement were performed in triplicate.

Western blot

Ten µg of protein and 10 µl of medium were added 2.5 µl of 5% SDS and 1.25 µl reducing agent (Invitrogen) and boiled at 100 ^{o}C for 10 minutes to denature the protein. After boiling, the samples, protein standard marker (Invitrogen) and Seeblue2 (Invitrogen) were centrifuged at 13000 rpm for 5 minutes and loaded to an 4-12% gradient gel for protein separation by SDS-PAGE. The gel was run for 60 minutes and blotted to PDVF membrane (Invitrogen) by electro-blotting. After 1.5 hour blotting, the membrane was washed in PBS. The membrane was incubated blocking solution (150 µl Tween, 7.5 g drymilk, 150 ml PBS). The membrane was incubated in blocking solution with 2 µl antibody (Zymenex provided by Denmark) against denaturated recombinant LAMAN diluted 1:7500 over night at 4 ^{o}C . The membrane was washed 3 times with PBST (0,1 % Tween (Sigma) in PBS) for 5 minutes, and then incubated in secondary antibody (Alkaline phosphatase-conjugated chicken-anti-rabbit IgG diluted 1:2000)(Santa Cruz Biotechnology) for 1 hour, and washed 4 times with PBST for 5 minutes. 3 ml of the CDP-star substrate (Roche Biochemicals) was added to the membrane and incubated in dark for 30 minutes. Finally, the immunocomplexes were visualized by using the FujiFILM Luminescence image analyser LAS-3000 instrument (Lifescience).

Deglycosylation

For *in vitro* studies of the glycan structure of glycoproteins, two different enzymes are usually used. Endoglycosidase H (endo H) is specific to high-mannose N-glycans. It digests within the chiobiose core and leaving one GlcNAc residue attached to the asparagines of the N-glycosylation sites (Figure 5). The second enzyme that has ability to cleavage the linkage between GlcNAC and asparagine to release N-linke glycans from glycopeptides called Peptide-N-glycan asparagine amidase F (PNGase F). Its enzymatic cleavage site is highly specific, with highly specific hydrolysis occuring between asparagine and GlcNAc of most high-mannose and complex type N-glycans. This liberates the oligosaccharide from the asparagine of glycosylation sites (Figure 5)

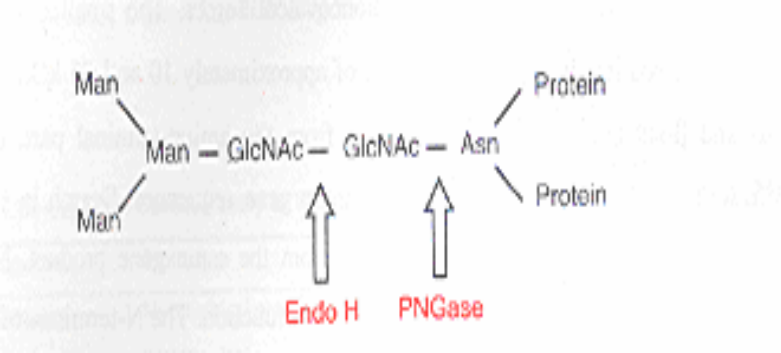


Figure 5. Lysosomal degradation of complex N-linked glycoproteins. Endo H cleaves within GlcNAc-GlcNAc while PNGase F between asparagine and the asparagine and the GlcNAc (Maley *et al*., 1989)

In order to study the type and structure of glycan attached to the LAMAN, the protein lysates were treated with Endo H (EC 3.2.1.96) and PNGase F(EC 3.5.1.52) (New England Biolabs). Generally 10 µg of cell lysates was added to a reaction sample of 10 µl contained 10x glycoprotein denaturate and H_2O. The reaction sample was heated for 10 min at 100 ^oC. For Endo H treatment, the heated sample was added 2 µl of 10x G5 reaction buffer (endo H buffer) , 3 µl of 500U/ml Endo H and H_2O to a total reaction volume of 20 µl. For PNGase F treatment, the sample was added 2 µl 10x G7 reaction buffer (PNGase F buffer), 2 µl of 10 % Nonidet P40, 1.5 µl PNGase F and H_2O to a reaction volume of 20 µl. The samples were incubated for 2 hours at 37 ^oC, added 5 µl of SDS and 2,5 µl reducing agent and boiled for 10 minutes at 100 ^oC. The samples were centrifuged, and supernatant was applied to an 4-12 % gradient SDS/PAGE gel and analysed as described above.

Immunofluorescence

COS-7 and BHK-21 cells were plated at a density of: 2 x 10^5 cells/well on coverslips in 6-well culture plates (4 coverslips per well) 1 day before transfection. The pcDNA3.1 containing wildtype lysosomal alpha-mannosidase construct was used as positive control and vector pcDNA3.1 lacking lysosomal alpha-mannosidase (mock construct) was used as negative control. Transfections were performed using 2.0 µg of plasmid DNA as described above.

After 40- 48 hours, cells were washed with 1x 2 ml PBS and 1x 1 ml serum free DMEM. Protein synthesis was stopped by incubation in 1 ml serum-free DMEM with 50µg/ml cyclohexamide (Sigma) and incubated for 2-3 hours at 37 ^oC. Cells were washed with 3x 2ml PBS, fixed in 1 ml ice-cold methanol by incubation for 6 minutes at -20 ^oC, followed by 2x PBS wash, incubated coverslips in blocking solution (0.5% BSA, 0.2 % saponin (Sigma) for 45 minutes at RT to block unspecific staining. Coverslips were dried on paper and transferred to 24-well plates and incubated for 45 min at RT in primary antibody diluted in blocking solution (40 µl primary antibody solution per coverslip). The following antibodies were used

-rabbit-anti-LAMAN denature bovine (1: 500 dilution) (Tollersrud *et al*., 1997)

-Mouse-anti-LAMP1 (H3A4, lysosomal marker, 1:200 dilution) (Thomas August, John Hopkins University , Baltimore, MD, USA)

-Mouse-anti-PDI (ER marker , 1:200 dilution) (Stressgen)

Cells were washed in 3 x 0.5 ml blocking solution and incubated with 40 µl of secondary antibody (molecular probes Alexa-488 goat-anti-rabbit IgG (Invitrogen) and Alexa-555 goat-anti-mouse IgG (Invitrogen) 1:500 dilution) for 45 minutes at RT. After washing with 3 x 0.5 ml PBS, the coverslips were dried and mounted upside-down in a drop of glycerol. The cells were examined by FISH microscope using two different filter sets: The “green“ called FITC (Fluorescein isothiocyanate) and the “red“ called TRITC (Tetramethylrhodamine 5-isothiocyanate). The cells were visualized and documented by using the CytoVision 3.9.11 (Applied Imaging).

Results

Optimization of transfection of COS-7, BHK-21 and CHO-k1 cells

The optimal growth time transfection conditions were established for COS-7 , BHK-21 and CHO-k1 cells using wild-type LAMAN and mock constructs. The cell lysates and medias were harvested after 12, 24, 36 and 48 hours post transfection, and enzyme activity, protein concentration measurement and Western blot analysis were performed. In COS-7 cells, the LAMAN activity was highest at 48 hours (20 fold higher than mock/background). In BHK the activity was 19 times higher than mock-transfected cells at 24 and 48 hours. Whereas, CHO cells produced highest alpha-mannosidase activity at 36 hours, but it was only 7x fold higher than in mock transfected cells. (Table 5 and figure 6)

Table 5 alpha-mannosidase activities in transfected CHO-k1, BHK and COS-7 cells.

Cell lines	Enzyme activity * (fold increase)			
	12 h	24h	36h	48h
CHO-k1	2	5	7	4
BHK-21	4	19	15	19
COS-7	2	7	7	20

* Enzyme activity is adjusted for protein concentration, OD405/OD750.

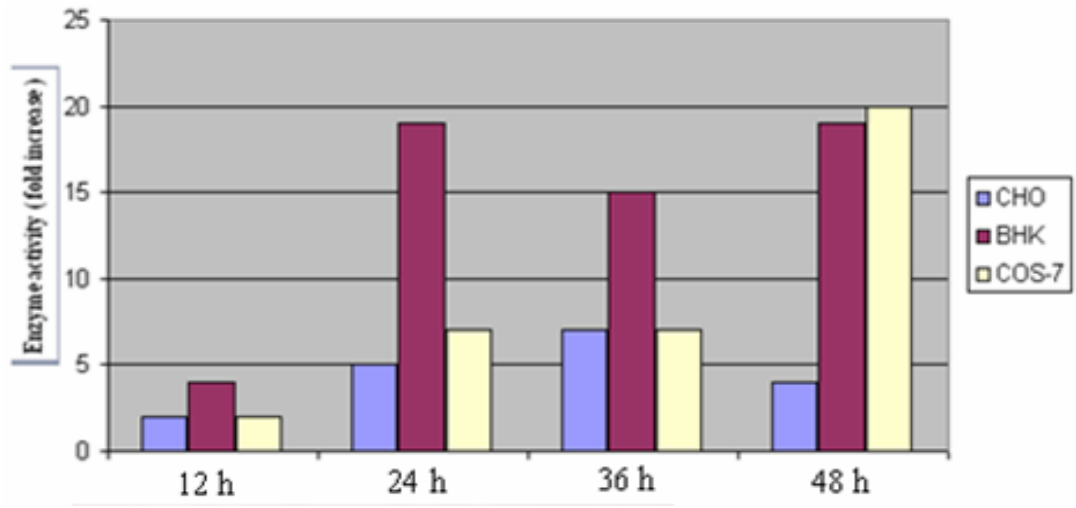


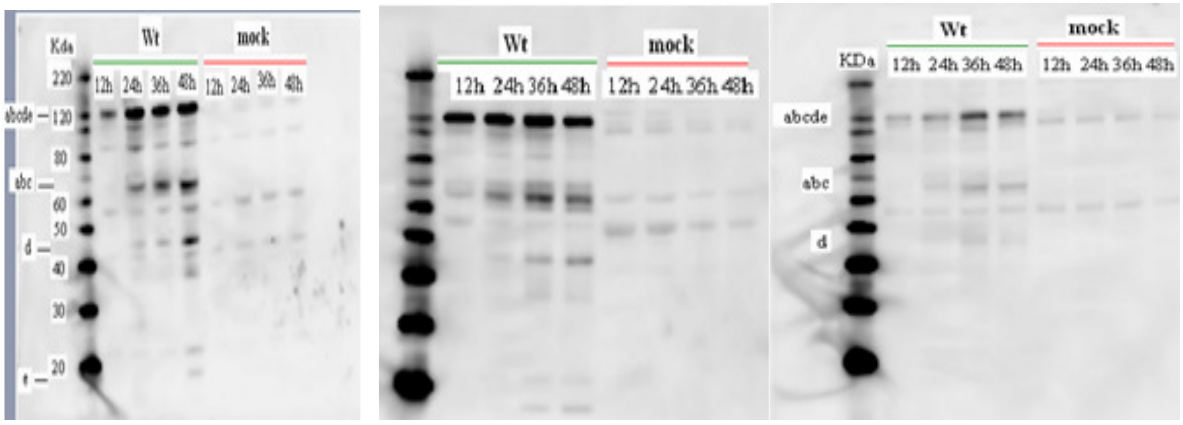
Figure 6 alpha-Mannosidase activities in cell-lysates

COS-7, BHK-21 and CHO-k1 cells were transfected with pcDNA3.1-LAMAN and mock- construct, and lysates were harvested after 12, 24, 36 and 48 h post transfection. Alpha-mannosidase activity is adjusted.

Western blot analyses showed that the processing of LAMAN in transfected COS-7 and BHK-21 cells is detectable after 24 hours, where proteolytically processing of the 120 kDa single-chain precursor into peptides abc, d and e is observed with highest band intensity after 48 hours (Figure 7). Secretion of the 120 kDa single-chain precursor into the media after highest at 48 hours. In transfected CHO cells, a slow proteolysis of LAMAN into peptides abc and d, with highest intensity after 36 hours. The extracellular form had reached the peak levels after 36 hours. Together the results of enzyme activity and western blot indicate that transfection incubation time for COS-7 BHK-21 cells should be at 36-48 hours, and 36 hours for CHO cells. The CHO cells were not used further due to the low expression of alpha-mannosiddase (7 fold) and slow proteolytically processing of LAMAN precursor.

