ISAS - INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

Human Lysosomal Acid Lipase: Functional characterisation by molecular genetic analysis and site-directed mutagenesis studies.

Thesis submitted for the Degree of
Doctor Philosophiae

Candidate: Rajalakshmi Pariyarathuparambil G.

Supervisor: Prof. Francisco Ernesto Baralle

Academic Year: 1995/96

SISSA - SCUOLA INTERNAZIONALE SUPERIORE DI STUDI AVANZATI
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ACKNOWLEDGEMENTS

This project was carried out in the Molecular Pathology laboratory of the International Centre for Genetic Engineering and Biotechnology, Trieste., under the supervision of Prof. F. E. Baralle during the period of 1992-1996.

I am deeply indebted to Prof. Baralle for accepting me as a PhD student in this laboratory. I'm extremely grateful to him for his patient guidance and inspiration throughout the period of last four years. I would like to express my sincere gratitude to my co-supervisor Dr. F. Pagani for giving me the opportunity to work in this particular project, and moreover, for his constant guidance and collaboration during the course of this work. I am grateful to Dr. R. Garcia for his collaboration and help regarding the protein chemistry part of the work and for critical reading of the manuscript. I am thankful to Ms. C. Stuani for excellent technical assistance offered as and when it was required.

A special vote of thanks goes to Dr. A. F. Muro for his timely advises and moral support that helped me to take this task to the end. I take this opportunity to extend my heartfelt gratitude to Dr. M. Romano for his help with computer programmes, and his infinite patience to bear with my mistakes. I'm also grateful to the rest of my colleagues of the Molecular Pathology laboratory and the scientific and administrative staff of ICGEB for their co-operation and friendship during these four years.

Last, but not the least, thanks to all my friends, inside and out side ICGEB, for their constant moral support that made my stay in Trieste less painful than it could have been without them.

ABSTRACT

Lysosomal Acid Lipase (LAL) is one of the key enzymes involved in the catabolism of triglycerides and cholesteryl esters entering the cells during the receptor mediated uptake of lipoprotein particles. The deficiency of LAL activity leads to variable levels of accumulation of cholesteryl esters and triglycerides in various tissues of the body leading to pathological conditions such as Wolman's Disease (WD) and Cholesteryl Ester Storage Disease (CESD). Unlike most of the well characterised mammalian lipases such as pancreatic, hepatic and lipoprotein lipases, LAL is capable of hydrolysing both triglycerides and cholesteryl esters. Moreover, primary amino acid sequence of LAL shows negligible homology with the members of lipase super family. The present study was designed to investigate structure-function relationships of LAL by analysing a variety of natural and site-directed mutants.

The molecular basis of Cholesteryl Ester Storage Disease (CESD) has been characterised in three Italian patients and these patients were found to be compound heterozygotes for mutations in the LAL gene. Altogether, three different missense mutations (P181L, G66V, L273S) and two splicing defects resulting in exon skipping due to mutations at the 3′ or 5′ splice site consensus (Δ205-253, Δ254-277 respectively) were detected. The effect of these mutations on the triglyceride and cholesteryl ester hydrolase activities of the enzyme has been analysed by in vitro expression of the mutant alleles in HeLa cells and the results confirmed that these mutations were detrimental for both catalytic activities. Moreover, some of these mutations altered the glycosylation pattern of the enzyme. In this context, glycosylation pattern of the recombinant LAL protein expressed in HeLa cells was studied by endoglycosidase H treatment followed by immunoblotting. The effect of deglycosylation on the catalytic activities of LAL has also been verified. These studies suggested that N-linked glycosylation is not essential for the catalytic activity of the enzyme but could be necessary for optimal rate of catalysis.

For further investigation of the functional aspects of LAL catalysis, site directed mutagenesis studies were performed in order to identify amino acid residues forming part of the putative Ser- Asp-His type of

catalytic triad of LAL. Convincing evidence is provided for the importance of Ser^{153}, His^{274} and D^{130} residues for both tri acyl glycerol lipase and cholesteryl ester hydrolase activities of LAL. Site directed mutagenesis of the residues in the vicinity of H^{274} showed that this region of the protein is of critical importance for optimal catalytic activity.

Part of this work has been published in the following articles.

Expression of lysosomal acid lipase mutants detected in three patients with Cholesteryl Ester Storage Disease.
F. Pagani, R. Garcia, R. Pariyarath, C. Stuani, B. Gridelli, G. Paone, F. Baralle
Human Molecular genetics (1996) 5:10, 1611-1617

L273S mutation in Human Lysosomal Acid Lipase creates a new N-glycosylation site.
R. Pariyarath, F. Pagani, C. Stuani, R. Garcia, F. E. Baralle
FEBS Letters (1996) in press.

INDEX

Chpater 1

Introduction.

1.1	General Overview of Lipid Metabolism	1
1.2	Co-ordinate regulation of exogenous and endogenous pathways of lipid metabolism	3
1.3	Role of Lysosomal Acid Lipase in cellular lipid metabolism	4
1.4	Biochemical characteristics of lysosomal acid lipase	6
1.5	Sequence homology and functional similarites with other mammalian lipases	8
1.6	Protein processing and targeting to lysosomes	11
1.7	Genomic organisation and chromosomal localisation of LAL gene	13
1.8	Clinical manifestations of LAL deficiency	
1.8.1	Wolman's Disease	14
1.8.2	Cholesteryl Ester Storage Disease	15
1.8.3	LAL deficiency and Atherosclerosis	15
1.9	Molecular defects in CESD and WD patients	
1.9.1	Molecular defects in Wolman's Disease	16
1.92	Animal model of Wolman's Disease	17
1.9.3	Molecular Defects in CESD patients	18

Chapter 2

Materials and methods.

2.1	Materials	
2.1.1.	Enzymes and Chemicals	19
2.1.2	Cell culture	19
2.2	Subjects	19
2.3	Methods	
2.3.1	Isolation of peripheral blood mononuclear cells	20
2.3.2	DNA extraction from blood	20
2.3.3	Rapid method for isolation of DNA from cultured cells	21
2.3.4	RNA extraction from blood and PBMNCs.	21
2.3.5	Reverse transcription and PCR amplification	22
2.3.5.1	Oligonucleotides used in RT-PCR analysis	22

2.3.5.2	Oligonucleotides used in Genomic DNA amplifications	23
2.6	Agarose gel electrophoresis and electro-elution of amplified DNA fragments	23
2.7	Direct sequencing of PCR products	24
2.8	Preparation of HSV tagged LAL construct for Vaccinia coupled expression in HeLa cells	25
2.9	Site directed mutagenesis	
2.9.1	S^{99} mutagenesis	26
2.9.2	S^{153} mutagenesis	27
2.9.3	D^{124} and D^{130} mutagenesis	27
2.9.4	H^{274} and L^{273} mutagenesis	27
2.10	Vaccinia virus expression system	
2.10.1	Preparation of Vaccinia virus stock	27
2.10.2	Plaque assay for titer determination	28
2.10.3	Vaccinia virus infection	29
2.10.4	Transfection of LAL and CAT constructs with lipofectin	29
2.10.5	Measurement of Transfection efficiency Phase extraction assay for CAT activity	29
2.11	Measurement of LAL activity	
2.11.1	Fluorimetric assay	30
2.11.2	Radioactive assay	30
2.12	Endoglycosidase H treatment	31
2.13	Immunoblotting	31

Chapter 3
Results

3.1	Identification of the molecular basis of CESD in Patients	
3.1.1	Patient 1 is a compound heterozygote for Ex.7 skipping and P181L mutation	33
3.1.2	Patient 2 is a compound heterozygote for Ex.8 skipping and G66V missense mutation	34
3.1.3	Patient 3 is a compound heterozygote for L273S mutation and a null allele	35
3.1.4	Analysis of XbaI polymorphism in the population	36
3.2	In vitro Expression and characterisation of lysosomal acid lipase	
3.2.1	Recombinant LAL expressed in bacteria is not catalytically active	36

3.2.2 Expression of recombinant LAL in mammalian cells 37
3.2.2.1 Tagged and non-tagged LAL were equally active 37
3.2.2.2 Recombinant LAL expressed in HeLa cells showed 4 different molecular weight forms 38
3.2.2.3 Different molecular weight forms of LAL originate from N-linked glycosylation 38
3.2.2.4 Effect of deglycosylation on the enzymatic activity of LAL 39
3.3 In vitro expression and characterisation of CESD mutants and polymorphic variants
3.3.1 CESD mutants expressed in HeLa cells produced inactive LAL protein 39
3.3.2 Some of the CESD mutants showed altered glycosylation pattern 40
3.3.3 L273S mutation introduces a new glycosylation site at N^{271} 40
3.3.4 Functional characterisation of the G2A polymorphic variant 41
3.3.4.1 Measurement of Acid lipase activity in the PBMNCs of subjects 41
3.3.4.2 In vitro expression of G2A mutants 41
3.4 Characterisation of putative catalytic triad residues of LAL
3.4.1 S^{153} is of critical importance for the catalytic activity 42
3.4.2 D^{130} may form part of the catalytic triad 43
3.4.3 H274 and neighbouring residues have a critical role in LAL catalysis 44

Chapter 4
Discussion
4.1 Identification of LAL mutations in CESD patients and predicted consequences of these mutations 44
4.2 Expression of recombinant lysosomal acid lipase in mammalian system 46
4.3 Putative catalytic triad of LAL: D^{130}-S^{153}-H^{274} 51
4.4 Conclusions 55
References 56

CHAPTER 1

INTRODUCTION

Inherited disorders of lipid metabolism, manifested as distinct pathological entities, are valuable keys for decoding the functional role of different factors in the maintenance of balanced lipid metabolism. This information, in turn, is necessary for better understanding of the origin, development and progression of complex pathological conditions such as various types of hyperlipidemiae and atherosclerosis. A complex picture of the overall regulation of exogenous and endogenous phases of lipid metabolism has emerged (Fig.1.1.1) from the studies based on human disease models showing specific blocks at different stages of lipid metabolism combined with information derived from extensive characterisation of similar animal models. The role of different species of lipoprotein particles plasma lipoprotein transport and the involvement of triacyl glycerol lipases and cholesteryl ester hydrolases in the processing and recycling of lipid components of these particles are evident from the figure.

1.1 General Overview of Lipid metabolism:

In general, dietary triglycerides are hydrolysed in the intestine to free fatty acids and monoglycerides by the action of pancreatic lipase and these fatty acids are reesterified to triglycerides in the intestinal mucosal cells (*Havel & Kane, 1995*). Subsequently, the triglycerides will be complexed with amphipathic apolipoproteins such as apoCs, apoE and apoB48 in the Golgi apparatus of these cells to form chylomicrons (CM) and then secreted into the lacteals. These complexes serve as ideal systems for the transport of hydrophobic lipids across the hydrophilic environment of circulatory system. In extra hepatic tissues, the chylomicrons are rapidly hydrolysed by lipoprotein lipase forming chylomicron remnants (CMrm) that retain their cholesteryl ester components. Meanwhile, the apolipoproteins and phospholipids are transferred to high density lipoprotein particles (HDL). Chylomicron remnants can then be internalised by specific receptors on the

surface of the hepatic cells and delivered to the lysosomes. Lysosomal acid lipase (LAL) hydrolyses the cholesteryl esters to free cholesterol and triglycerides to free fatty acids.

Surplus fatty acids are exported from the liver in the form of very low density lipoprtoein particles (VLDL) containing apoB100, apoE and apoC apoproteins as major protein components. VLDL particles loaded with triglycerides and low amounts of cholesteryl esters will then undergo partial lipolysis catalysed by lipoprotein lipase giving rise to VLDL remnants.