A) Cell lysate

COS-7 **BHK** **CHO-K1**



B) Cell Media

COS-7 **BHK** **CHO-k1**

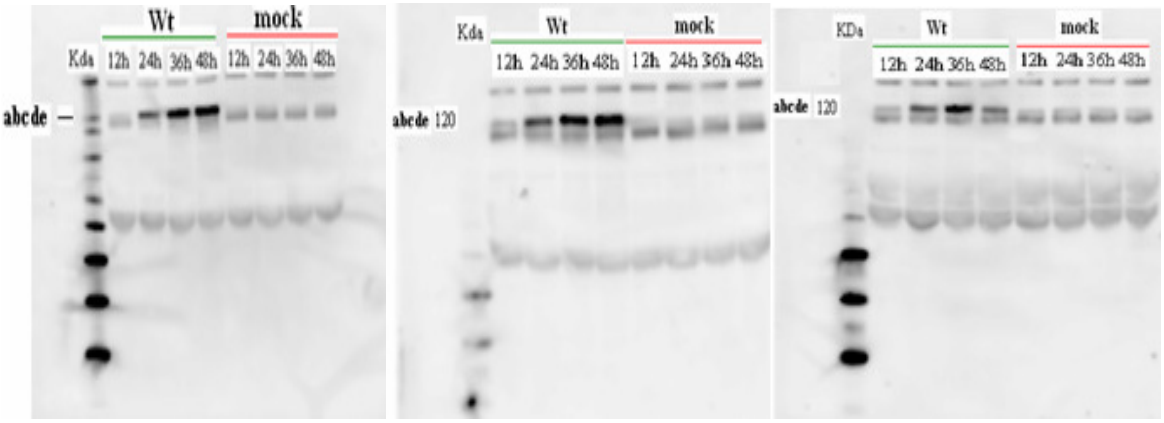


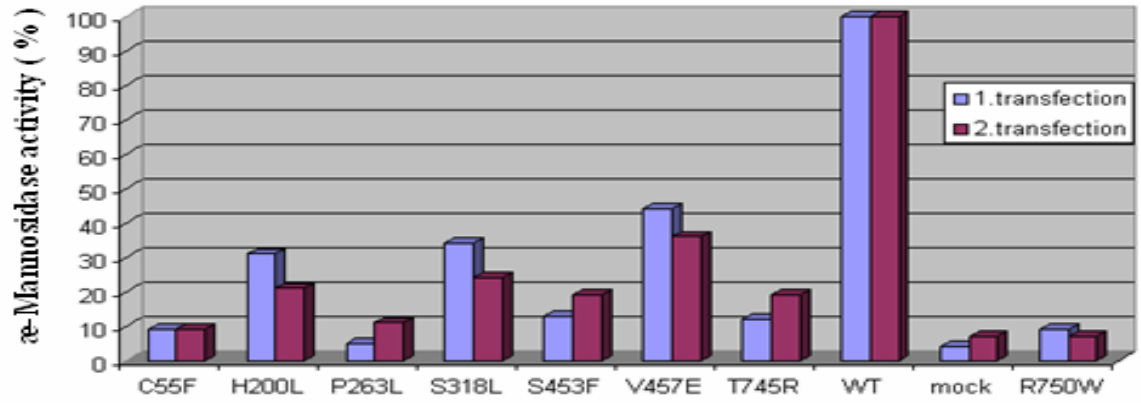
Figure 7 Western-blot analyses of wild-type (WT) human LAMAN

Ten µg of (COS-7 BHK), 20 µg of CHO protein lysates and 10 µl media from three cell lines transfected with wildtype LAMAN and mock were subjected to Western blot analysis using the primary antibody against denatured human LAMAN Molecular mass standards (Invitrogen) are indicated on the left side.

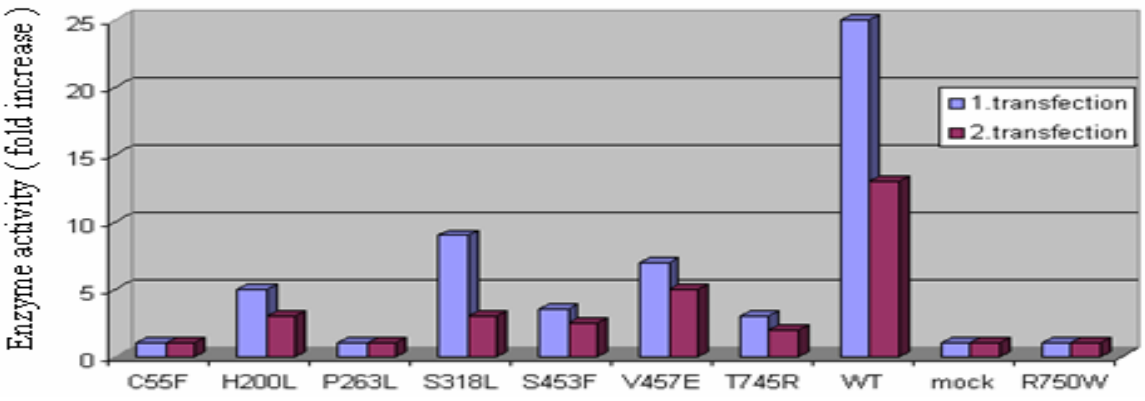
Alpha-mannosidase activity of mutant LAMANs in transfected COS-7 and BHK-21 cells

To establish whether the mutations p.C55F, p.H200L, p.P263L, p.S318L, p.S453F, p.V457E ,p.T745R, constructs affect alpha-mannosidase activity, we transiently expressed these mutant in COS-7 and BHK-21 Cells. The construct contained p.R750W was used as a negative controll as it shows no activity in transfected COS-7 cells (Hansen *et al*., 2004) Expression of the pcDNA3.1-p.H200L, p.S318L, p.S453F, p.V457E and p.T745R in COS-7 cells produced alpha-mannosidase activity that was 4-8 fold higher than that in mock-transfected cells (Figure 8 b). In BHK transfected cells alpha-mannosidase activity was 2-6 fold higher in mutant compared mock-transfected cells, respectively (figure 8 d). The activity of p.C55F, p.P263L and p.R750W showed no signficant increase over background levels (Figure 8). The LAMAN activity in media was higher than the intracellular level. Activity of mutant LAMANs (p.H200L, p.S318L, p.S453F, p.V453E) are about 2 fold higher than p.P263L in media (Figure 9), suggesting that these mutant enzyme were secreted into media more than p.P263L.

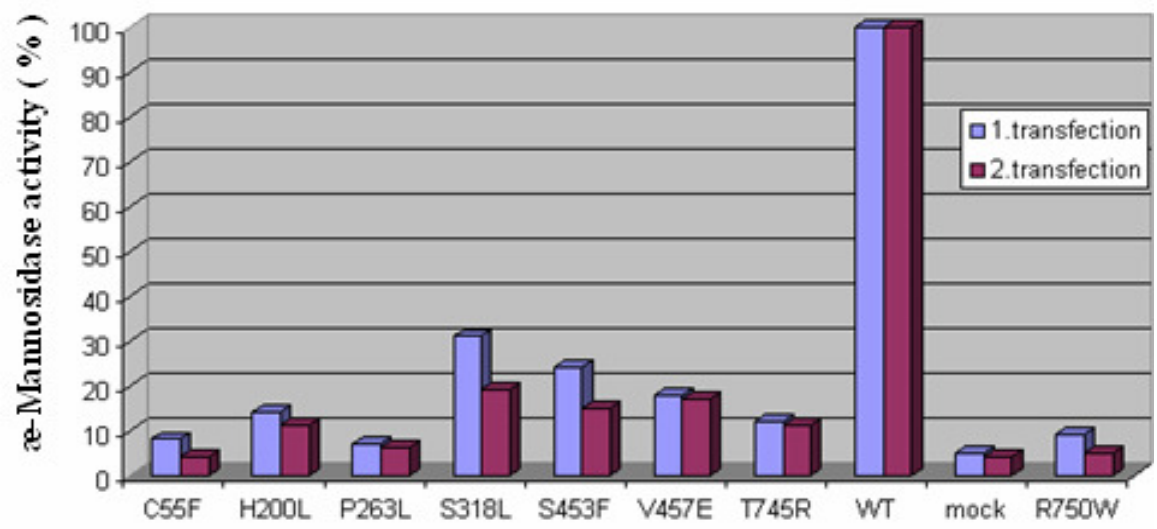
A) COS-7



B) COS-7



C) BHK



D) BHK

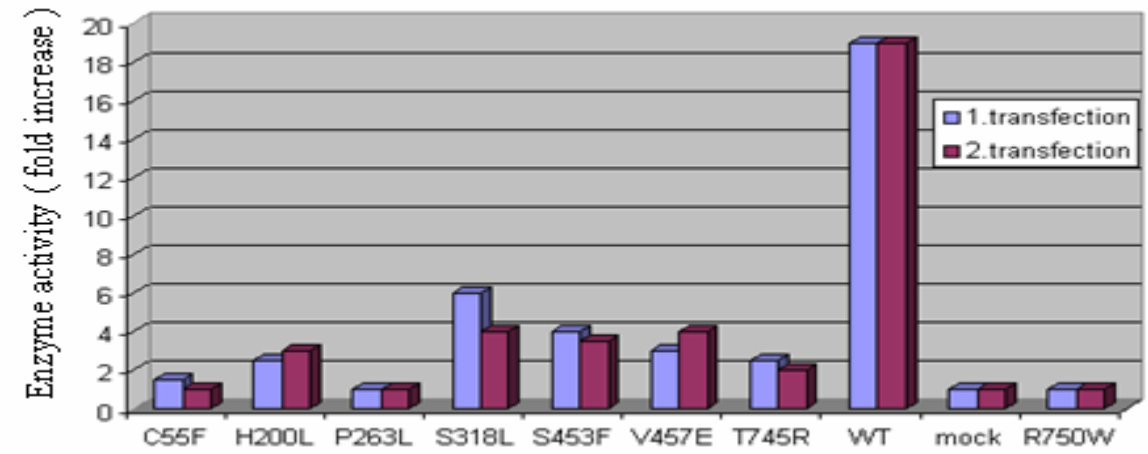
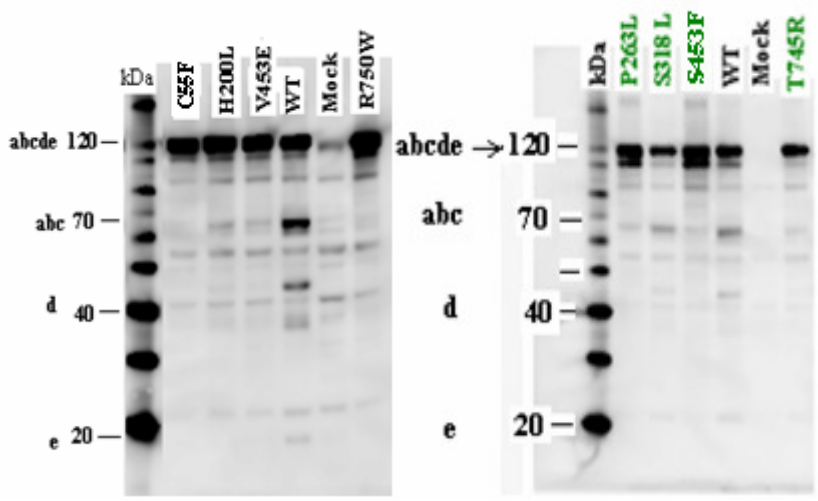


Figure 8 Alpha-mannosidase activities in transfected COS-7 and BHK-21 cells. COS-7 and BHK-21 transfected with 2µg control plasmid or plasmid containing mutant plasmid. Alpha- Mannosidase activity was measured in triplicate.