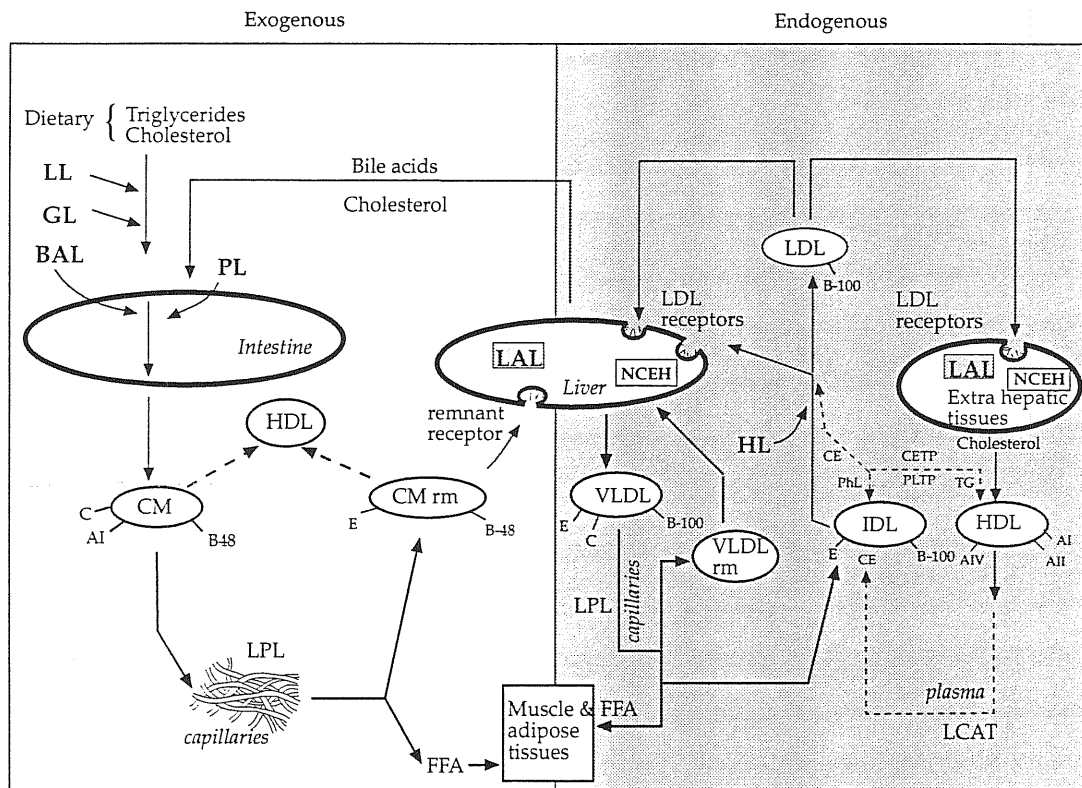

Fig.1.1.1: Schematic representation of the exogenous and endogenous pathways of lipid metabolism. Involvement of different lipases at different stages of lipid metabolism are indicated. LL: Lingual lipase., GL: Gastric lipase., PL:Pancreatic lipase., BAL: Bile salt activated lipase., LPL: Lipoprotein lipase., LAL: Lysosomal acid lipase., HL: Hepatic lipase., NCEH: Cellular neutral cholesteryl ester hydrolase.Various lipoprotein particles involved in lipid transport are also indicated. CM: chylomicron., CM rm: chylomicron remnant., HDL: high density lipoprotein., LDL: low density lipoprotein., VLDL: very low density lipoprotein., VLDL rm: VLDL remnant. The major protein components of lipoprotein particles are indicated in association with respective particles. The dotted lines indicate the pathway of reverse cholesterol transport. CETP: Cholesteryl ester transfer protein, PLTP: Phospho lipid transfer protein., LCAT: Lecithin - cholesterol acyl transferase. FFA indicates free fatty acids.CE : Cholesterol, PhL: phospholipid.

Larger VLDL remnants bind to LDL receptors with high affinity by means of multiple copies of apoE present on the surface of these particles and will be internalised by receptor mediated endocytosis ultimately leading to hydrolysis by lysosomal hydrolases. Smaller VLDL remnants have lesser

affinity to LDL receptors and therefore remain in the blood for longer time in the form of intermediate density lipoprotein particles (IDL). The IDL particles may undergo further lipolysis by hepatic lipase to form Low density lipoproteins (LDL) containing apoB100 as the main protein component. Major part of cholesterol in circulation is found in the form of LDL that has to be cleared off from the plasma by LDL receptor mediated endocytosis pathway leading to terminal catabolism of these particles by lysosomal hydrolases.

Excess cholesterol from extra hepatic tissues gets complexed with A type apoproteins forming HDL particles in which the cholesterol will be trapped as cholesteryl esters by the action of lysolecithin:cholesterol acyl transferase (LCAT). Identification of cholesteryl ester transfer protein (*Drayna et al., 1987*) and phospholipid transfer protein (*Day et al.,1994*) involved in the exchange of cholesteryl esters and phospholipids between different species of HDL or between HDL and triglyceride rich lipoprotein particles including IDL and LDL, provided the missing links in the process of reverse cholesterol transport whereby excess cholesterol from extra hepatic cells is transported to liver and subsequently delivered into bile.

1. 2 Co-ordinate regulation of exogenous and endogenous pathways of lipid metabolism:

Studies on the regulation of expression of LDL receptors provided further insight into the mechanism by which exogenous and endogenous pathways of lipid metabolism are co-ordinately regulated (*Goldstein &Brown ., 1990*). The free cholesterol derived from lysosomal hydrolysis of LDL, modified and oxidised LDL or chylomicron remnants will be released in the cytoplasmic compartment.

This free cholesterol and its oxidised derivatives act as second messengers that regulate the expression of a series of genes involved in the biosynthesis of cholesterol and its derivatives by virtue of sterol regulatory elements (SREs) present in the upstream regions of these genes (Fig.1.2.1). Sterol regulatory element binding proteins (SREBPs) have been recently identified (*Hua et al., 1993; Yokoyama et al., 1993*) as basic loop helix- leucine zipper type of transcription factors and their role in sterol mediated transcriptional regulation of genes involved in the biosynthetic pathway of cholesterol has been well documented (*Guan et al., 1995; Ericsson et al., 1996*). It has been shown that these proteins are involved in the regulation of genes of the fatty acid biosynthesis pathway as well (*Bennett et al., 1995; Lopez et al., 1996*). Thus SREBPs can be considered as molecular co-ordinators responsible for the synchronous regulation of the complex pathways of lipid metabolism.

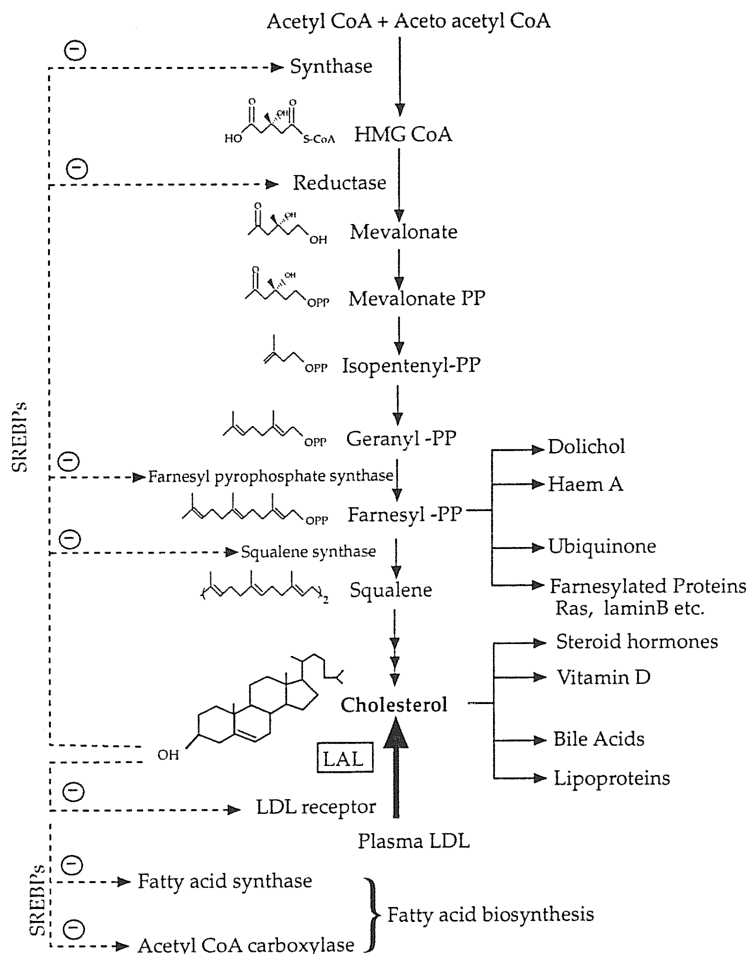

Fig.1.2.1: Coordinate regulation of lipid pathways. Regulatory role of cholesterol at rate limiting steps in the biosynthetic pathways of cholesterol and fatty acids are indicated. Free cholesterol released from LDL particles by the action of lysosomal acid lipase can influence the rate of transcription of mRNAs for HMGCoA synthase, HMG CoA reductase, LDL receptor, Fatty acid synthase and Acetyl CoA carboxylase by means of sterol regulatory element binding transcription factors.

1. 3 Role of Lysosomal Acid Lipase in Cellular Lipid Metabolism.

As early as in 1969, it was known that an acid hydrolase activity was deficient in the liver and spleen of patients with Wolman's disease characterised by a massive storage of cholesteryl esters and triglycerides in various tissues of the body (*Patrick et al., 1969*). Though this observation was confirmed by other studies, the importance of this hydrolysis step in the regulation of cellular cholesterol homeostasis remained obscure till *Goldstein and Brown, (1974)* unravelled the mistery of terminal catabolism of low density lipoproteins analysing the molecular and biochemical basis of Familial Hypercholesterolemia.

During the course of these studies, they discovered the presence of a cell surface receptor that facilitates receptor mediated endocytosis of plasma LDL and delivery to lysosomes. Their studies using radioactive LDL

unambiguously verified the deficiency of an acid lipase activity in the fibroblasts of patients with Cholesteryl Ester Storage Disease and Wolman Disease (*Goldstein et al., 1975*). Subsequently, they showed that this activity was sensitive to chloroquine treatment implicating lysosomal localisation of the enzyme. *Sando & Rosenbaum (1985)* purified this activity from fibroblasts in culture and demonstrated that the CESD fibroblasts regained acid esterase activity when the purified lysosomal acid lipase was added to the medium.