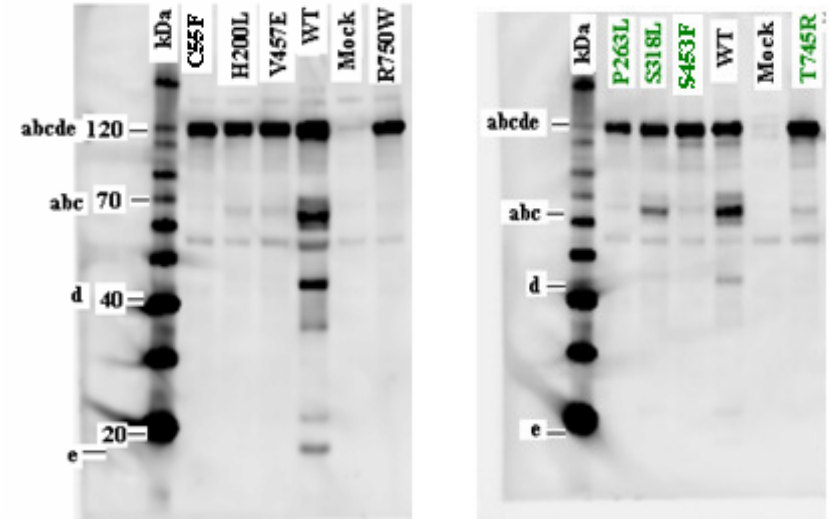
Processing of mutant LAMANs in transfected COS-7 and BHK-21 cells

In order to study intracellular processing of different LAMAN-variants, COS-7 and BHK cells were transfected with the 7 mutant pcDNA3.1-LAMAN constructs as well as p.R750W was used a a negative control of processing (Hansen et al., 2004). The lysates and mediums were harvested from transfected cells and subjected to Western blot analysis using LAMAN antibodies raised against human LAMAN (Figure 9). The LAMAN 120 kDa band, corresponding to the precursor abcde form of LAMAN, was detected in all lysates, except in the mock transfected cells (Figure 9). No further processing of LAMAN peptides abc and d could be observed for p.C55F and p.R750W (Figure 9a and b), and they were not secreted (Figure 9c and d). In contrast, the WT-, p.H200L, p.P263L, p.S318L, p.S453F, p.S457E and p.T745R –LAMANs were proteolytically processed as judged from the appearance of the 70 kDa peptide abc. The 40 kDa peptide d was only observed in WT and p.S318L. Mutant p.T745R was not secreted into media to any extent as judged from the very low abundance of 120 kDa single-chain precursor (Figure 9). The transfected COS-7 cells showed better LAMAN proteolytically processing than in BHK cells. The 15 kDa peptide e was observed only in wild-type. This result indicates that the mutant LAMAN p.C55F did not home to the lysosomes and was probably accumulated in the ER. Whereas, the mutant LAMAN, p.H200L, p.P263L, p.S318L, p.S453F, p.V457E and p.T745R were partly transported to the lysosomes as judged from appearance of peptide abc. The same pattern of processing was observed in BHK-21 (Figure 9d and b). COS-7 lysate was used for further analysis.

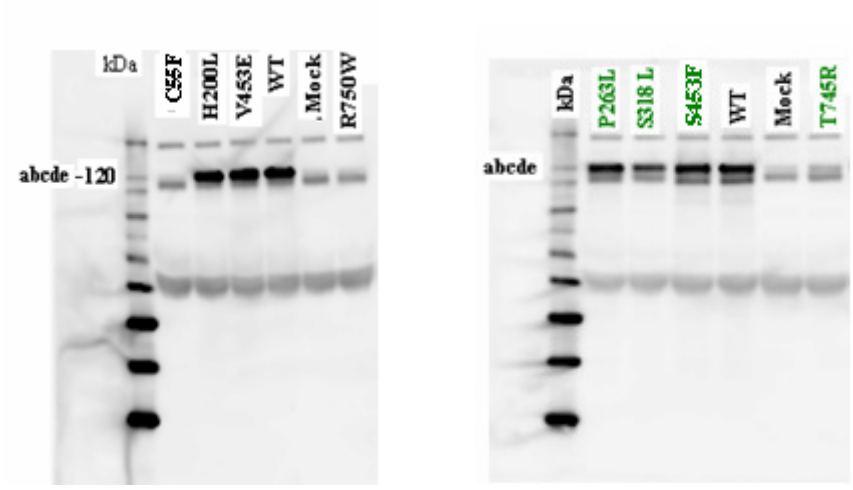
A) COS 7 lysates



B) BHK lysates



C) COS-7 media



D) BHK media

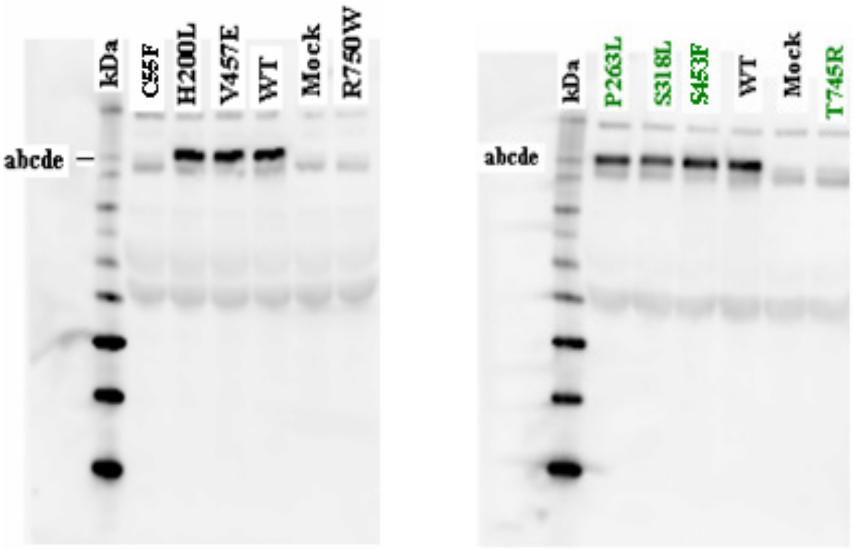


Figure 9 Western-blot analyses of mutant human LAMANs

Ten µg cell lysate and 10µl medium from COS-7 and BHK cells transfected with various mutant LAMAN constructs were subject to SDS/PAGE and electroblotted on to a PDVF membrane as described in Material and Methods section. The molecular masses of the LAMAN peptides are shown on the left side.

Glycosylation of mutant alpha-mannosidases

The human LAMAN enzyme is N-glycosylated by both high-mannose and complex type glycans (Nilssen *et al*., 1997; Berg *et al*., 1997 ; Faid *et al*., 2006, Hansen *et al*., 2004). In order to study the type of N-linked glycans on mutant LAMANs**,** the COS-7 cell lysates were treated with Endo H and PNGase F and separated by SDS-PAGE. The result showed a decrease in molecular weight of the precursors after treatment PNGase F, due to its removal of N-glycans. The 120 kDa precursor and 70 kDa peptide abc of mutant LAMANs, p.H200L, p.P263L, p.S318L, p.S453F and p.V457E were partially deglycosylated by endo H, indicating that these mutant LAMANs were occupied by both high-mannose and complex N-glycans (Figure 10). The presence of complex N-glycans indicates that these mutant LAMANs have been transported through Golgi.

The complete reduction of precursor abcde-petides of p.C55F after treatment with Endo H, indicates that this peptide contained mainly of high-mannose N-glycans, it had not reached the complex glycan-synthesizing enzymes of the trans-Golgi network.

The precursor of p.T745R was also endo H sensitive, but the 70 kDa peptide abc was partially deglycosylated by endo H, indicating that this abc peptide contain both high-mannose and complex N-glycans. The untreated protein showed a double band pattern could be caused by heterogenous glycosylation, or the antibody could have reacted with some LAMAN precursors that the host cells have produced.

A) COS-7 lysates

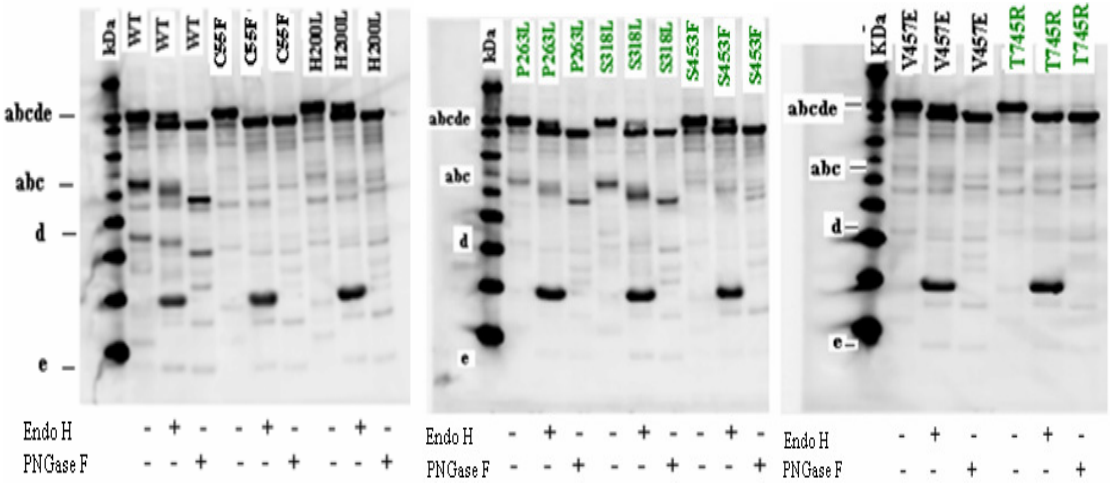


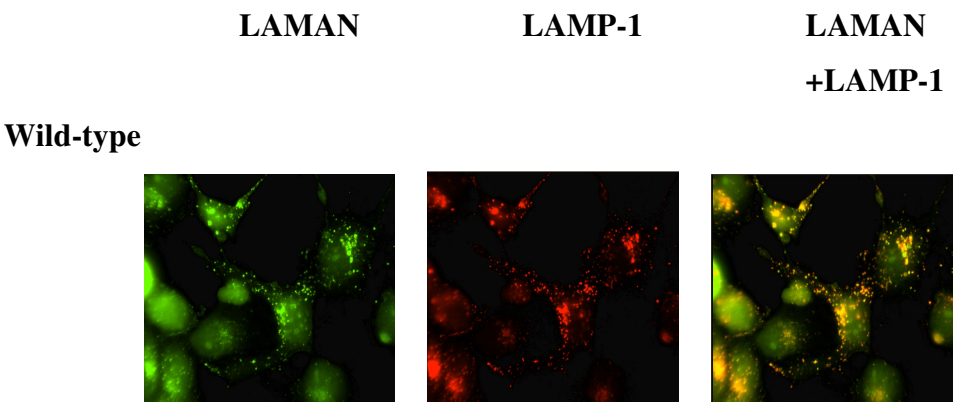
Figure 10 Molecular-shift analysis after Endo H and PNGase F treatments

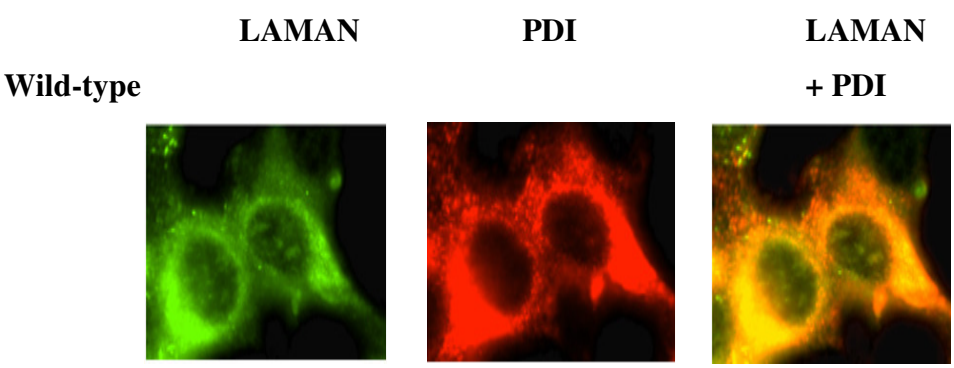
COS-7 cells were transfected with pcDNA containing wild-type and various mutations, the cells were harvested for 48 hours. The lysates were treated with endo H and

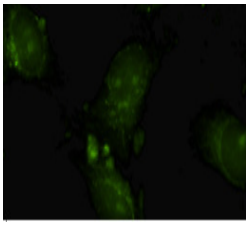
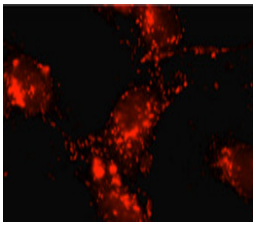
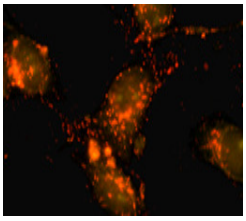
PNGase F, separately by SDS/PAGE and analysed by Western Blot. The positions of the respective LAMAN peptide fragments are indicated on the left.