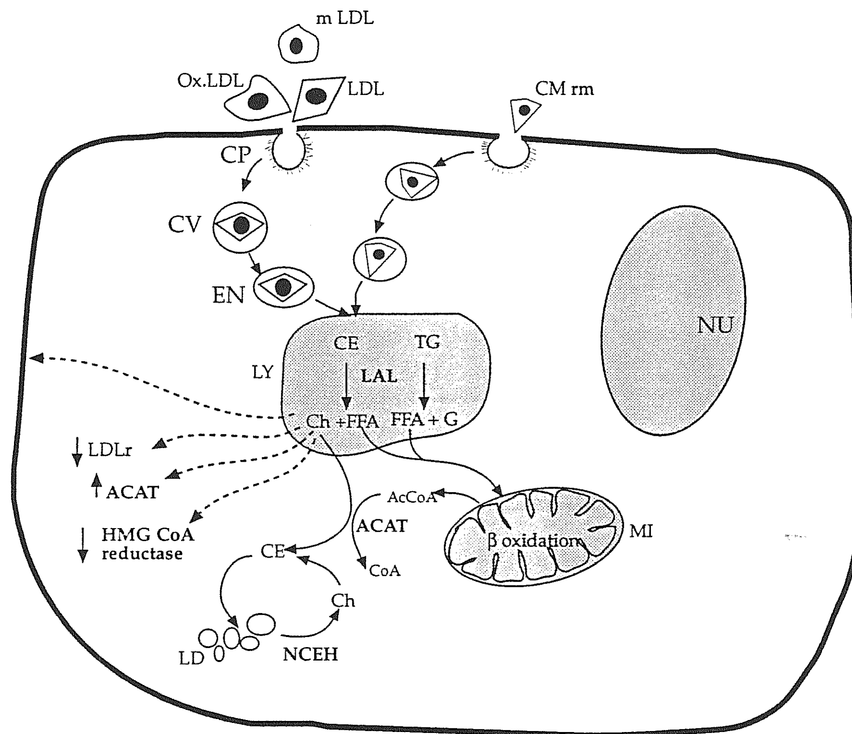

Fig.1.3.1: Schematic representation of the role of Lysosomal acid lipase inthe regulation of intra cellular lipid metabolism. Different sources of cholesteryl esters (CE) and triglycerides (TG) entering the cell are shown. LDL: lowdensity lipoprotein., OxLDL: oxidised LDL., mLDL: modified LDL., CMrm: chylomicron remnant. These particles enter the cell through the receptor mediated endocytosis via coated pits (CP) and get entrapped into the coated vesicles(CV)that get transformed into endosomes (EN) and subsequentlly, become lysosomes(LY) by acidification. Inside the lysosomes, lysosomal acid lipase (LAL) hydrolyse these cholesteryl esters and triglycerides to cholesterol (Ch) , free fatty acids(FFA) and glycerol(G). Free fatty acids proceed to the mitochondrial β oxidation pathway while cholesterol may bedirected to membrane biosynthesis pathway or alternatively, enter the cytoplasmic cholesteryl ester cycle by getting esterified with Acetyl coenzyme (AcCoA) by the action of Acetyl CoA: cholesterol acyl transferase (ACAT). These cholesteryl esters may be stored as lipid droplets (LD) in the cytoplasm or may undergo hydrolysis by the Neutral cholesteryl ester hydrolase (NCEH). Amount of free cholesterol in the cytoplasm inturn regulates the availability of LDL receptors, HMG CoA reductase and ACAT in the cytoplasm. MI indicates mitochondria and NU indicates nucleus.

These studies confirmed the role of lysosomal acid lipase in the hydrolysis of cholesteryl esters and triglycerides forming the core of low density lipoprotein particles. When there is a deficiency of this hydrolase activity, cholesteryl esters and triglycerides accumulate in the lysosomal compartment of cells of different tissues. Moreover, the level of free cholesterol

in the cytoplasm drops down resulting in the activation of genes in the biosynthetic pathway of cholesterol (Fig.1.3.1). As a consequence, a deficiency in the activity of lysosomal acid lipase perturbs the fine tuning of cellular lipid metabolism ultimately resulting in pathological conditions such as Wolman disease and Cholesteryl Ester Storage Disease (CESD).

1. 4 Biochemical characteristics of Lysosomal Acid Lipase:

Distinct from the well known mammalian lipases of the lipase super family that are tri acyl glycerol lipases, LAL shows a dual catalytic activity, being capable of hydrolysing both triglycerides and cholesteryl ester substrates. Early reports on Lysosomal Acid lipase (LAL) deficiency were based on measurement of LAL activity in PBMNCs of the subjects using 4-methyl umbelliferyl oleate (4-MUO) or palmitate as substrate by means of a fluorimetric detection method (*Kelly & Bakhrau-Kishore 1979*). This method took advantage of the broad substrate specificity of esterases. There were no chances of interference from other lipases present in the cell extract since this enzyme has a pH optimum of 4.5-4.8 that is detrimental for neutral lipases. But acid phospholipases could still interfere since they are also able to hydrolyse umbelliferyl esters.

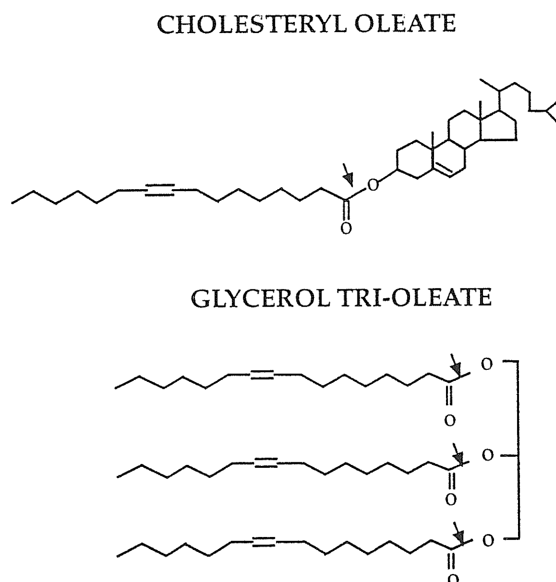

Fig.1.4.1: Dual nature of LAL catalysis. LAL is capable of hydrolysing both cholesteryl esters and triglycerides. The chemical structure of two model substrates used for measuring these two activities are shown in the figure. Arrow head indicates the site of cleavage.

Therefore, near natural substrates such as ^{3}H or ^{14}C labelled cholesteryl oleate and tri oleyl glycerol (Fig.1.4.1) have been used as substrates for further characterisation of the dual nature of the catalytic property of

purified LAL. In this method, the ^{14}C oleate released during hydrolysis can be separated by partitioning using organic solvents. The radioactivity in the upper aqueous phase can be counted and enzyme activity can be expressed as pmol/µg protein/hr.

Further analysis of the biochemical properties of this enzyme was still limited by the availability of purified material. Attempts have been made to purify LAL from different tissues including human liver (*Warner et al.,1981*), placenta (*Burton et al., 1980 ; Chen et al., 1971*) aorta (*Sakurada et al., 1976*), and leukocytes (*Rindler -Ludwig et al., 1977*). There is no general agreement among these reports regarding the molecular weight of the purified enzyme. However, in most of these cases the specific activity of the purified enzyme was relatively low. Therefore the estimation of molecular weight seems to be less reliable. Purification of LAL to higher specific activity was achieved from cultured fibroblasts (*Sando & Rosenbaum, 1985*) and according to these authors, fibroblast LAL migrates as a band of 47- 49 kDa on 11% SDS-polyacrylamide gels while estimation of the molecular weight by glycerol gradients suggested an apparent molecular mass of 47 kDa.

Though some of the early reports indicated that triglyceride esterase activity and cholesteryl esterase activity reside in two different proteins, purification of the enzyme from cultured fibroblasts proved that the two activities are present in the same protein (*Sando &Rosenbaum., 1985*) migrating as a 49 kDa band on 11% SDS-polyacrylamide gel. Later reports of purification of LAL from different sources attributed different molecular weights to the LAL protein. *Ameis et al (1994)* have reported two major molecular weight forms of human hepatic LAL migrating at 41 kDa and 56 kDa bands. These authors have performed N-terminal sequencing of the 41 kDa form and showed that a stretch of 49 amino acids corresponding to the N-terminus of mature LAL is missing in this form. It has been postulated that tissue specific proteolytic processing may be involved in the maturation of the LAL polypeptide and that the 41 kDa hepatic form may represent the result of such a proteolytic event, specific for hepatic tissue.

During the course of the studies reported in this thesis, *Sheriff et al., 1995* have reported expression of LAL in SF9 cells by means of the Baculovirus expression system and showed that LAL protein expressed in this system was active towards both ^{14}C labelled cholesteryl esters and ^{14}C labelled triglycerides, diglycerides and monoglycerides. Two major molecular weight forms of 41 kDa and 46 kDa were observed in this study and the authors suggest that the higher molecular weight forms may represent heavily glycosylated forms of the protein. Their studies indicated that 7-9 carbon fatty

acyl chain lengths may be the ideal size for enzyme binding and catalysis by proper accommodation in the active site. It is likely that *cis* unsaturation in the fatty acyl chain is better tolerated than trans unstauration (*Sheriff et al., 1995*).

1.5 Sequence homology and functional similarities with other mammalian lipases:

Anderson et al, in 1991 described a 2.6 Kb LAL cDNA with an open reading frame of 1197 bp predicting a resultant polypeptide chain of 399 amino acids including a signal peptide. Expression of this cDNA in COS cells confirmed that it codes for a functional Acid Lipase. Comparison of the amino acid sequence with known sequences in the protein data bank revealed 58% homology to Human Gastric Lipase and 57% homology to Rat Lingual Lipase (*Anderson et al., 1991*) while there was no significant homology with the mammalian lipases belonging to the super family of lipases (*Hide et al., 1992*). Recently, the rat homologue of LAL was cloned(*Natagawa et al., 1995*) and it was shown that the amino acid sequence was 87 % identical to that of human LAL. A partial cDNA clone for mouse LAL was obtained by RT-PCR on mouse liver RNA and this sequence was found to be similar to rat LAL amino acid sequence. Alignment of the amino acid sequences of all these related acid lipases is shown in Fig. 1.5.1. These sequence comparison studies indicate that LALs, along with other two pre-duodenal acid lipases, form a new family of acid lipases distinct from the super family of lipases including pancreatic, hepatic and lipoprotein lipases. It can be noted that the arrangement of cysteine residues in this family is completely different from that of members of lipase super family suggesting that the overall folding of these lipases could be completely different from that of the known lipase three dimensional structures.