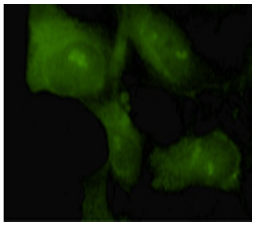
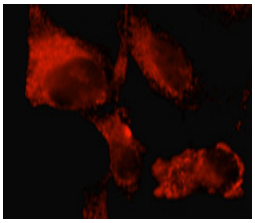
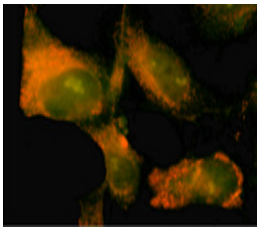
Intracellular localization of mutant LAMAN

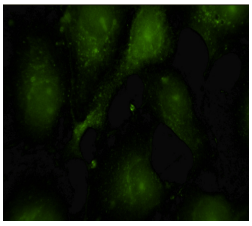
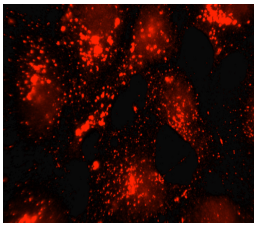
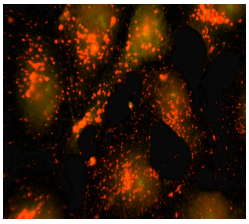
In order to study the the levels of LAMAN protein expression and intracellular distribution, the immunofluorescence studies were carried out in COS-7 cells transfected with the pcDNA3.1-LAMAN, the seven mutants, mock, and p.R750W constructs. The cells were fixed and stained with anti-LAMAN abc peptide, LAMP-1 and PDI and examined by immunofluorescence microscopy as described in Materials and Methods section. As seen in the figure 11, p.H200L, p.P263L, p.S318L, p.S453F, p.V457E and p.T745R LAMANs were co-localized with the lysosomal marker LAMP-1 and ER marker PDI, hence indicating that these mutant LAMANs were localized in ER and partially sorted to lysosomes similar to wildtype. Whereas, p.C55F and p.R550W were only co-localized with PDI, indicating that they were folded abnormally and prevented to pass through the ER control system.

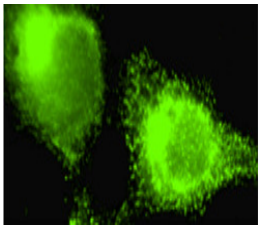
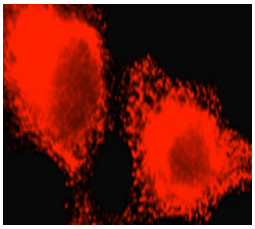
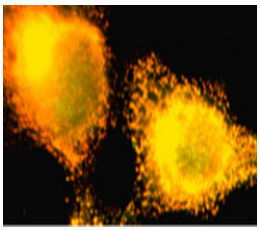


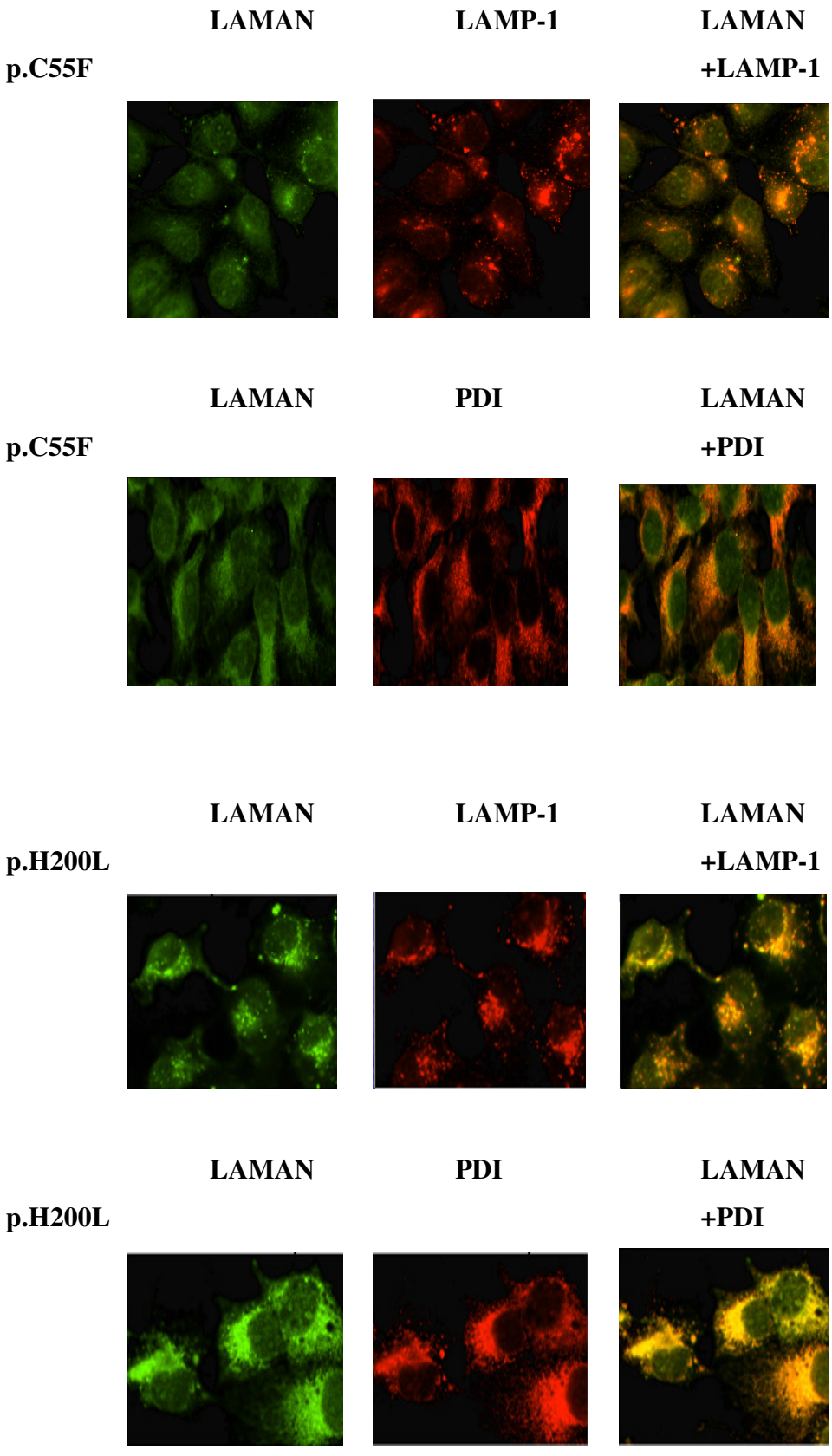


	LAMAN	LAMP-1	LAMAN +LAMP-1
Mock			

	LAMAN	PDI	LAMAN +PDI
Mock			

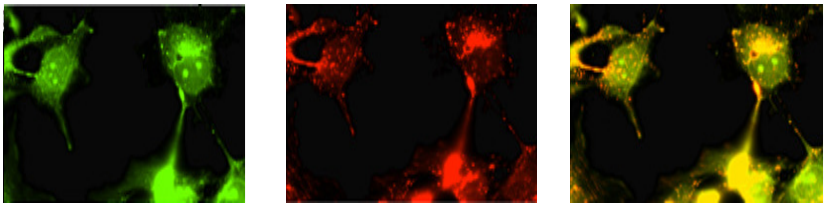
	LAMAN	LAMP-1	LAMAN + LAMP-1
p.R750W			

	LAMAN	PDI	LAMAN +PDI
p.R750W			


LAMAN
LAMP-1
LAMAN
+LAMP-1
p.C55F
LAMAN
PDI
LAMAN
+PDI
p.C55F
LAMAN
LAMP-1
LAMAN
+LAMP-1
p.H200L
LAMAN
PDI
LAMAN
+PDI
p.H200L

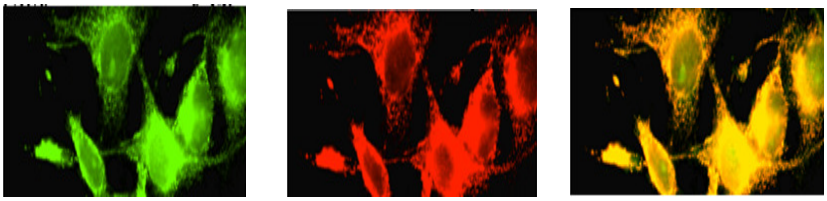
LAMAN **LAMP-1** **LAMAN +LAMP-1**

p.P263L



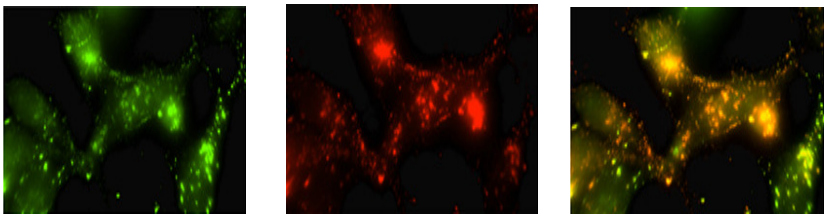
LAMAN **PDI** **LAMAN +PDI**

p.P263L



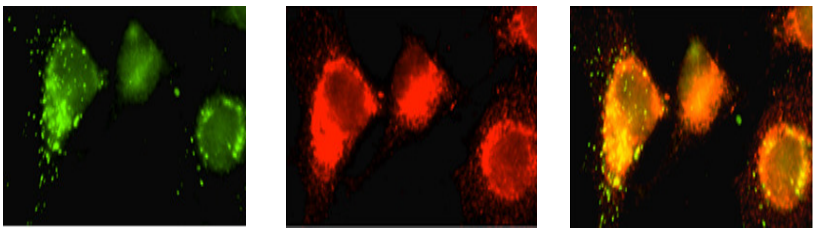
LAMAN **LAMP-1** **LAMAN +LAMP-1**

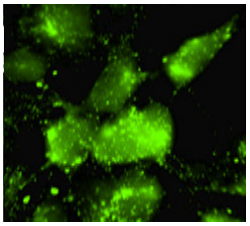
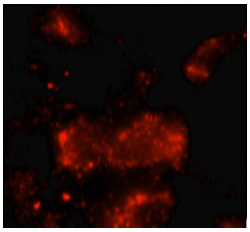
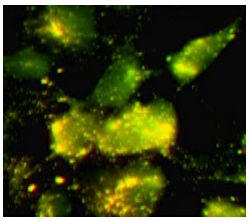
p.S318L

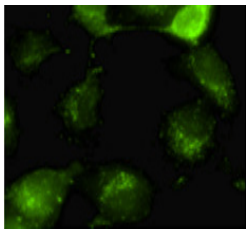
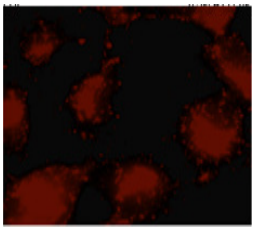
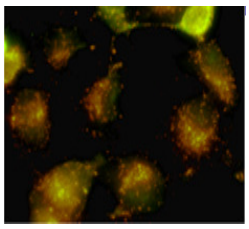