In spite of scarce overall homology with other lipases, LAL conserves the GXSXG motif common for esterases in general. Two such esterase associated pentapeptide motifs (GXSXG) surrounded by a stretch of predominantly hydrophobic amino acids can be identified in the primary amino acid sequence of LAL (Fig. 1.5.1). In spite of poor homology among their amino acid sequences, it has been assumed that the central serine of a GXSXG motif may form part of the catalytic triad of different classes of lipases along with an Aspartate/Glutamate and a Histidine (*Brady et al., 1990*). This prediction turned out to be true for a number of mammalian lipases for which site directed mutagenesis and crystallisation data are available.

```
mlal   1   ..........................................................................................
rlal   1   MqlLGrViCfVvgiLlSgGptGtisAVDPEaNMNVtEIImhWG  ypEhsVqTgDGYILgvhRIPHGRKNqfDKGPKPVVyLQgeHGfLADSSNWVTN
hlal   1   MkMrfLGlVvClVLwpLhSeGsgGKLtAVDPEtNMNVSEIIsYWGfPsEEYlVeTEDGYILclNRIPHGRKNhsDKGPKPVVFLQ  HGLLADSSNWVTN
hgl    1   MWLLLtmaSlISVL  GttHGLFGKLhPGsPEvtMNISQMITYWGYPnEEYEVVTEDGYILeVNRIPyGKKNSgNtGqRPVVFLQ  HGLLASATNWIsN
rll    1   MWLLL itSvIS  tfGgaHGLFGKLgPGnPEanMNISQMITYWGYPcqEYEVVTEDGYILgVyRIPhGKnNSeNiGkRPVVyLQ  HGLiASATNWIaN

mlal  97   ..........................WSlKHKTLSVSQDEfWAFSFDEMAKYDLPASINYILNKTGQEQiYyVGHSQGCTIGFIAFSQMPELAKKiKMFl
rlal  97   idNnSLGFILADAGFDVWMGNSRGNTWSRKHKTLSVSQDEyWAFSFDEMAKYDLPASINYILNKTGQEQlYnVGHSQGCTIGFIAFSQMPELAKKvKMFF
hlal  99   LaNsSLGFILADAGFDVWMGNSRGNTWSRKHKTLSVSQDEFWAFSyDEMAKYDLPASINFILNKTGQEQvYYVGHSQGTTIGFIAFSQiPELAKRIKMFF
hgl   97   LPNNSLAFILADAGYDVWLGNSRGNTWaRrNlYYSPDSVEFWAFSFDEMAKYDLPATIdFIVkKTGQkQlHYVGHSQGTTIGFIAFSTNPsLAKRIKTFY
rll   97   LPNNSLAFmLADAGYDVWLGNSRGNTWsRkNvYYSPDSVEFWAFSFDEMAKYDLPATInFIVqKTGQekiHYVGHSQGTTIGFIAFSTNPtLAKkIKTFY

mlal 198   vLAPVLSLNFASGPllqLrRLPDpLLkDmFGQKQFLPQSAMlKWLSiHvCTHVIMKELCANvFFLlCGFNEKNLNMSRVDVYTTHC..............
rlal 198   ALAPVLSLNFASGPMvKLGRLPDlLLeDLFGQKQFLPQSAMvKWLSTHiCTHVIMKELCANiFFLiCGFNEKNLNMSRVDVYTTHCPAGTSVQNMvHWtQv
hlal 200   ALgPVASVaFctSPMaKLGRLPDhLiKDLFGDKeFLPQSAFlKWLgTHVCTHVILKELCgNlcFLlCGFNErNLNMSRVDVYTTHsPAGTSVQNMlHWsQA
hgl  198   ALAPVATVKYTkSlinKLrFvPqsLFKfiFGDKiFyPHnfFDqFLaTEVCSREmLnLLCSNaLFIiCGFDsKNfNtSRlDVYLsHNPAGTSVQNMfHWtQA
rll  198   ALAPVATVKYTqSplkKisFiPtfLFKlmFGkKmFlPHtyFDdFLgTEVCSREvLdLLCSNtLFIfCGFDkKNlNvSRfDVYLgHNPAGTSVQdflHWaQl

mlal       ..........................................................................................
rlal 299   VKyhKlQAFDWGSSdKNYFHYNQSYPPlYsiKDMqlPTAlWSGGkDWLADtsDiNILLTeIptLVyHknIPEWdHLDFIWGLDAPWRLYNevvsLMkKYQ
hlal 301   VKfqKFQAFDWGSSaKNYFHYNQSYPPtYNVKDMlVPTAVWSGGhDWLADvyDVNILLTqItNLVfHesIPEWeHLDFIWGLDAPWRLYNkIinLMrKYQ
hgl  299   VKSGKFQAyDWGSPvQNrmHYdQSqPPyYNVtAMnVPiAVWNGGkDlLADPQDVglLLPKLpNLiyHKEIPfYNHLDFIWAMDAPQEVYNdIvsMISedkk
rll  299   VrSGKFQAfnWGSPsQNmlHYnQktPPeYdVsAMtVPvAVWNGGnDiLADPQDVamLLPKLsNLlfHKEIlaYNHLDFIWAMDAPQEVYN   eMISmm
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Fig.1.5.1. Amino acid sequence alignment of different members of the Acid lipase family. Alignment was performed using the Genalign programme of Intelligenetics package. **mlal**: mouse LAL., **rlal**: rat LAL., **hlal**: human LAL., **hgl**: human gastric lipase., **rll**: rat lingual lipase. Except for mouse LAL, all the other sequences were retrieved from Genbank. Mouse LAL sequence shown here is derived from the nucleotide sequence of a partial mLAL clone obtained by PCR amplification from mouse liver RNA using oligonucleotide primers in the highly conserved region of human LAL. Homology searches against the whole databank revealed that this partial sequence has highest homology with hLAL and rLAL sequences. The two esterase associated pentapeptide motifs (GXSXG) are shown in bold letters along with two conserved Aspartates and a Histidine that were subjected to site directed mutagenesis. The cysteine residues are typed in bold and indicated with asteriks. The putative N-glycosylation sites (N-X-S/T) are underlined.

Pancreatic lipase:

Three dimensional structure of pancreatic lipase determined by X-ray crystallography (*Winkler et al., 1990*) suggested that S^{152} can be the nucleophilic residue essential for catalysis. The location of this residue in the large N-terminal domain situated at the C-terminal edge of a doubly wound β sheet, forming part of an Asp-His-Ser triad chemically analogous to that of serine proteases strongly suggested it's status as the catalytic nucleophile. Functional evidence in this regard was provided by site directed mutagenesis of S^{153}, D^{177} and H^{274} residues (*Lowe, 1992*). These constructs produced catalytically inactive protein when expressed in COS cells. Co crystallisation of pancreatic lipase-pro colipase complex in the presence of mixed micelles provided further evidence for the role of these residues in the conformation of the catalytic site (*Tilbeurgh et al., 1993*). These studies provided experimental evidence for the long sought phenomenon of inter facial activation of lipases upon binding of lipid substrates and established the role of disulphide bonded cysteines (C237-C261) in the modulation of these changes.

Lipoprotein lipase:

Extensive site directed mutagenesis studies by *Faustinella et al., (1991a)* suggested that S^{132} of lipoprotein lipase may have a critical role in the catalytic activity. Their experimental results were strongly supported by the homologous position of this Serine to that of S^{152} of pancreatic lipase. Identification of an LPL mutation D156G as the basis of Familial Type I Hyper lipoproteinemia in a patient suggested that this residue may be critical for catalysis by LPL (*Faustinella et al., 1991b*). *Emmerich et al., 1992* have extended these studies to identify D^{156} and H^{241} as the putative partners of S^{132} in this catalytic triad. Comptuer modelling of the three dimensional structure of lipoprotein lipase based on the crystal structure of pancreatic lipase further strengthened the conclusion that these residues form part of the functional catalytic site of the enzyme (*Tilbeurgh et al., 1994*).

Hepatic lipase: Site directed mutagenesis of different serine residues in rat hepatic lipase showed that S^{147} is critical for catalytic activity. (*Davis et al., 1990*)

Hormone sensitive lipase : In spite of lack of significant homology with members of any of the known lipase families, primary amino acid sequence of HSL has a GXSXG motif around the S^{423}. Site directed mutagenesis of this serine abolishes the catalytic activity of the enzyme (*Holm etal., 1994*) while

mutating other residues S^{185}, S^{320}, S^{420} or S^{533} didn't affect the catalytic activity significantly.

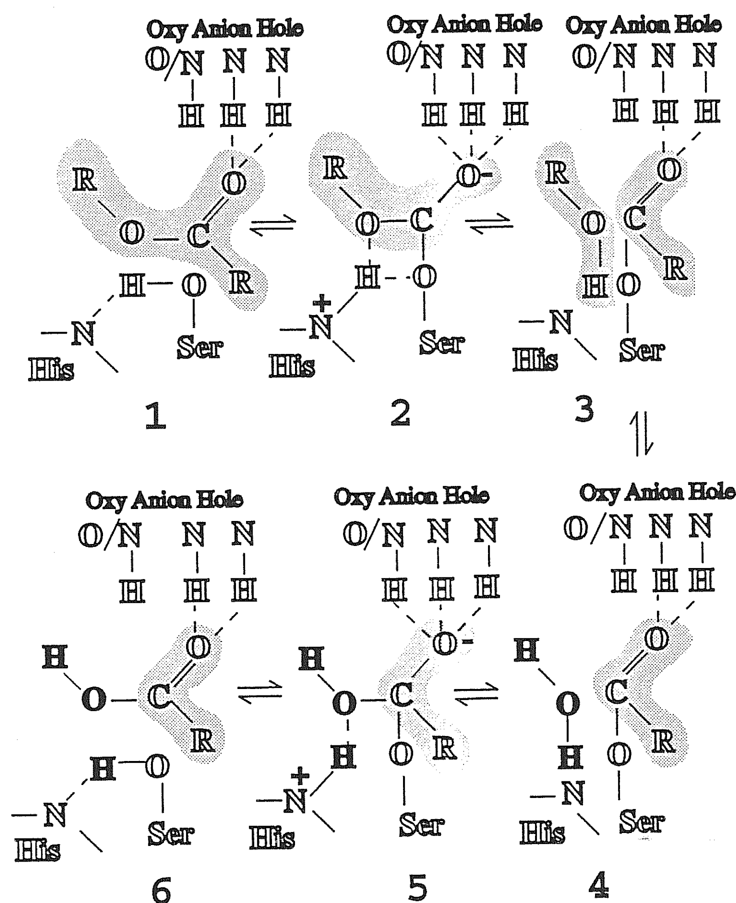


Fig.1.5.2: Proposed model of the the lipase ester cleavage pathway. The role of catalytic triad residues in the charge relay system leading to the cleavage of ester bond is verified by molecular dynamics simulation of the lipase reaction based on the three dimensional model of *Rhizopus oryzae* lipase derived from the crystal structure of *Rhizomucour miehei* lipase. This model also suggest that even residues located far away from the catalytic Serine, Aspartate/glutamate and Histidine in the primary structure may also participitate in catalysis by donating hydrogen bonds that help to stabilise the tetrahedral intermediate. Shaded area represents the substrate molecule. The water molecule is shown bold.
Step 1: Noncovalent Michaelis complex. Step 2: First tetrahedral stage. Step 3: Formation of acyl enzyme and release of an alcohol molecule. Step 4: Attack of water molecule on the acyl enzyme. Step 5: Second tetrahedral transition state. Step 6: Release of fatty acid.

The role of these residues in lipase catalysis has been further confirmed by a model of lipase reaction (Fig. 1.5.2) proposed by *Beer et al., (1996)* based on molecular dynamics simulation of *Rhizopus oryzae* lipase (ROL) models bound to substrate analogues. This model emphasises the role of catalytic serine and histidine residues in the charge relay system leading to the cleavage of ester bond. In ROL, the Oγ of S^{144} forms a covalent bond via a nucleophilic attack on the carbonyl carbon of the scissile ester bond, which becomes sp^3 hybridised. H^{257} acts as a general base catalyst and attracts a proton from S^{144} Oγ. This Nε - bound proton forms hydrogen bonds to the S^{144} Oγ and to the substrate's bridging oxygen. The resulting tetrahedral oxyanion