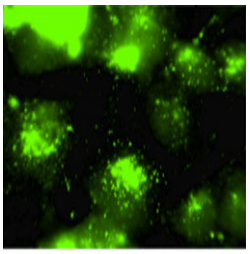
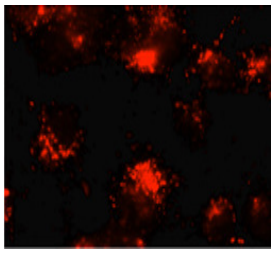
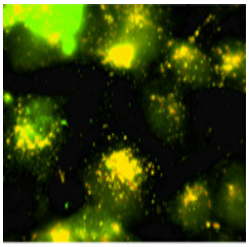
LAMAN **PDI** **LAMAN +PDI**

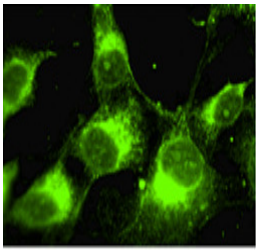
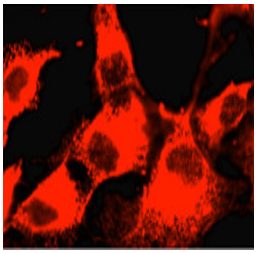
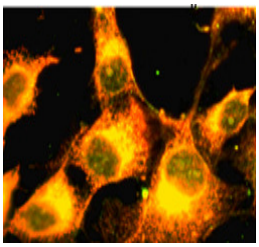
p.S318L



	LAMAN	LAMP-1	LAMAN +LAMP-1
p.S453F			

	LAMAN	PDI	LAMAN +PDI
p.S453F			

	LAMAN	LAMP-1	LAMAN +LAMP-1
p.V457E			

	LAMAN	PDI	LAMAN +PDI
p.V457E			

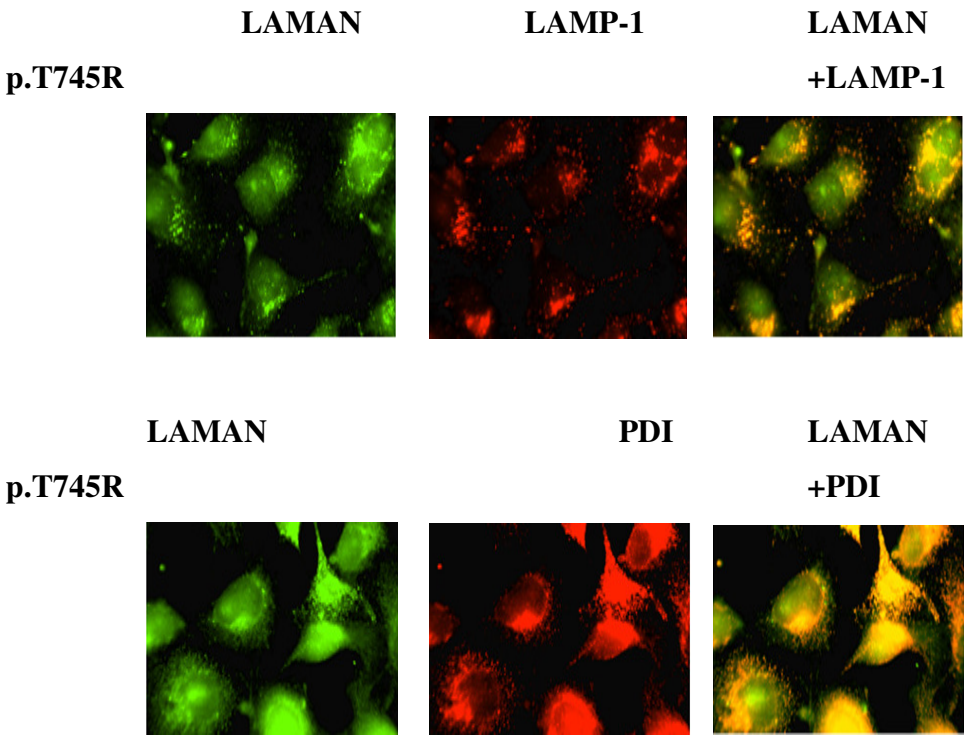


Figure 11 Immunofluorescence microscopy of LAMAN mutants in transfected COS-7 cells.

Double immunofluorescence staining was performed using antibodies against LAMAN abc peptide, LAMP-1 and PDI. Yellow indicates overlap of the LAMAN peptides (green) , and LAMP-1 (red) or PDI (green).

Discussion

In my previous project work, I identified 3 novel putative disease causing mutations in 3 independent mannosidosis patients. The mutations were p.C55F, p.H200L and p.V457E. A set of 55 of normal norwegian control subjects were used to screen for each of the missense mutations. None of these mutations were identified in the 55 normal control subjects, confirming that these mutations do not represent frequent polymorphisms, suggesting that they could be the disease-causing mutations.

In the present work, I have further characterized these mutations along with 4 other putative disease-causing mutations (see table 2). In order to evaluate their biochemical and biological consequences, seven missense mutations were introduced into pcDNA3.1 containing wild-type LAMAN and expressed in transfected COS-7, and BHK-21 cells. The results are summarized in table 6.

In vitro expression of LAMAN mutants

Cell extracts and culture medias were assayed for alpha-mannosidase activity both on transfected COS-7 and BHK cells. The enzyme activity varied in two independent transfection experiments. The variation in enzyme activity could be due to the transfection efficiency or it might have been affected by the process of harvesting the lysates. The COS-7 and BHK cells transfected with the following mutant LAMAN constructs expressed activities higher than the background : pcDNA-WT, pcDNA-H200L, pcDNA-S318L, pcDNA-S453F, pcDNA-V457E and T745R. Alpha-Mannosidase activity in the mutant plasmids containing p.C55F and p.P263L or p.R750W showed no increase over background levels (Figure 8). Mutation p.C55F disrupts the disulphid brigde of Cys55-C358 important for enzyme conformation (Heikinheimo *et al*., 2003 and Hansen *et al*., 2004). Sequence alignment studies with alpha-mannosidase sequences from other species (e.g human, cow, rat, pig, mouse and Drosophila, see appendix) had shown that these aa residues mutations are conserved within the class 2 alpha-mannosidase family indicating that they probably serve important physiological functions.

Intracellular processing of mutant LAMANs

To study the expression and proteolytic maturation of the mutant enzymes, COS-7 and BHK-21 cells were transfected with mutated plasmid constructs. The cell lysates and medias were harvested after 48 hours. The result showed that the mutants, p.H200L, p.P263L, p.S318L, p.S453F, p.V457E and p.T745R were partly transported to the lysosomes as judged from proteolytic processing into the peptides abc and d (see figure 9) and partly secreted as single chain forms (Figure 9). As proteolytic cleavage of abc and d was observed, indicates that processing was taken place in the lysosomes (Hansen *et al*., 2004). Thus, it appeared that these mutant could have an intracellular transport similar to wild-type LAMAN. The appearance of 42 kDa peptide d observed in COS-7 but not in BHK, may be the result of a slow proteolysis between peptides abc and d in in BHK cells. Further partial processing of peptide abc into peptides a, b, c could not be observed in our expression system. Proteolytically processing of peptide abc into a, b and c is a late lysosomal processing event (Nilssen *et al*., 1997). These LAMAN-variants except p.T745R were, like the wild-type, secreted into the medium in the 120 kDa precursor form. This may be caused by saturation of the sorting system in the trans Golgi network (Hansen *et al*., 2004) or it could be a fraction LAMAN protein that was not disassociated from the Mannose-6-phosphate receptor in the acidic environment. The high level of proteolytic processing and secretion correlated with residual activity.

In contrast, other mutants, p.C55F and p.R750W were neither proteolytically processed nor secreted as a single-chain form. This result suggests that these mutants did not enter the endosomes/lysosomes. They were probably retained or degraded in ER. The result could be related to the low of alpha-mannosidase activity of these mutants. The mutant p.T745R was proteolytically processed as judged from the appearance of peptides abc and d, but the 120 kDa single-chain precursor was not secreted into the extracellular environment (Figure 9). The reason could be explained by that p.T745R enzyme did not enter the secretory pathway. Probably, it was completely disassociated from the receptor in the acidic environment and transported to the lysosomes. As jugded from the band-intensity, all the LAMAN-variants were expressed at approximately similar amounts.

N-linked glycans of mutant enzymes

Glycoproteins are generated in the ER, and N-glycosylation of polypeptides is followed by a series of trimming reactions. The correctly folded proteins are further transported to the Golgi for additional modifications (Plemper and Wolf 1999). Lysosomal proteins are generally modified by addition of the M-6-P marker for targeting to the lysosome. Many lysosomal proteins undergo their final proteolytic cleavage in the lysosomes. The human LAMAN amino acid sequence contain 11 potential N-glycosylation sites. These N-glycosylation sites are occupied by high mannose and complex type N-linked (Nilssen *et al*., 1997). The result of deglycosylation (see figure 10) demonstrated that wild-type LAMAN precursor contained both of endo H-sensitive and resistant N-glycans. The LAMAN mutants , p.H200L, p.S318L, p.S453F and p.V457E precursors contained both of endo H sensitive and resistant N-glycans as judged from the partially deglycosylation by endo H, suggesting that a small fraction of these mutant proteins have reached Golgi network. The intracellular level of the single-chain form had decreased and two peptides of 70 and 40 kDa had appeared, corresponding in molecular masses to peptides abc and d. The 70 and 40 kDa peptides were partially endo H-resistant, indicating that the N-glycans consist of high-mannose and complex type and had been transported through the trans-Golgi network, indicating that they followed the same route through the trans-Golgi network as the wild-type. The weak intensity of the abc peptides of p.H200L, p.S453F and p.V457E as compared to p.P263L and p.S318L may due to the fact that only a small fraction of LAMAN is being transported to the lysosomes.

Mutant p.C55F and p.R750W (Hansen *et al*., 2004) remained as intracellular single-chain forms and were not secreted. They were endo H and PNGase F sensitive, this was probably a form that was retained in the ER (endoplasmic reticulum). They may have been folded incorrectly and thus trapped in the ER by the folding control system.

Especially, the mutant p.T745R precursor showed to be endo H sensitive, but surprisingly, the result showed weak proteolytical processing of this mutant into peptides abc and d and they were partially endo H-resistant. Since the precursor of this mutant contained only endo H sensitive N-glycans type, we may expected that it has not reached the complex glycan synthesizing enzymes of the trans-Golgi network. Likely a large fraction of the precursor is arrested in ER. This is consistent with a very low level of secretion into the media. However, a the small fraction of proteolytically

processed peptides of this mutant are occupied by both of high-mannose and complex type glycans, suggesting that this fraction follow the same route through Golgi as the wild-type enzymes. This mutant is not affected in any of the N-glycosylation sites.

Intracellular localization of mutant LAMANs

The intracellular localization of LAMAN polypeptides was studied by immunofluorescence microscopy using COS-7 cells transfected with the different pcDNA-LAMAN constructs. As seen in Figure 11, the WT-, p.H200L, p.P263L, p.S318L, p.S453F, p.V457E and p.T745R-LAMANs were co-localized with the lysosomal marker LAMP-1. This indicate that these mutants were processed and targeted to the lysosomes similar to that of wild-type LAMAN, therefore confirming the normal sorting of these mutants.

In contrast, the p.C55F- and p.R750W –LAMANs were co-localized with the ER marker, but not with LAMP-1. This demonstrated that these mutants were expressed at significant levels as evidenced by immunofluorescence studies. Probably, these mutants folded abnormally and quickly degraded in ER. Such misfolding and ER retention were consistent with the fact that the precursor contained only high-mannose N-glycans and did not secrete the single-chain precursor. It also possible that a very small fraction of p.C55F escaped from the quality control system in the ER and were transported into the lysosomes. The method is not sensitive enough for detection a very small fraction of protein. The results suggest that p.H200L, p.P263L, p.S318L, p.S453F, p.V457E and p.T745R-LAMANs were partially processed, transported and localized to the lysosomes, but p.C55F and p.R750W were misfolded and arrested in ER.