intermediate is stabilised by the N^{82} γ-OH (giving up its hydrogen bond to D^{91}) together with the backbone amides of N^{82} and L^{145}, which interact with the three free electron pairs of the substrate's carbonyl oxygen.

In the next step, Nε bound proton from H^{257} is transferred to the bridging oxygen in the substrate, releasing the alcohol component. The acid moiety of the substrate is still covalently bound to the enzyme. For the change of the carbonyl carbon back to sp^2 hybridisation stage, one oxygen electron pair has to be easily released from the hydrogen bond stabilisation by the oxyanion hole. This becomes possible when the N^{82} re-establish its hydrogen bond with N^{91}. At this point, the lipase exists as a stable acyl-enzyme, a reaction intermediate which is also found in protease reactions. The two electron pairs of the carbonyl oxygen are stabilised by the backbone amides of N^{82} and L^{145}. The hydrogen bond to the leucine becomes more ideal if the planar carbonyl geometry is tetrahedrally distorted. The acyl-enzyme can be attacked by a water or alcohol molecule, running through the above steps in the reverse direction. Thus the above model establishes that some residues situated far from the catalytic residues can also influence catalysis by donating hydrogen bonds that stabilise an oxyanion intermediate as in the case of N^{82}, N^{91} and L^{145} of ROL.

1.6 Protein Processing and targeting to lysosomes:

Primary amino acid sequence of LAL also indicates the possibility of a highly hydrophobic, 27 amino acid long, amino terminal signal peptide. (Fig.3.1.1). This type of signal sequences are common to almost all proteins that are targeted to the extra cytosolic side of the endoplasmic reticulum, Golgi complex, lysosomes and nuclear and plasma membranes. In general, these common signal sequences consist of a stretch of 15-30 mainly hydrophobic amino acids localised at the N-terminus of the proteins.

These signal sequences direct the protein synthesising ribosomes to rough endoplasmic reticulum. During translation, this polypeptide stretch that protrudes from the ribosome, first forms a complex with a cytosolic ribonucleoprotein called signal recognition particle (*Walter et al., 1984*). Subsequently, this complex binds to a receptor at the surface of the rough endoplasmic reticulum known as SRP receptor or the docking protein (*Meyer et al., 1982*), and the nascent protein is transferred into the lumen of the rough endoplasmic reticulum. Most of the soluble proteins that are released into the endoplasmic reticulum lose the signal peptide before the synthesis of their polypeptide is completed.

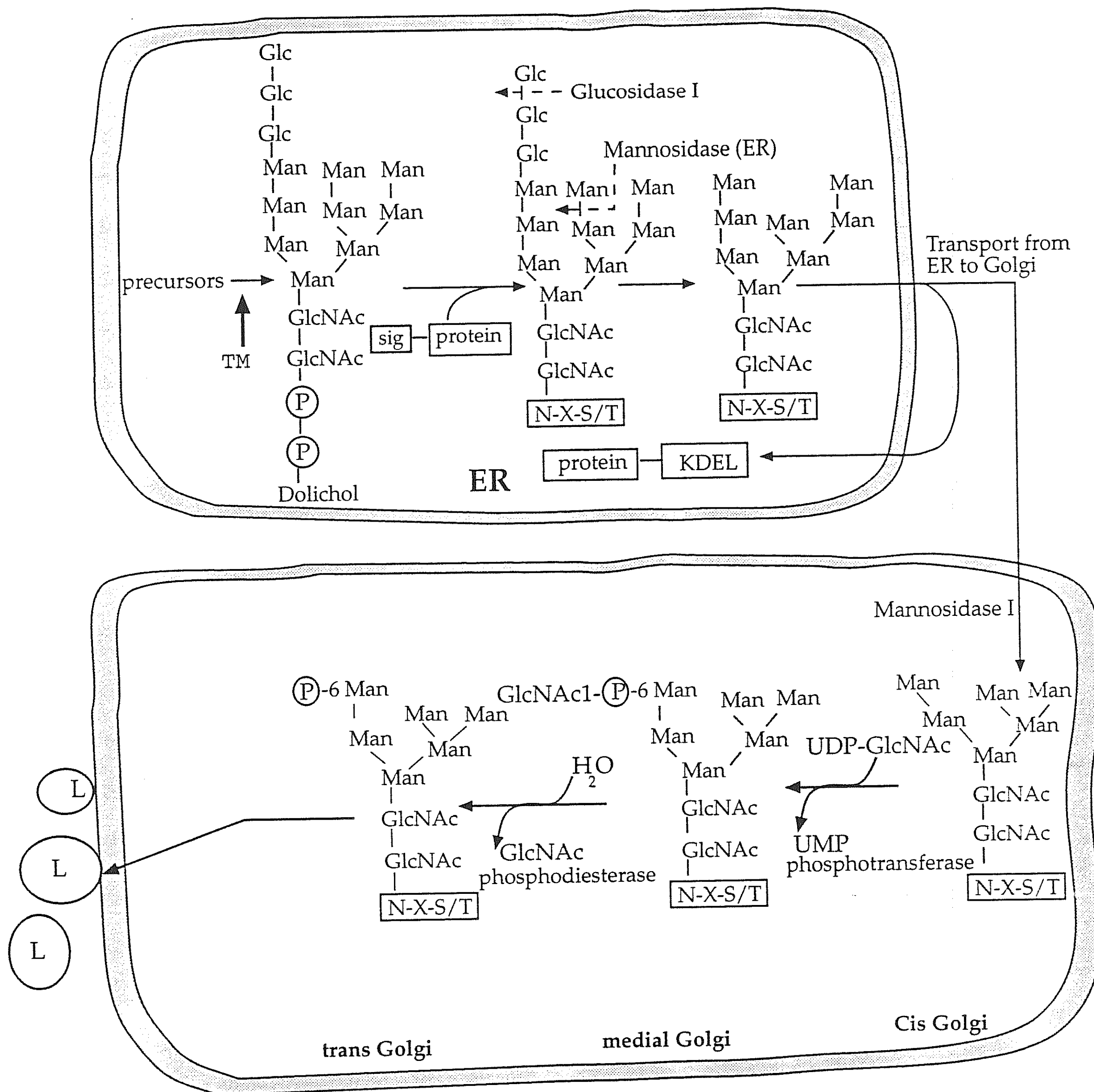


Fig.1.6.1: General pathway of post translational processing of newly synthesised lysosomal enzymes. Different steps involved in the synthesis and trimming of N-linked carbohydrate chains, transport from endoplasmic reticulum (ER) to Golgi apparatus and addition of Man-6phosphate are shown. sig- protein refers to proteins with a signal peptide that dictates the targeting of the protein to ER. Protein-KDEL stands for proteins with KDEL signal(ER retension signal). N-X-S/T indicates the N-glycosylation consensus. Man: mannose ., GlcNAc: N-acetyl glucosamine., Glc: Glucose., UDP: uridine diphosphate., UMP: uridine monophosphate. TM: the point of inhibition with Tunicamycin.

At this point, glucosylated oligosaccharides are transferred to certain Asparagine residues in the polypeptide marked by the consensus N-X-S/T. The oligosaccharide transferred to asparagine residue is pre-assembled in an activated form as a derivative of Dolichol pyrophosphate. The asparagine -linked oligosaccharides undergo further processing by a series of trimming reactions catalysed by glucosidase I, glucosidase II and α mannosidase specific to ER (Fig. 1.6.1). The transport of nascent lysosomal enzymes from ER to Golgi is likely to be mediated by smooth vesicles formed in transitional elements of the endoplasmic reticulum. The enzymes undergo further

trimming reactions in the cis Golgi region catalysed by mannosidase I capable of hydrolysing high mannose oligosaccharides.

Further processing include the addition of Man-6 Phosphate to the terminal carbohydrate residue. This reaction takes place in two steps. In the first step, N-acetyl glucosaminyl phosphotransferase transfers an N-acetyl glucosaminyl phosphate from an activated UDP-N-acetyl glucosamine to the terminal α1-2mannose residue. In the second step, a specific phosphodiesterase removes N-acetyl glucosamine creating the Man-6-Phosphate recognition marker specific for lysosomal targeting mediated by Man6-Phosphate receptors (*Sahagian et al., 1981 ; Holflack et al., 1985*).

Six putative N-glycosylation sites were identified in the primary amino acid sequence of LAL. Three of these are conserved among the different members of the acid lipase family while the others are present only in LAL. Curiously enough, gastric lipase and rat lingual lipase are secreted proteins while LAL is targeted to lysosomes. It has been demonstrated that N-linked glycosylation is necessary for the receptor mediated endocytosis of extra cellularly added lysosomal acid lipase suggesting that LAL follows Man-6 Phosphate dependent targeting pathway (*Sando & Henke 1982*).

Sequence comparison of the known lysosomal enzymes suggest that there are no consensus sequence signals around the glycosylation sites that may dictate the action of a phosphotransferase on the carbohydrate chain attached to a particular Asparagine (N). The lysine residues flanking the consensus glycosylation sites, especially dileucine motifs, were thought to be important for the recognition of a particular carbohydrate chain by this phosphotransferase.

Recent crystallographic studies on lysosomal enzymes such as Cathepsin D (*Metcalf et al., 1993*) and Acid β glucoronidase (*Jain et al., 1996*) have shown that the recognition by phosphotransferase depends on the local conformation of the polypeptide chain rather than a particular amino acid moiety. A putative lysosomal targeting motif has been identified based on these studies. Only 9 of the known lysosomal proteins possess this structural motif indicating that different classes of recognition motifs exist. Since LAL doesn't belong to this group, further studies on processing and sorting of LAL may help to identify novel recognition motifs for phosphotransferase.

1.7 Genomic organisation and chromosomal localisation of LAL gene:

LAL gene was localised on chromosome 10q23 by Fluorescent In Situ Hybridisation (*Anderson et al., 1993*) distinct from pancreatic lipase locus (10q 26.1) and proposed locus for Sphingolipid activator proteins (10q 21-22).

Analysis of genomic clones revealed that LAL locus spans a 36kb region consisting of 10 exons and 9 introns (Fig. 1.7.1) (*Aslanidis et al., 1994a*).

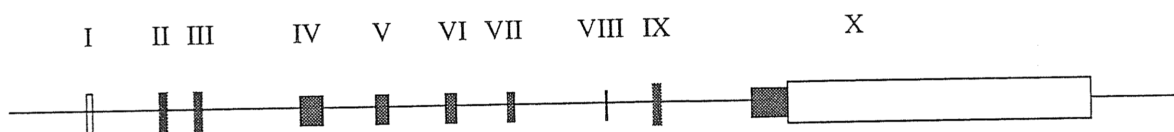


Fig.1.7.1: Schematic representation of the structure of LAL gene. The empty boxes indicate noncoding exons. The grey boxes indicate coding exons.

Comparison of the genomic organisation of the different members of the Pancreatic lipase family have revealed the interesting possibility of evolution of a multiple gene family by means of exon shuffling (*Kirchgessner et al., 1989*). Deatiled analysis of phylogenetic relationships between the different members of Acid lipase family is not yet available.

1.8 Clinical Manifestations of LAL Deficiency:

1.8.1 Wolman's Disease:

Wolman's disease represents the most severe clinical manifestation of Lysosomal Acid lipase deficiency. The disease shows an autosomal, recessive pattern of inheritance. Symptoms appear as early as the first week of life and generally the infants don't survive beyond the first year of their life. Major clinical symptoms include Hepatomegaly, abdominal distension, continuous vomiting and diarrhoea, anemia and general weakness.

Presence of lipid laden foam cells in peripheral blood and bone marrow and lower plasma lipid levels indicate abnormalities in the lipid metabolism. Impairment of fat absorption in Wolman's Disease patients has been clearly demonstrated by feeding ^{131}I labelled fats (*Eto & Kitagawa , 1970*). Intestinal damage can be so severe that the possibility of enteral nutrition can be virtually excluded. Vacuolisation of peripheral blood leukocytes can be observed very frequently. Vacuoles can be intracellular or intra nuclear. Adrenal calcification is the most striking diagnostic feature of Wolman's disease. Symmetrically enlarged adrenals appear to be full of calcific deposits. The density of the enlarged liver and the cortical calcification of the adrenals monitored with high sensitivity by computer aided tomography (CT) can be of use in correct diagnosis (*Hill et al., 1983*). Specific symptoms related with central nervous system are uncommon while neurologic development may not be normal. In spite of being alert and active at birth, infants show a progressive

mental disorientation within few weeks after the onset of symptoms (*Assman & Seedorf., 1995*)

1.8.2 Cholesteryl Ester Storage Disease (CESD):

CESD is less severe with a late onset of symptoms. Patients show Hepatomegaly, elevated LDL cholesterol levels and lower HDL cholesterol levels in addition to massive accumulation of refractile granules in liver biopsy. Low level of LAL activity measured in the peripheral blood leukocytes helps to distinguish CESD from other intracellular lipid storage diseases such as Tangier disease and Niemann-Pick disease type C. Tangier Disease is also characterised by accumulation of cholesteryl esters in lysosomes but these patients show a characteristic pattern of plasma HDL catabolism that helps to distinguish them from CESD patients. Unlike in Wolman's disease, adrenal calcification is not common in CESD patients. Patients may survive into adulthood without serious complications except for Hepatomaegaly and hyper beta lipoproteinemia. Liver transplantation is performed in cases of severe liver cirrhosis.

As it can be noted from the above descriptions, in spite of having a common biochemical basis, Wolman's Disease and Cholesteryl Ester Storage Disease follows completely different clinical courses. Identification of the cDNA (*Anderson, 1991*) and genomic sequences (*Klima et al., 1993*) of human lysosomal Acid lipase gene provided the hope that molecular defects leading to these two diseases will explain the differences in the two phenotypes.

1. 8. 3 LAL deficiency and Atherosclerosis:

Preliminary studies by *Bonner et al., (1974)* aortic acid lipase was lower in those species relatively susceptible to atherosclerosis such as rabbit and swine than in relatively resistant species such as rat, dog, guinea pig etc. Studies of *Goldfischer et al., (1974)* suggested that deficiency of acid lipase activity in the rabbit aorta might explain the susceptibility of some species to cholesterol-induced atherosclerosis. In humans, evidences suggest that LAL deficiency may lead to accumulation of lipoprotein derived cholesteryl esters and triglycerides in the lysosomes of arterial tissue as well (*Dincsoy et al., 1984*). Reduced level of LAL activity was shown to be one of the risk factors for premature atherosclerosis (*Coates et al.,1986*).

1.9 Molecular defects CESD and WD patients

Previously reported LAL mutations in WD and CESD patients have been summarised in Table.1.9.1. The mutations detected in our study are

not included in the Table.1.9.1.

Reference	Clinical Phenotype	Geographic origin	Mutation	Predicted consequences
Anderson et al., 1994.	WD	?	T->C (639) / T insertion (634)	L179P / Premature stop codon
Aslanidis et al., 1996.	WD	?	G->A +1 5' SS / G->A +1 5' SS	Δ254-277
Klima et al., 1993 Aslanidis et al., 1996.	CE SD	Polish-German	G->A -1 5' SS / Z F186L L179P	Δ254-277 / abnormal mRNA processing!!
Pagani et al., 1994	CE SD	Italian	C->T (923) / C->T (923)	H274Y / H274Y
Ameis et al., 1995	CE SD	East European	G->A -1 5' SS / G->A -1 5' SS	Δ254-277
Muntoni et al., 1995	CE SD	Spanish	G->A -1 5' SS / G->A -1 5' SS	Δ254-277
Ameis et al., 1995	CE SD	?	G->A -1 5' SS / Δ967-968	Δ254-277 / Δ296-330 abnormal mRNA processing!!
Seedorf et al., 1995	CE SD	Canadian-Norwegian	G->A -1 5' SS / T->C (1008)	Δ254-277 / L336P

Table.1.9.1: Summary of previously reported LAL mutations in Wolman's Disease (WD) and Cholesteryl ester Storage Disease(CESD). The grey and white triangles indicate the two different alleles. The stop codon is indicated with symbol Z. 5'SS and 3'SS refer to 5' and 3' splice sites respectively. ? indicates that the geographic origin is not mentioned in the references.

1.9.1 Molecular defects in Wolman's Disease:

In 1994, *Anderson et al.* reported an insertion of a Thymidine at pos 634 in Ex.6 of the LAL cDNA from one allele and a T to C substitution at pos 639 of the LAL cDNA from the other allele in a fibroblast cell line derived from a Wolman's Disease patient. Insertion mutation creates an inframe stop codon 12 amino acids downstream predicting a truncated translation product of 190 amino acids. This mutation may affect the stability or proper processing of the mRNA explaining low levels of LAL mRNA observed by Northern blot analysis on the RNA extracted from the fibroblasts of this patient. Missense mutation resulting in L179P coding change is expected to affect the enzymatic activity. It is located 26 amino acids downstream of the putative catalytic serine of the enzyme. Later this mutant allele was expressed in insect cells by means

of Baculovirus expression system and it was demonstrated that intact protein is produced but is catalytically inactive (*Sheriff* et al., *1995*).

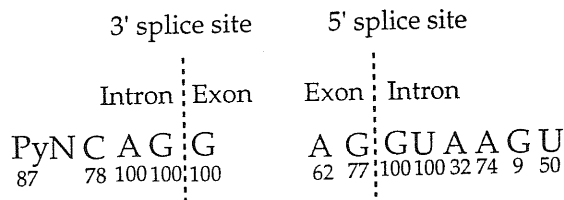


Fig.1.9.1: Consensus sequences at the 3' and 5' splice sites of mammalian genes

Aslanidis et al. (1996) have recently described two children with Wolman disease homozygous for a G->A mutation at the +1 position of the 5′ splice donor site of Ex.8 of LAL gene. This mutation resulted in skipping of Ex.8 in the LALcDNA in these patients giving rise to a protein lacking 24 amino acids that correspond to Ex.8. Since this position is absolutely conserved in at splice sites mammalian exons (Fig. 1.9.1), there is little chance that a low amount of correctly spliced mRNA can be derived from this allele (*Shapiro et al., 1987*).

These authors provide evidence for absence of correctly spliced mRNA in these patients by RT/PCR analysis. In this study, cDNA constructs with Ex.8 deletion were expressed in SF9 and H-5 cells using Baculovirus expression system it was seen that the enzyme with 24 amino acid internal deletion was inactive while the normal LAL was active when expressed in this system. Authors conclude that severity of Wolman's Disease compared to CESD is due to absence of residual enzyme activity in the case of Wolman's disease as described in these patients.

A 10 allele VNTR polymorphism in the first intron of the LAL gene (*Muntoni et al., 1995*) was proved to be useful for prenatal diagnosis of Wolman Disease (*Aslanidis et al., 1996*) in an East European Family.

1.9.2 Animal model of Wolman Disease:

Yoshida et al ., (1990) reported an animal model of LAL deficiency in a colony of Donryu rats. Based on the severe manifestations, short life span, accumulation of cholesteryl esters and triglycerides and acute deficiency LAL. This animal model is considered as the rat counterpart of Wolman's Disease. *Natagawa et al., (1995)* reported cloning of rat LALcDNA and subsequent characterisation of a genomic deletion in the LAL gene in Wolman rats. Nucleotide sequence analysis of the cDNA from Wolman rats had the same sequence as normal rat LALcDNA from the 5′ untranslated region till the nucleotide position 1101. A 60 bp replacement was observed from nucleotide

position 1102 to 1161 with a new poly A signal followed by a 1.8kb deletion. The deduced amino acid sequence of the 60bp replacement region demonstrated a missense substitution, I367N and a nonsense mutation (P368Z). Thus this replacement leads to a net loss of 29 amino acids from the LAL polypeptide. Southern blot analysis of the genomic DNA indicates the possibility of about 4.5 kb deletion in the rLAL gene in Wolman rats.

1.9.3 Molecular Defects in CESD patients:

Following the identification and characterisation of the human gene for LAL, a number of mutations has been detected in the LAL gene from CESD patients (Table.1.9.1). It is clear from this table that Ex.8 deletion due to A->G substitution at the -1 splice donor site is more frequent in CESD patients from Europe compared to other mutations. *Seedorf et al ., 1995* suggest that it could be due to a founder effect. Two patients homozygous for this mutation have been detected. Expression of this mutant allele in Baculovirus system showed that recombinant LAL protein carrying this deletion is catalytically inactive (*Sheriff et al., 1995*). This fact, in combination with the observation that Ex.8 skipping due to a mutation at the +1 position of 5′ splice site leads to Wolman phenotype, strengthens the hypothesis that the drastic differences in the phenotypic expression of the disease in Wolman and CESD patients is due to residual LAL activity. However, as in the case of other lysosomal enzymes, mutations affecting LAL gene may have diverse consequences other than their affect on the catalytic activity of the enzyme, such as alterations in the glycosylation pattern and defective targeting. These possibilities have not yet been explored in the case of LAL mutations.