Alpha-mannosidosis-causing mutations can be divided into two groups according to their effect on intracellular processing, localization an enzyme activity of LAMAN

Taken together, the results from enzyme activity, western blot, deglycosylation, as well as the results from studies of immunofluorescence (Table 6), we divided the mutants into two main groups according to their intracellular routes: 1) Lysosomal sorting and secrection and 2) Transport arrest in the endoplasmic reticulum. p.C55F and p.R750W mutations resulted in inactive LAMAN-variants that probably did not pass the folding control system and were thus arrested in the ER in a one-chain precursor form. The

observed precursor polypeptides were retained in the ER, likely due to incorrect folding. These mutants could be categorized into group 2 (Hansen *et al*., 2004).
The group 1: mutants H200L, p.P263L, p.S318L, p.S453F, p.V457E and p.T745R resulted in LAMAN variants that were proteolytically processed and targeted to the lysosomes and resulted either in no residudal activity or residual activity. A small fraction of enzymes could have folded sufficiently to pass the ER control system. The mutant p.H200L were previously reported and predicted to be an active site mutations (Sharagli *et al*., 2005). The distortion of the active site in this mutant did not affect intracellular sorting and stability of the mutant enzyme in this study. The p.P263L variant displayed lysosomal sorting, but had decreased activity towards the artificial substrate p-nitrophenyl- alpha-mannopyranoside.

We performed the experiments in an artificial system. We forced the cells to massively express mutant LAMAN enzymes, and the artificial enzyme substrate was used in order to study their activity towards the more complex natural oligomannosidic substrates. Over-expression of enzymes could result in saturation of the ER system such that a fraction of enzyme could escape the folding control system of the ER. It is conceivable that these mutants could have decreased *in vivo* stability, or zero activity towards the more complex natural substrates in the alpha-mannosidosis patients. Furthermore, residual activity may be caused by other acidic alpha-mannosidases being misinterpreted as residual activity (Nilssen *et al*., 1997). At present, we do not know if the residual activity we observed for LAMANs carrying human p.H200L, p.S318L, p.S453F, p.V457E and p.T745R muations could reflect the *in vivo* situation. LAMAN enzymes, even though containing residual activity upon testing at appropiate pH, may be mislocalized to non-lysosomal compartments and therefore functionally inactive *in vivo*.

Table 6 Summary of Western blot experiments, immunofluorescence , activity measurement and deglycosylation

Variant	Western blot		Immunofluorescence		Enzyme activity (fold increase)				Group	Deglycosylation			
					Transfection experiments					Precursor		peptide abc	
	Intracellular	Medium	Location		COS-7		BHK			Endo H	PNGse F	Endo H	PNGase F
					1	2	1	2		Sensitive	Sensitive	Sensitive	Sensitive
	Proteolysed	One-chain	ER	Lysosomes									
Wild-type	+	+	+	+	16	24	17	25	**1**	-/+	+	-/+	+
Mock	-	-	-	-	1	1	1	1	-				
p.C55F	-	-	+	-	1	1	1	1	**2**	+	+		
p.H200L	+	+	+	+	5	3	3	3	**1**	-/+	+	-/+	+
p.P263L	+	+	+	+	1	1	1	1	**1**	-/+	+	-/+	+
p.S318L	+	+	+	+	9	3	6	4	**1**	-/+	+	-/+	+
p.S453F	+	+	+	+	3	2	4	3	**1**	-/+	+	-/+	+
p.V457E	+	+	+	+	7	5	3	4	**1**	-/+	+	-/+	+
p.T745R	+	-	+	+	3	2	2	2	**1**	+	+	-/+	+
p.R750W	-	-	+	-	1	1	1	1	**2**				

Group 1 is defined as being transport to the lysosomes, while group 2 is accumulating in the ER

+ : sensitive

-/+: non sensitive and sensitive (see glycosylation of mutant LAMAN section for more details)

Conclusions

Missense mutation p.C55F resulted in no ezyme activity in transfected cells. This missense-mutation probably resulted in misfolding and ER-retention. This was consistent with non lysosomal localization in transfected cells. The mutant is most probably disease-associated mutation. The six other mutant variants resulted either in no significant residual or low activity, but they were parially proteolytically processed and partially sorted to the lysosomes. However, in this study we used an artificial expression system, the tranfected cells were forced to producing these mutant LAMANs in large quantities scales. This might not reflect *in vivo* situation, where mutant enzymes may have lower stability and affinity toward to the more complex natural substrate in tissues, compared to in the overexpressing cells. Finally, I am aware of the possibility that there could be another mutations localized in the non-codings region that cause down-regulation of transcription in the patients.

Future perspectives

Recently, only p.H200L was successfully modelled into the three-dimensional structure of bovine LAMAN (Sbaragli *et al*., 2005). A further goal is to the determine the 3-D structure of the mutant LAMANs caused by p.P263L, p.S318L, p.S453F, p.S457E and p.T745R. The information obtained from the 3-D structure and from this work together will contribute to increase understanding of the mutant LAMANs, and also extend the understanding of the biochemical and cellular consequences of these suggested disease-causing sequence variants. Future work should also involve more specific experiments, such as using the natural enzyme substrate for activity measurements..

Additional materials

Table A Primer used for contruction of mutants

Mutation			
cDNA level	Primer protein level	Direction	sequence
c.788C>T	p.P263L	Forward	5’-CCCAATGGTTACAACCTGCCAAGGAATCTG-3’
		Reverse	5’-GCACAGATTCCTTGGCAGGTTGTAACCATT-3’
c.909+731del6272 (IVS6+731del6272)	p.S318L	Forward	5’-GATGACCATGGGCTTGGACTTCCAATATG-3’
		Reverse	5’-CATATTGGAAGTCCAAGCCCATGGTCATC-3’
c.1358C>T	p.S453F	Forward	5’-GTCAGCGGCACCTTCCGCCAGCACGTG-3’
		Reverse	5’-CACGTGCTGGCGGAAGGTGCCGCTGAC-3’
c.2234C>G	p.T745R	Forward	5-GGGACGCTTCTACAGAGACAGCAATGGC-3’
		Reverse	5’-GCCATTGCTGTCTCTGTAGAAGCGTCCC-3’

Appendix ! Sequence alignment

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Drosophila          ----------------------MSAKTSVWLVFFCALGCLIHDADLRSIQPKASQCGYQS 38
Tribolium           ----------------------MFRRTLVPAWIFA--GVCFHLCATAAIKS-ATQCGYQA 35
Cow                 MVGDARPSGVRAGG-CRGAVGSRTSSRALRPPLPPLSSLFVLFLAAPC----AWAAGYKT 55
Cat                 MGADARPLGVRAGGGGRGAARPGTSSRALPPPLPPLS-FLLLLLAAPG----ARAAGYET 55
Human               MGAYARASGVCARG-CLDSAGPWTMSRALRPPLPPLC-FFLLLLAAAG----ARAGGYET 54
Guinea  pig         MGASVLPLGLGAGD-CQSSSGRRMSACLPR---TALSFLLSLLLATPG----ARAAGYET 52
Xenopus             MER-----------------------------VELLLLPLLLLGAAGRLKQAEGCGEQS 30
Nematostella        -------------------------MGVLGKKCDILAVLYLTCFISSS----LCFQCAEK 31
Ciona               ----------------------------MLG----LFIVILASCCTASA----DVSCGYHS 25
Acyrthosiphon       -----MVVLLTGMAVRTFDRSRVVRFRFAAAVCVAVFALLARTTAADAAESSSSGCAPKS 55
                                                                              .

Drosophila          CHPTKPNMLNVHLVAHTHDDVGWLKTVDQYYYGSETKIQKAGVQYIIDSVVEALLRDPEK 98
Tribolium           CNPIKDGFINVHLVPHTHDDVGWLKTVDQYFYGANNSIQNAGVQYIIDSVIDELKKDPKR 95
Cow                 CPKVKPDMLNVHLVPHTHDDVGWLKTVDQYFYGIYNNIQPAGVQYILDSVISSLLANPTR 115
Cat                 CPMVHPDMLNVHLVAHTHDDVGWLKTVDQYFYGIHNDVQHAGVQYILDSVISSLLVEPTR 115
Human               CPTVQPNMLNVHLLPHTHDDVGWLKTVDQYFYGIKNDIQHAGVQYILDSVISALLADPTR 114
Guinea  pig         CPIVQPGMLNVHLVAHTHDDVGWLKTVDQYYWGIHNDLQQAGVQYILDSVISALLAEPTR 112
Xenopus             CPQAPDGFLNVHLIPHTHNDVGWLKTVDQYYYGAHNDIQHAGVQYILDSVVSQLLHDPSK 90
Nematostella        GAGKDAEKLQIHIVPHTHDDVGWLKTVDEYFYGANNSIQHAGVQYILDSVIPALNEDPNK 91
Ciona               CNLGEAEKLNVHLVPHTHDDVGWLKTVDQYYYGSNSSIVIAGVQYILDSVIPQLLSDPSK 85
Acyrthosiphon       CNAIDPNKLNVHLIAHTHDDVGWLKTVDQYYYGSNKAHAPYGVQYIMDSVVSELLKNKNR 115
                            :::*::.***:*********:*::*  .     *****:***:  *  :  :

Drosophila          RFIYVESAFFFKWWKEQKPKVQEAVKMLVEQGRLEFIGGAWSMNDEATTHYQSVIDQFSW 158
Tribolium           KFIYVETAYFWKWWIHQHDFVKHQVKTYVNNGQLEFIGGAWTMNDEATTHYQSIIDQFTW 155
Cow                 RFIYVEIAFFSRWWRQQTNATQKIVRELVRQGRLEFANGGWVMNDEATTHYGAIIDQMTL 175
Cat                 RFIYVEIAFFSRWWHQQTNATQEVVRDLVRQGRLEFANGGWVMNDEAATHYGAIIDQMTL 175
Human               RFIYVEIAFFSRWWHQQTNATQEVVRDLVRQGRLEFANGGWVMNDEAATHYGAIVDQMTL 174
Guinea  pig         RFVYVEMAFFSRWWHQQTNETQEVVRRLVRQGRLEFANGGWVMNDEAATHYGAIVDQMTL 172
Xenopus             RFIYVESAFFWRWWKQQDELVRRDVTRLVEEGRLEFINGGWSMNDEAATHYSSIIDQMTL 150
Nematostella        RFIYVEIAFFKRWWDQQNVEMQEIVKKLVLQKRLEFINGGWCMNDEAATHYNAIIDQMTV 151
Ciona               RFIYVEIAFFSRWWEEQNDAMKENVKQLVQEGRLEFILGGWCMNDEASTHYNAIIDQMTH 145
Acyrthosiphon       KFVFVETAFLWRWWEEQDEWNRNSLKTLVHEGRLQLLHGGWVMSDEAVPHYSTLIDQMTL 175
                    :*::** *:: :** .*    :. :   * : :*::  *.* *.*** .** :::**::

Drosophila          GLRLLNDTFGECGRPRVGWQIDPFGHSREMASMFAQMGFDGMFFGRLDYQDKDERLMTKN 218
Tribolium           GLRKLNDTFGDCGRPKIGWQIDPFGHSREMASIFAQLGFDGLLLGRIDYEDKDKRFESKT 215
Cow                 RLRFLEETFGSDGRPRVAWHIDPFGHSREQASLFAQMGFDGFFFGRLDYQDKKVRKKTLQ 235
Cat                 GLRFLEDTFGKDGRPRVAWHIDPFGHSREQASLFAQMGFDGLFFGRLDYQDKRVREENLG 235
Human               GLRFLEDTFGNDGRPRVAWHIDPFGHSREQASLFAQMGFDGFFFGRLDYQDKWVRMQKLE 234
Guinea  pig         GLRFLEDTFGSDGRPRVAWHIDPFGHSREQASLFAQMGFDGVFFGRIDYQDKLVRKKRRE 232
Xenopus             GLQFLKSTFGDCGRPRVAWHIDPFGHSREQALLFAQMGYDGFFFGRLDYQDKANREKTKQ 210
Nematostella        GLKFIEETFGSDARPRIAWHIDPFGHSNEQASLFAQMSFDGFFFGRIDYDDKTLRLKQQR 211
Ciona               GLRYLNDTFGACARPRVAWQIDPFGHSREQASLFAQMGFDGLFFGRLDYEDKENREKIRN 205
Acyrthosiphon       GLKFLNETFGECARPRVAWQIDPFGHSSEVAAEFAEMGFDGLVLGRIDHEDLALRKQQKT 235
                     *: ::.***  .**::.*:******* * *  **::.:**..:**:*::*   *