The studies described in this thesis were aimed at the characterisation of molecular basis of CESD in patients from Italian population. Following the identification of mutations in LAL gene in three patients with clinically evident CESD, these studies were extended to verify the effect of these mutations on the catalytic activities of the enzyme, by in vitro expression of mutant LAL constructs in HeLa cells taking advantage of the Vaccinia T7 expression system. Subsequently, the potential of site directed mutagenesis technique was exploited for further characterisation of the molecular and biochemical aspects of LAL function.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Enzymes and Chemicals.

General chemicals were purchased from Sigma Chemical Co., or MERK FR. Germany. Restriction enzymes were from Pharmacia Biotech, (Sweden) or New England Biolabs, Inc. (USA). The Radioactive chemicals were purchased from Amersham International plc, (UK). DNA modifying enzymes such as Taq Polymerase, Klenow fragment of *E.coli* DNA Polymerase I, T4 DNA Polymerase and T4 DNA ligase were obtained from Boehringer Manheim GmbH (Germany). T4 polynucleotide Kinase was from New England Biolabs, Inc. and Calf Intestinal Alkaline Phosphatase was from Pharmacia Biotech, (Sweden). Synthetic oligonucleotides were from PRIMM (Milan, Italy).

2.1.2 Cell culture

HeLa cells for Vaccinia infection experiments were provided by the Tissue culture Division of ICGEB. HeLa cell cultures were maintained in Dulbecco's Modified Eagle Medium supplimented with 10% FBS. Fetal Bovine Serum was from Biochrom (Berlin). The recombinant Vaccinia virus strain, vTF7-3 was a generous gift from *Dr. Moss* & *Dr. Fuerst* . Transfectan reagent used in transfection experiments was from Boehringer Manheim. All sterile plasticware used in Tissue Culture were purchased from Falcon, (Becton Dickinson Co., NJ. USA).

2.2 Subjects:

Patient 1 was a male subject from South Italy characterised by clinical manifestation of CESD. The patient is the second child of healthy unrelated parents. His sister and parents were asymptomatic. At the age of 5 years the patient presented with hepatomegaly, elevated liver function test, hypercholesterolemia and hypertriglyceridemia. At the age of 10 years a liver biopsy revealed cirrhosis with vacuolisation and massive storage of

birifrangent material in hepatocytes. The enzyme activity was measured in leukocytes isolated from the blood of family members. Both parents had reduced LAL activity. At the age of 11, orthotopic liver transplantation was performed and the patient is doing well 2 years after the procedure.

Patient 2 was a male from Sicily and presented with hepatomegaly at the age of 6 years. Hypercholesterolemia and hypertriglyceridemia were observed at the age of 21 years and a liver biopsy revealed widespread vacuolized hepatocytes. LAL deficiency was diagnosed in liver biopsy, cultured skin fibroblasts and peripheral leukocytes. Only cultured fibroblasts were available for subsequent analysis.

Patient 3 presented with hepatomegaly at the age of 5 years. Progression of the disease was evident by the age of 15 with the appearance of hypercholesterolemia and hypertriglyceridemia in association with mildly elevated liver function tests. The diagnosis of CESD in this patient was confirmed by the observations that cholesteryl ester content of liver biopsy was abnormally high and LAL activity in cultured fibroblasts was below the average. The parents were asymptomatic. Subsequent analysis was carried out on DNA and RNA extracted from frozen blood samples from the patient and parents.

2.3 Methods.

All standard enzymatic modifications of DNA were performed according to the protocols described by *Sambrook et al., 1989.*

2.3.1 Isolation of Peripheral Blood Mononuclear Cells (PBMNCs):

Blood samples in 0.5M EDTA were mixed with dextran to a final concentration of 1%. Mixed gently and allowed to sediment at room temperature for about 30 min. Decanted plasma was layered on a bed of 3vol. of Lympho trap (a mixture of Ficoll 400 and PEG 8000; purchased from Nycomed-AS, Oslo Norway). This mixture was Centrifuged at 1000g for 15 min. and the interphase was recovered taking care not to mix with the granulocytes that remain at the bottom. 10-15 ml of PBS was added and centrifuged at 1200rpm for 5min. The supernatant was aspirated and the cell pellet containing the PBMNCs were stored at -80^{o}C.

2.3.2 DNA Extraction from Blood :

5ml blood sample collected in 10mM EDTA (pH 8) was mixed with 45 ml of Lysis buffer (0.32M Sucrose, 10 mM Tris.Cl pH 7.5, 5mM $MgCl_2$, 1% Triton X 100). Mixed gently and incubated on ice for 10 min. Centrifuged at 1500g in a Benchtop centrifuge (Sigma 4-10). Pellet was washed several times

with Fisio Buffer (0.075M NaCl, 0.025M EDTA pH 8). After each washing centrifuged as mentioned above. Once the pellet is pretty white, it is resuspended in 5ml of Fisio Buffer. 250μl of SDS (10%) and 100μl of proteinase K (10 mg/ml) were added, mixed well and incubated at 37°C for 3-4 hrs. After incubation, 5 ml of Phenol/Chloroform (1:1) was added, mixed gently and centrifuged for 10 min. at 1500g in order to separate the phases. Aqueous phase was collected and transferred to a fresh tube. The phenol phase was back extracted with 5 ml of Fisio Buffer and the upper phase was pooled together with the upperphase from the first extraction. Extracted once more with 10μlof Chloroform and the upper phase was transferred to a new Falcon tube and DNA was precipitated with 1ml of 3M Sodium Acetate and 2.5vol. of Absolute Ethanol. White filamentous pellet can be spooled out with a hooked pasteur pipette and transferred to 1.5ml eppendorf. The pellet was washed with 70% Ethanol and redissolved in 1ml of TE buffer.

2.3.3 Rapid method for isolation of DNA from cultured cells:

DNA was prepared from cultured fibroblasts by a simplified procedure (*Laird eat al.,1991*). Cells were scraped off in PBS (NaCl 136mM; KCl 2.7mM; Na_2HPO_4 8.1mM; KH_2PO_4 1.47mM) and pelleted at 1000g in an eppendorf centrifuge. 500μl of modified Lysis buffer (100mM Tris.HCl pH 8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl, 100μg Proteinase K/ml). Incubated at 37°C for 2-3 hrs with agitation. 1 vol. of Isopropanol was added and the tube was swirled gently till the precipitation is complete. White filamentous precipitate was collected with a sterile pasteur pipette and transferred to a fresh tube. The pellet is washed with 70% ethanol and redissolved in 0.5ml of TE buffer.

2.3.4 RNA Extraction from Blood and PBMNCs.

Total RNA was extracted from blood and PBMNCs according to the acid phenol chloroform extraction method (*Chomczynski et al., 1987*) with slight modifications. Frozen blood samples collected in 0.5M EDTA were thawed and equal volume of an extraction buffer containing 4M Guanidium Thiocyanate, 25mM Sodium Citrate and 0.5% Sarcosine and 100mM β-mercaptoethanol. For PBMNCs, the cell pellets were resuspended in appropriate vol. of extraction buffer. Mixed well and 0.1 vol. of Sodium Acetate was added followed by equal vol. of water saturated phenol pre-warmed at 65°C and 0.2vol of chloroform. Mixed well after adding each reagent and finally vortexed for 30secs. at max.speed and incubated on ice for 15min. Centrifuged at 4000g in an SW14 rotor in a sorvall centrifuge at 4°C for

10min. The upper phase is collected and an equal vol. of a 1:1 mixture of Acid Phenol and Chloroform. Vortexed as before and incubated again on ice for 10min. Centrifuged for 10min. at 4000g at 4°C. Clear upper phase was collected and equal vol. of Isopropanol was added and incubated on ice 30min. Centrifuged at 8000g for 20min. at 4°C in order to collect the precipitate. The precipitate was washed with 80% ethanol, air dried and dissolved in RNAse free H_20.

2.3.5 Reverse Transcription and PCR amplification:

1-2µg of total RNA was reverse transcribed in to first strand complementary DNA (cDNA) in a 50µl reaction containing 50mM Tris HCl. pH 7.6, 60mM KCl, 10mM DTT, 250nM dNTPs, 25pM of a random hexanucleotide primer, 40U of RNAse Block (Strategene) and 250U of Moloney Leukemia Virus Reverse Transcriptase (BRL) at 37°C for 1hr. 2µl of this reaction mixture was used as template in the Polymerase Chain reaction (Saiki et al., 1988) in order to amplify LAL cDNA. 70µl PCR reactions contained 1x Taq buffer (Boerhinger), 40pM each of both forward and reverse primers specific for LALcDNA and 2U of Taq DNA polymerase (Boehringer). 35 cycles of amplification including denaturation at 94°C for 1min., primer annealing at 56°C for 1min., and chain extension at 72°C for 1min were performed in a Perkin Elmer DNA Thermal Cycler 480.