Drosophila          AEMIWHGSAN--LGEEADLFSGALYNNYQAPDGFCFDILCNDAPIIDGKHSPDNNVKERI 276
Tribolium           PEMIWKGSDN--L--DADIFTGVMYNTYAPPKGFCFDIMCDDEPLVDDKKSPLYNIERKV 271
Cow                 MEQVWRASTS-LKPPTADLFTSVLPNMYNPPEGLCWDMLCADKPVVEDTRSPEYNAKELV 294
Cat                 LEQVWRASAS-LKPPAADLFTSVLPNIYNPPEKLCWDTLCADKPFVEDRRSPEYNAEELV 294
Human               MEQVWRASTS-LKPPTADLFTGVLPNGYNPPRNLCWDVLCVDQPLVEDPRSPEYNAKELV 293
Guinea  pig         MELVWRASAS-LKAPAADLFTGVLPNNYGPPEGLCWDVLCADPPVVDDPRSPEYNAKKLV 291
Xenopus             MEMLWRASDD-ISPPYADLFTGVLPNGYNPPDGFCWDQLCDDTPIIDDPSVEDYNADVIV 269
Nematostella        MEMVWRGSKSGNLGKNSDIFTGVLYNGYNPPPGFCFDQFCSDQPIQDDPRLYDENVKQRV 271
Ciona               MEEIWRGSQS-LTSPHADLFTGVDENGYNPPDGFCFDAFCRDQPIMDNPALEDYNVEEKV 264
Acyrthosiphon       MEMVWRPDVN--MGQGGELFTSVLYNLYVAPEGFCFDAFCNDDPILDNPKLHGYNVNAKV 293
                     * :*: . .      .::*:..  * * .*  :*:* :* * *. :.      * .  :
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Drosophila          DTFLDFAKTQSQYYRTNNIIVTMGGDFTYQAAQVYYKNLDKLIRYGNERQ-ANGSNINLL 335
Tribolium           DDFFAYLDNVTKAYTTSNVIITMGEDFNYQNAHTWFKNLDKLIYYANQRQ-INGSKYNLL 330
Cow                 RYFLKLATDQGKLYRTKHTVMTMGSDFQYENANTWFKNLDKLIQLVNAQQRANGIRVNVL 354
Cat                 NYFLQLATAQGQHFRTNHTIMTMGSDFQYENANMWFRNLDRLIQLVNAQQQANGSRVNVL 354
Human               DYFLNVATAQGRYYRTNHTVMTMGSDFQYENANMWFKNLDKLIRLVNAQQ-AKGSSVHVL 352
Guinea  pig         SYFLQLATAQGRYYRTNHTVMTMGSDFQYENANTWFKNLDKLIQLVNMQQ-ANGSRVHVL 350
Xenopus             QKFLKAATQQAQKYLSSHIVMTMGSDFQYENAIMWFKNMDRLIKNVNMQQ-INGSKVNVF 328
Nematostella        EEFIKLACEQGSQYKSNNIMMTMGSDFMYENANLWYKNLDKLIAHVNQDS----R-VRAF 326
Ciona               DKFVAAALKQANHFQTNHIMMTMGSDFEYSNSNVWYKNLDKLIKYVNAAD----KNMTLF 320
Acyrthosiphon       ENFANHVKRYASAFKTNNIMITMGGDFSYSVASSWFRNMDKLIKHVNILK----PDLNVL 349
                      *          : :.: ::*** ** *. :  :::*:*:**   *  .        :

Drosophila          YSTPSCYLKSLHDA--GITWPTK-SDDFFPYASDPHAYWTGYFTSRPTLKRFERDGNHFL 392
Tribolium           YSTPSCYTKAVHDS--NQKFVSK-TDDFFPYSSDGNSFWTGYFTSRPTLKRFERQGNNFL 387
Cow                 YSTPACYLWELNKA--NLSWSVK-KDDFFPYADGPYMFWTGYFSSRPALKRYERLSYNFL 411
Cat                 YSTPACYLWELNKA--NLTWSVK-QDDFFPYADGPHQFWSGYFSSRPALKRYERLSYNFL 411
Human               YSTPACYLWELNKA--NLTWSVK-HDDFFPYADGPHQFWTGYFSSRPALKRYERLSYNFL 409
Guinea  pig         YSTPACYLWELNKA--NLTWPVK-EDDFFPYADGPHMFWTGYFSSRPALKRYERLSYNFL 407
Xenopus             YSTPSCYLQSLHRA--NLTWPMK-MDDFFPYADGPHMFWTGYFTSRPAFKGYERLSNNFL 385
Nematostella        YSTPTTYLEALHAA--NLTWGLK-TDDFFPYADCPHCYWTGYFTSRPALKRYSRLNNNLL 383
Ciona               YSTPSCYLYALNHA--NVMWNIK-KDDFFPYADAPHQYWTGYFTSRPGLKGYVRESNKYL 377
Acyrthosiphon       YSTPECYLSALQMSSKNVTWPLKDSDDFFPYAHDEHSYWTGYFTSRSNLKYMICKANNLL 409
                    ****  *   :: :  .  :  *  ******:     :*:***:**. :*       : *

Drosophila          QVCKQLSALAPKKPEEFDP---HLTFMR---ETLGIMQHHDAITGTEKEKVALDYAKRMS 446
Tribolium           QVCKQLYALVDLGPEDWV----DLNALR---EAMGVMQHHDAITGTEKQHVAEDYARILQ 440
Cow                 QVCNQLEALAGPAANVGPYGSGDSAPLN---EAMAVLQHHDAVSGTSRQHVANDYARQLS 468
Cat                 QVCNQLEALAGPAANVGPYGSGDSAPLN---QAMAVLQHHDAVSGTSKQHVADDYARQLA 468
Human               QVCNQLEALVGLAANVGPYGSGDSAPLN---EAMAVLQHHDAVSGTSRQHVANDYARQLA 466
Guinea  pig         QVCNQLEAQVGPAANVGPYGHGDSSPLN---QAMAVLQHHDAVSGTSKQHVADDYARQLA 464
Xenopus             QVCNQMEALSGLEARNGPYGQSSSTVMR---RAMGVAQHHDAVTGTAKQHVNNDYSLRLS 442
Nematostella        QACKQLEVLN---GPAQSGSPSSDLLR----RALAVAQHHDAVSGTSKQHVADDYAKRLA 436
Ciona               QVCNQLETFMHFKSTSHKLTSSSKVLISFPGDAMGVAQHHDAVSGTSKQHVANDYAKRLY 437
Acyrthosiphon       QAVKQIGSILGGELNEHVQTLAIAVAQS---------QHHDAITGTEKQHVSDDYAQYLD 460
                    *. :*:                                *****::** :::*  **:  :

Drosophila          VAFRACGATTRNALNQLTVQSKDNVKDTSAKYVFEFKTCALLNITSCPVSEANDRFALTL 506
Tribolium           GGIDECQFIVNTALSKIITGENSTGPDPGPK--FPINTCWLTNTSSCPFSEDQDNFLVTV 498
Cow                 EGWRPCEVLMSNALAHLS-----GLKE-------DFAFCRKLNISICPLTQTAERFQVIV 516
Cat                 AGWDPCEVLLSNALARLS-----GSKE-------DFTYCRNLNVSVCPLSQTAKNFQVTI 516
Human               AGWGPCEVLLSNALARLR-----GFKD-------HFTFCQQLNISICPLSQTAARFQVIV 514
Guinea              AGWGPCEVLLSNALAKLS-----GSKE-------TFLFCRDLNISICPFSQTSERFQVLV 512
Xenopus             EGWDSCQVVISNSLSSLT-----GTKE-------NFAFCNLLNISVCHVTETANNFKVYL 490
Nematostella        IGAADCQALMSNVIGKKSIKSKGNAPP-------VFSSCNLLNVSSCPSTEDSKSFVVNA 489
Ciona               EGREECNKVISTALTGN------NEQL-------IF--CDYLNITLCDLTQSSNQFVVMV 482
Acyrthosiphon       EGIGESQKVLTAAYRKWFG---KDFPE--------QQYCKMLNISECDVSENNSKFVITL 509
                     .   .                                  *   * : *  ::    * :

Drosophila          YNPLAHTVNEYVRIPVPYSNYRIIDNKGVTLES----QAVPIPQVLIDIKHRNS----TA 558
Tribolium           YNPLSRPVTKYVRLPVIGEAYNVKCPQGKELLT----QLIPIPDAVKNMPGRVS----KA 550
Cow                 YNPLGRKVDWMVRLPVSKHVYLVKDPGGKIVPS----DVVTIPSSDSQ------------ 560
Cat                 YNPLGRKIDWMVRLPVSKHGFVVRDPNGTVVPS----DVVILPSSDGQ------------ 560
Human               YNPLGRKVNWMVRLPVSEGVFVVKDPNGRTVPS----DVVIFPSSDSQAHPP-------- 562
Guinea  pig         YNPLGRKVDRMVRLPVRKGLFLIKDPGNNTVPS----TVVELTSSGNP------------ 556
Xenopus             YNPLGRSVTWTVRLPVNGHAYKVIGPNDETVPS----EVVDVSDFTKALRFQQ----GGA 542
Nematostella        YNPIARDITSYIRVPVNLP-MSVYNPQGAAIKS----QLLPISQETMTLRRMQKLSASNS 544
Ciona               YNPLARGVSKYLRIPVNCDDHYVYVVDDLATSTRLTVQLVPVSEATLSVRRNR----GSA 538
Acyrthosiphon       YNPLSRAVTTPVRIPVKYADYKVTGPNGANVPY----ELVFLPGQIFRLGGRTS----NA 561
                    ***:.: :   :*:**      :    .           : ..
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Drosophila          KYEIVFLATNIPALGYRTYYVEKLDSTEGNTRSK--ALPKRTSSVTVIGNSHIQLGFDTN 616
Tribolium           TVELIFEAPFVPPLGFKTFTVSKKTGNE----------VLKAEKITKIADGEVGFTLDPN 600
Cow                 --ELLFSAL-VPAVGFSIYSVSQMPNQRP---------QKSWSRDLVIQNEYLRARFDPN 608
Cat                 --ELLFPAS-VPALGFSIYSVSQVPGQRPHAHKPQPRSQRPWSRVLAIQNEHIRARFDPD 617
Human               --ELLFSAS-LPALGFSTYSVAQVPRWKPQARAPQPIPRRSWSPALTIENEHIRATFDPD 619
Guinea  pig         --ELLFPAL-VPALGFSVYSVTRVSDQNPQTRSQHSRPQKYSSPVLSIKNEYLRASFHPD 613
Xenopus             ERELIFQGQ-IPAVGFSSFTVGKLSLPDRFVIK----GKRGKKQPDKIQNQYYRVDFDPE 597
Nematostella        KYELIFKVK-LPPLGFASYFVNTSKTSSKMYGKS-FAVDPST----VIQNEFIKLEFSRD 598
Ciona               NCELVFLAK-LPALGHNTFQVTKHKTSKTVSLLK-TKVEKVINQDVTITNEFYKVKFDGN 596
Acyrthosiphon       THELLFIASEVSPLGLVNYHVERINEPEPPPRP----MPYNSTEDVTIDNGKLKIGFNGI 617
                      *::*    :..:*   : *                          * :      :

Drosophila          -GFLSEVT--ADGLTRLVSQEFLFYEGAVGNNAEFLNRSSGAYIFRPNENK-IHFATD-- 670
Tribolium           SGLLKTVI--LNGKTVDVNQEFLYYEGFVGDNEEPKNRSSGAYIFRPIPGKDPVVVAD-- 656
Cow                 TGLLMELENLEQNLLLPVRQAFYWYNASTGNNLS--SQASGAYIFRPNQNKPLFVSHW-- 664
Cat                 TGLLVEMENLDQNLLLPVRQAFYWYNASVGNNLS--TQVSGAYIFRPNQEKPLMVSHW-- 673
Human               TGLLMEIMNMNQQLLLPVRQTFFWYNASIGDNES--DQASGAYIFRPNQQKPLPVSRW-- 675
Guinea  pig         TGLLSMIEVLDRKLTLPVNQAFFWYNASVGDKRS--SQASGAYIFRPSQQWPFPVSHL-- 669
Xenopus             TGLISGIHNLEKKISLPLKQSFYWYNASVGNEES--SQPSGAYIFRPNNSDSVPVSQH-- 653
Nematostella        TGRLTSMTDLVSEVTTQVDQQFLWYNASVDQG-----QPSGAYIFRPNTSSTTPVNAGGK 653
Ciona               TGLMASIENIASGITVPVKQDMLWYNASMGNDES--KQQSGAYIFRPNVSTPFQLIIG-- 652
Acyrthosiphon       SGLVQWIE--KNGTRHQLQQNFFFYESMKGYNFNADNRASGAYIFRPTKSQPTAISEK-- 673
                     * :  :          : * : :*:.  .       : ********       .

Drosophila          QVEIEVYKGDLVHEVHQKFNDWISQVVRVYNKDSYAEFEWLVGPIPIDDGIGKEVITRFN 730
Tribolium           KVDYKIFRGNLVSEVQQVFNEWITQTIRVYRTESYIEFDWIVGPIPDTDANGKEIITRFT 716
Cow                 -AQTHLVKASLVQEVHQNFSAWCSQVVRLYPRQRHLELEWTVGPIPVGDGWGKEVISRFD 723
Cat                 -AQTRLVKTPLVQEVHQNFSAWCSQVVRLYRGQRHLELEWTVGPIPVGDGWGKEIISRFD 732
Human               -AQIHLVKTPLVQEVHQNFSAWCSQVVRLYPGQRHLELEWSVGPIPVGDTWGKEVISRFD 734
Guinea  pig         -ARTRLVKTALVQEVHQNFTAWCSQVVRLYSGQRHLELEWTVGPIPVGDKWGKEIISRFD 728
Xenopus             -VRSYLVQNSLVQEVHQNFSSWCSQVVRLYRDQRYIELEWTVGPVPIGDEWGKEVISLIE 712
Nematostella        ATFTVYQG-PLVQEVRQVFSPYVSQVVRLYYGQKHAEFEYTVGPIP--DDLGREVITRFD 710
Ciona               CVFNIQGSNLLVQEVYQKFSDWVWQVIRLYAGKKQIEVEWTVGPIPVQDKWGKEIISRYE 712
Acyrthosiphon       -INLTIYRGKNVHEVHQSFSSWLSQVVRIYDQQESIEFEWLVGPIPIMEWIGKEVITKYA 732
                              * ** * *. :  *.:*:*  .   *.:: ***:*  :  *:*:*:

Drosophila          SDIASDGIFRTDSNGREMIKRKINHRDTWSVKINEAVAGNYYPITTKIDVEDDT--ARMA 788
Tribolium           TPLNTKSTFYTDSNGREMLKRVRNARPTWTLTLEEPVSGNYYPVTSKIVLVDESQDLELA 776
Cow                 TALATRGLFYTDSNGREILERRRNYRPTWKLNQTEPVAGNYYPVNSRIYITDGN--MQLT 781
Cat                 TVLETKGLFYTDSNGREILERRRDYRPTWKLNQTETVAGNYYPVNSRIYIRDGN--MQLT 790
Human               TPLETKGRFYTDSNGREILERRRDYRPTWKLNQTEPVAGNYYPVNTRIYITDGN--MQLT 792
Guinea  pig         TPLETGGVFFTDSNGREVLERRRDYRPSWKLNQTEPVAGNYYPVNSRIYITDGK--MQLT 786
Xenopus             TNLKTDGVFYTDSNGRQILKRRRDSRETWKLNQTEPIAGNYYPVNSRIYIKGKN--TQVT 770
Nematostella        SDIKSNKVFYTDANGREMQLRTRDFRPTWKLNNTEPVAGNYYPVNSRIFVKDSN--RQLT 768
Ciona               TNLETNGYFYTDSNGREILERRKDYRPTWHLNQSESVAGNYYPVNSRIYIHDNQ--VQLT 770
Acyrthosiphon       TKIASNGTFYTDSNGRRWMQRKRNQRSSWNLTLTEPVSSNYYPITSSIAIRDAI--HQAT 790
                    : : :   * **:***.   *  : * :* :.  *.::.****:.: * : .     . :

Drosophila          ILTDRAQGGSSLKDGSLELMVHRRLLKDDAFGVGEALNET-EYGDGLIARGKHHLFFGKS 847
Tribolium           VLTDRAQGGTSLQDGQLELMVHRNCLHDDAFGVAEALNET-AFGKGLVARGSHFLTLGPH 835
Cow                 VLTDRSQGGSSLRDGSLELMVHRRLLKDDARGVGEPLNKE---GSGLWVRGRHLVLLDKK 838
Cat                 VLTDRSQGGSSLRDGSMELMVHRRLLKDDGRGVGEALLED---GLGRWVRGRHLVLLDKV 847
Human               VLTDRSQGGSSLRDGSLELMVHRRLLKDDGRGVSEPLMEN---GSGAWVRGRHLVLLDTA 849
Guinea pig          VLTDRSQGGSSMSDGSLELMVHRRLLKDDGRGVGEALQEP---GSGGWVRGRHLLLLDTA 843
Xenopus             VLTDRSQGGSSIRDGSLELMVHRRLLRDDYRGVGEPLLENGLLGEGIVVRGRHLLLLDHA 830
Nematostella        LLTDRSLGGSSLKDGSVELMLHRRLLVDDKRGVDEPLNESGISGKGLIVRGKVNVVLAPP 828
Ciona               VLNDRSQGGSSLSTGQLELMIHRRLLGEDSKGVGEALNETGQFGDGLISRGKHWLLLDTV 830
Acyrthosiphon       VITDRPQGGTSIEDGTLELMLHRRLLYDDSQGVSEPLDEN-QYGEGMVTRGKHILHFNEL 849
                    ::.**. **:*:  * :***:**. * :*  ** *.* :    * *   **   : :
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Drosophila          TDREG-VSLKGIERLTQLEKLLPTWKFFSNMEDYSADEWQTAFTNIFSGISLVLPKPVHL 906
Tribolium           FKKTGNVSTAALERDIAQRKVLDSWVFIS------APYNDSKYIKEFSGLKRALPANVQI 889
Cow                 ETAAARHRLQAEMEVLAPQVVLAQGGGARYR-------LEKAPRTQFSGLRRELPPSVRL 891
Cat                 RTAATGHRLQAEKEVLTPQVVLAPGGGAPYH-------LKVAPRKQFSGLRRELPPSVHL 900
Human               QAAAAGHRLLAEQEVLAPQVVLAPGGGAAYN-------LGAPPRTQFSGLRRDLPPSVHL 902
Guinea              REAAAEHRLLAEKELLAPQLVLAPGQGPSYHH----DHHEAVPRKQFSGLRRQLPPSVRL 899
Xenopus             DEAADTHRTLALQQYMSPQVVLSSGDGIPYS-------QSGTPKRKFSALNGELPQNGHL 883
Nematostella        ATSAATHRELGEQMMLEPIVSFAQNPSTVEK-------WLAKYNSLYTGVARQLPSNVHM 881
Ciona               TSSAKQHRLLAEEIYMSPLVAFQHH---------------APSTTHESHVTSPLPPNIHL 875
Acyrthosiphon       DKAAKAHRLSALHTAMQPVVTLAPTHMGSNE-------WVSKFSATHKLLNNSLPLNIHL 902
                              .          :                        . :   **   ::

Drosophila          LTLEPWHENQLLVRFEHIMENGEDAS--YSQPVQFNVKNVLSAFDVEGIRETTLDGNAWL 964
Tribolium           LTLEPWKGFSFLLRLEHVLEKGEDAD--LSQPAIVNLQNLFTPFEIKSIRETTLGANQWL 947
Cow                 LTLARWGPETLLLRLEHQFAVGEDSGRNLSSPVTLDLTNLFSAFTITNLRETTLAANQLL 951
Cat                 LTLARWDQKTLLLRLEHQFAVGEDSG-NLSSPVTLDLTDLFSAFTITYLQETTLVANQLR 959
Human               LTLASWGPEMVLLRLEHQFAVGEDSGRNLSAPVTLNLRDLFSTFTITRLQETTLVANQ-R 961
Guinea  pig         LTLARWGPDTLLLRLEHQFALGEDSSRNLSLPVTLDLQDLFSTFTITRLQETTLAANQLR 959
Xenopus             LTFAMHAADKILLRLEHPFQS--QESKNNSQPITINLNTLFSSVSLSNFEETTLGANMEK 941
Nematostella        LTLETSNQY-ALIRLEHQFAD-EDSK--LSMPVNVSLQGLFTDLEVSKVEELNLAANQLL 937
Ciona               LTLSTTTDGGYIVRVEHQFEKNEDST--LSKPATVSLKNLLTDFHVSSCEELLLGGNAFK 933
Acyrthosiphon       LTLEHWRKDQVLLRIEHIFEKDEDRF--LSLPETINLQQLFSQLEVLEYKELTLSANLAK 960
                    **:        ::*.** :    :     * *  ..:  ::: . :   .*  * .*

Drosophila          DESRRLQFAPDPEEAAFNTYATFSQPAESVHLLSAEKPMLGVKYADEALPAGQLGAESNR 1024
Tribolium           EKNERLEFEAK---------DLFRKNE--------------------------KRATL 970
Cow                 AYASRLQWTTDTGP-----------------------------------------TPH 968
Cat                 ASASRLKWTPNTGP-----------------------------------------TPL 976
Human               EAASRLKWTTNTGP-----------------------------------------TPH 978
Guinea  pig         ASASRLKWTTEIDP-----------------------------------------ISR 976
Xenopus             SRLKRLQWRTRTGSPL---------------------------------------QMDS 961
Nematostella        KDKHPMQWNIKSVRKSRVRVSGSAKQPS---------LRLANTPVELRPMQIRTFKATLR 988
Ciona               QDVTRLNWETTEKLKFAPGIDSVSN------------------------FEKPYDVTNG 968
Acyrthosiphon       ADLDRYRWNYSDKP-----------------------------------------QRPE 978
                          .:

Drosophila          IRRETETRQEKKDEGRSSKSTEGPYNSFKSDSSNQEYIIELSPMEIRTFIVYLTPA---- 1080
Tribolium           IR-----------------------------ALDDYQITLNPMQIRTFVIEIEKN---- 996
Cow                 PSP----------------------------SRPVSATITLQPMEIRTFLASVQWEEDG- 999
Cat                 PSP----------------------------SRLDPATITLQPMEIRTFLASVQWEEHG- 1007
Human               QTP----------------------------YQLDPANITLEPMEIRTFLASVQWKEVDG 1010
Guinea pig          PAV----------------------------PRLDPSSITLQPMEIRTFVASVQWEENS- 1007
Xenopus             PRA----------------------------PVVDPSNITLSPMEIRTFLANVRYKAALH 993
Nematostella        MKK----------------------------KSKDSVQMTKIRKAKRVYRLLTSRNG--- 1017
Ciona               LR-----------------------------QQLDSCDVTLEPMQIRTFLIK-------- 991
Acyrthosiphon       LN-----------------------------APLPDNLLTPMAIKTYLLTVKPR---- 1003
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Figure B : Sequence comparison of LAMAN proteins from different species. The sequennces were aligned using the ClustalW 2.0.10 . The amino acid one-letter code is used. The human LAMAN amino acid sequence is shaded with light blue colour. The conserved residues of LAMAN sequences which had mutated and were identified in patients in previous work are indicated by green colour shades. "*" means that the residues are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed and "." indicates that semi-conserved substitutions are observed.

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