2.3.5.1 Oligo nucleotides used in RT-PCR analysis.

Name	Sequence	Location
1. LAL F1	ACTGCGACTCGAGACAGCGGC	Exon 1
2. LAL 46F	AATGCGGTTCTTGGGGTTG	Exon 2
3. LAL F209	GATGGATATATTCTGTGCC	Exon 3
4. LAL RC306	AGCAAGCCATGTTGCAGGA	Exon 4
5. LAL F424	ACATAAGACACTCTCAGTT	Exon 4
6. LAL F555	GTCATTCTCAAGGCACCAC	Exon 5
7. LAL RC684	TTGGCCATAGGGCTAGTAC	Exon 6
8. LAL F782	TGCACTCATGTCATACTGAA	Exon 7
9. LAL RC801	TTCAGTATGACATGAGTGCA	Exon 7
10. LAL F826	TCTTCTGTGTGGATTTAA	Exon 7
11. LAL F895	TGCTGGAACTTCTGTGCA	Exon 8
12. LAL RC912	TGCACAGAAGTTCCAGCA	Exon 8
13. LAL RC1057	ACTGCAGTCGGCACAAGCAT	Exon 10
14. LAL F1081	TGCAGATGTCTACGACGTCAAT	Exon 10

15. LAL RC1274 ATAAGCTTGGTGGTACACAGCTCAAGT 3'UTR

2.3.5.2 Oligonucleotides used in Geonomic DNA amplifications:

1.	LAL Ex.1R	TGCTGAAGGCACCAGCTTC	5'UTR
2.	LAL Ex.1bis	CCTGCTGAAGGCACCAGCT	5'UTR
3.	LAL Pro1	GTGCAGCCTGCAGACTCGG	Promoter
4.	LAL Ex.2F	GTGGGAGCATTAAGTTACC	Int.1/2
5.	LAL Ex.2R	TGGATCGGGAAATAGATGC	Int.2/3
6.	LAL Ex.4F	GAAGCTTGGTGCTACTGCC	Int.3/4
7.	LAL Ex.4R	CTGGAAGCCTGTTGTCTGC	Int.4/5
8.	LAL Ex.7F	TATGCACCAGAGTGAAATGC	Int 6/7
9.	LAL Ex.7R	AGTTCTGATGAGGTCATTCC	Int.7/8
10.	LAL Ex.8F	TCAATGCCACCTTAATGC	Int.7/8
11.	LAL Ex.8R	GGAAAGGGTTTTGCATGCC	Int.8/9

2.6 Agarose gel Electrophoresis and electro elution of amplified DNA fragments:

Products of PCR amplification were fractionated on 1% agarose gel run in 1x TBE (0.09 M Tris - Borate, 0.002 M EDTA) buffer inorder to verify the size of the amplified DNA fragments. 1kb DNA ladder (BRL) was used as size standard. DNA bands are visualised by Ethidium Bromide fluorescence. Bands of interest were cut from the gel and the DNA was electro eluted. The gel slices were placed in a pre-wet cellogel dialysis tubing along with 400µl of 0.5x TBE and the tubing was sealed with clips. After 10min. of electrophoresis at 200volts in 0.5x TBE, the solution inside the tubing was collected and extracted once with equal vol. of 1:1 mixture of Tris saturated phenol (pH 7.6) and Chloroform and once with Chloroform. The DNA was then precipitated by adding Sodium Acetate (pH 5.6) to a final concentration of 0.3 M and 2.5 vol. of absolute ethanol and incubating at -20^{o}C for 20min. The pellet was collected by centrifugation at 14000 rpm at 4^{o}C in an eppendorf centrifuge. The pellet was washed with 70% ethanol, dried and resuspended in TE buffer (10mM Tris, 1mM EDTA)

2.7 Direct sequencing of PCR products.

PCR products were purified on sephacryl 400HSR microspin columns (Pharmacia). Column eluates were concentrated in a Speedvac apparatus (Savant). 10µl of this concentrated sample containing ~ 100ng of DNA was denatured by incubating with 1µl of 1M NaOH at 37^{o}C for 10min

and then neutralised by adding 1µl of 1M HCl. Dideoxy sequencing (*Sanger et al., 1977*) was performed using T7 DNA polymerase (Pharmacia) following the instructions of the manufacturer. α S^{35} dATP (Amersham) was used as the label.

2.8 Preparation of HSV tagged LAL construct for Vaccinia coupled Expression in HeLa cells.

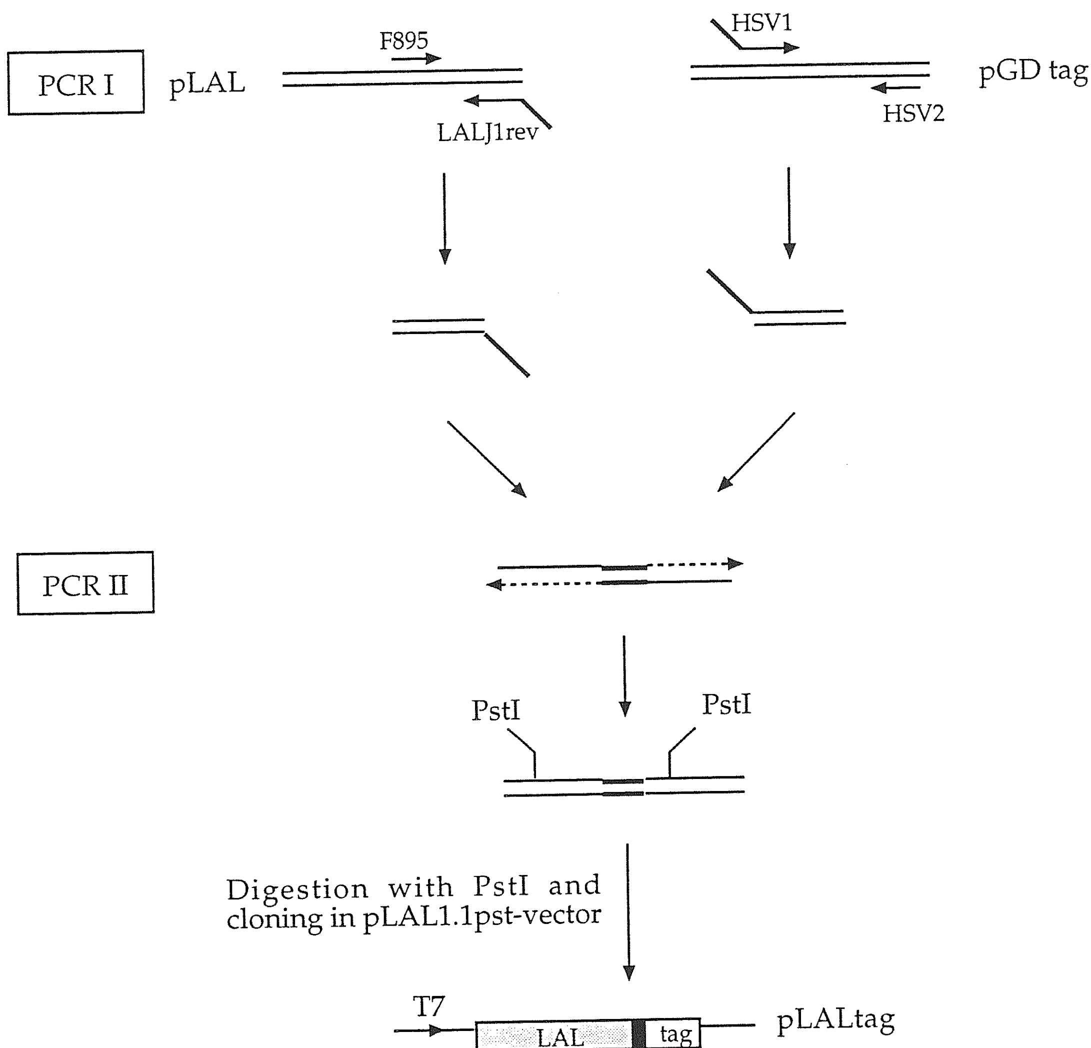


Fig.2.8.1: Preparation of HSVtagged LAL construct. PCR based sew-in method used for adding the glycine rich hinge region and HSV tag to the 3' end of LLA coding region. Primers LALJI Rev and HSV 1 possess the nucleotide sequence for hinge region as overhangs complimentary to each other (thick line). In the second PCR step, these complimentary overhangs anneal and extend the chain in both directions integrating this new sequence in the resulting fragment.pGDtag refers to the original plasmid containing the HSV tag sequence. Blackened region in pLAL tag indicates the glycine rich hinge region.

Normal LAL cDNA was cloned downstream of T7 promoter in pBluescript vector taking advantage of the unique XhoI and HindIII sites in the multiple cloning site. This plasmid, pLAL1.1 was digested with PstI, the vector was electroeluted from the gel and religated creating the plasmid pLAL1.1pst-.

Meanwhile, HSV tag coding fragment was amplified from the plasmid pGDtag using primers HSV1 and HSV2 and tailored to the 3' end of LALcDNA amplified using primers F895 and J1Rev (Fig. 2.8.1). The end product of the joint amplification was digested with PstI and cloned back in PstI digested pLAL1.1pst- creating the parent construct pLALtag that had 1179bp LAL coding region till the second last codon followed by a spacer region (GSGGG) and 11 aminoacids of the tag, terminated by the TGA stop codon.

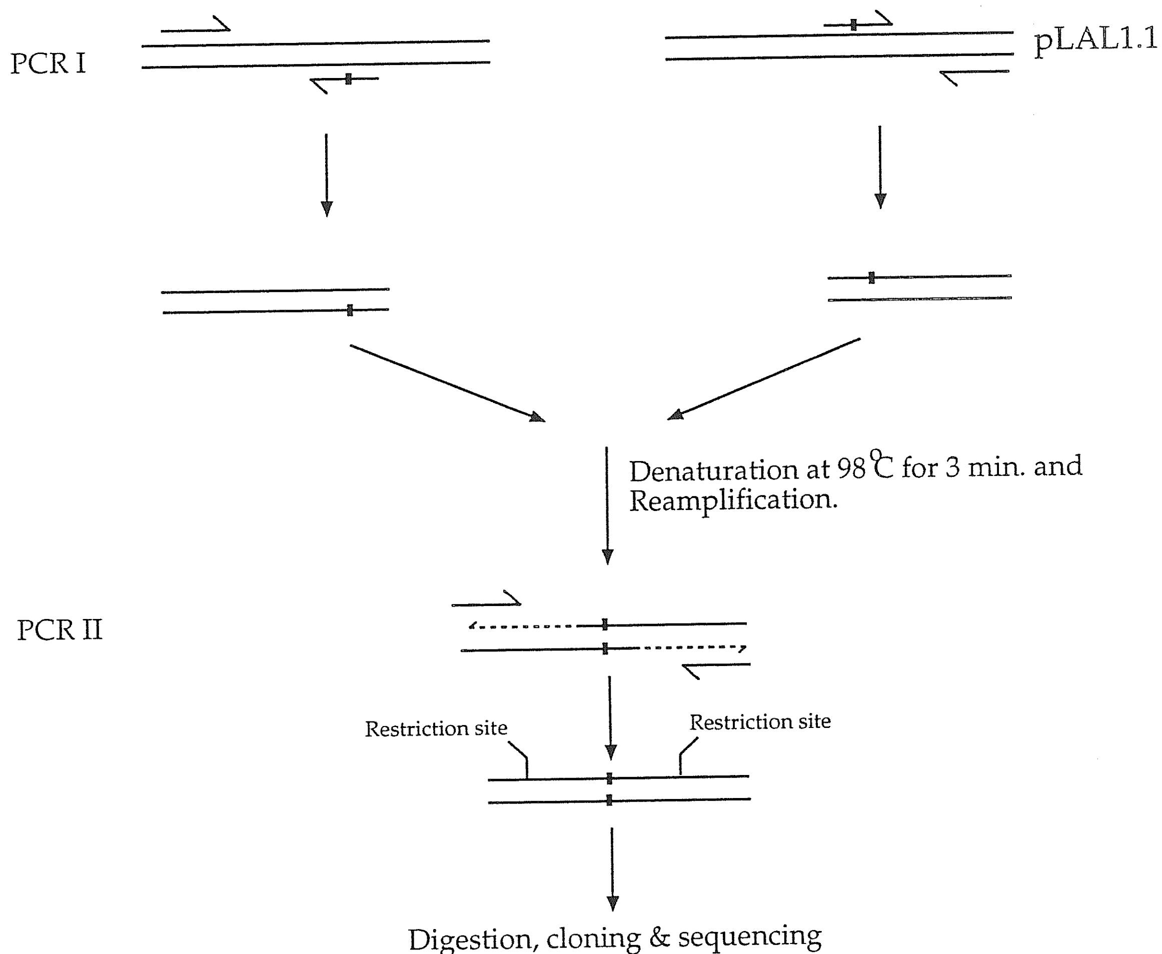


Fig.2.9.1: PCR based strategy used for sitedirected mutagenesis.The region of the LAL cDNA to be mutagenised was amplified in two fragments using pLAL1.1 as template. Mutated oligonucleotides are used in the first amplification (—▪—>).

2.9 Site directed mutagenesis :

PCR based overlap extension method was used for site directed mutagenesis (Fig. 2.9.1) (*Higuchi et al., 1987*). Normal pLAL1.1 was used as template for amplifications. Table.2.9.1 presents the sequences of the oligonucleotide primers used in for mutagenesis and construction of HSV tag.

2.9.1 S^{99} mutagenesis:

A region of LAL cDNA spanning nucleotides 209 - 597 was amplified from PLAL1.1 as two fragments using primer pairs (F209 - S99T/G 3′) and (S99T/G 5′ - S153G 3′). Inorder to avoid carry over contamination of the original plasmid in the subsequent reactions, PCR products were size fractionated on agarose gel, DNA band was excised and the DNA was electroeluted from the gel. Equivalent amounts of the two fragments were mixed together and coamplified with the external primers F209 and S153G 3′. This PCR product was digested with EcoRI and cloned in pLAL1.1 Eco- replacing the normal fragment with the modified one. 5 clones were sequenced for each series to ensure the presence of the desired mutation (eg., G->C at position 399 for S99T and A->G at position 398 for S99G) and absence of additional PCR errors.

Table.2.9.1

Oligonucleotides used for site directed mutagenesis and pLALtag construction:

LALJ1Rev	ACCTCCGCCAGATCCCTGATATTTCCTCATTAGA
HSV1	GGATCTGGCGGAGGTCAGCCTGAACTCGCTCCAG
HSV2	GATCCCTGCAGAGGTCCAGG
S99T 5′	GTGGATGGGCAACACCAGAGGAAATACC
S99T 3′	GGTATTTCCTCTGGTGTTGCCCATCCAC
S99G 5′	GTGGATGGGCAACGGCAGAGGAAATACC
S99G 3′	GGTATTTCCTCTGCCGTTGCCCATCCAC
S153T 5′	TTATGTGGGTCATACTCAAGGCACCAC
S153T 3′	GTGGTGCCTTGAGTATGACCCACATAA
S153G 5′	TTATGTGGGTCATGGTCAAGGCACCAC
S153G 3′	GTGGTGCCTTGACCATGACCCACATAA
D124N 5′	GGCTTTCAGTTATAATGAGATGGCAA
D130N 5′	GATGGCAAAATATAACCTACCAGCTTC
D130N 3′	GAAGCTGGTAGGTTATATTTTGCCATC
D130E 5′	GATGGCAAAATATGAACTACCAGCTTC
D130E 3′	GAAGCTGGTAGTTCATATTTTGCCATC
H274N 5′	GCAAAACATGTTAAACTGGAGCCAGGCTG
H274N 3′	CAGCCTGGCTCCAGTTTAACATGTTTTGC
L273I 5′	GCAAAACATGATACACTGGAGCCAGGCTG
L273I 3′	CAGCCTGGCTCCAGTGTATCATGTTTTGC
L273W 5′	GCAAAACATGTGGCACTGGAGCCAGGCTG
L273W 3′	CAGCCTGGCTCCAGTGCCACATGTTTTGC

2.9.2 S^{153} mutagenesis:

Part of LAL cDNA covering nucleotides 1 to 912 was amplified in two fragments using primer pairs ($M13_{20}$ - S153G/T 3′) and (S153G/T5′-LAL RC912) from pLAL1.1 template. The products were gel purified, mixed together and re amplified with the external primers $M13_{20}$ and LAL RC912. The resultant PCR product was digested with KpnI and cloned in pLAL KpnI- replacing the normal KpnI/KpnI fragment with the modified fragment. The presence of expected mutations were verified by sequencing.

2.9.3 D^{124} and D^{130} mutagenesis:

The cloning strategy used for the mutagenesis of these two residues were the same as the strategy adopted for S_{153} mutagenesis. Corresponding region of the cDNA was amplified as two separate fragments using pLAL1.1 as template and primer pairs ($M13_{20}$ - D124N/E3′) and (D124N/E5′ - LAL RC912) for D^{124} mutagenesis and ($M13_{20}$ - D130N/E3′) and (D130N/E5′ - LAL RC912) for the D^{130} mutagenesis. In both cases, the products of the first PCR reaction were gel purified and the corresponding fragments were mixed and re amplified with external primers $M13_{20}$ and LAL RC912.The products of the second PCR were digested with KpnI and cloned in pLAL1.1 Kpn-. The clones were verified by sequencing.

2.9.4 H^{274} and L^{273} mutagenesis:

The 3′ end of LAL coding region was amplified with primer pairs (F555 - H274N 3′) and (H274N 5′ - RP) or (F555 - L273I/W 3′) and (L273I/W 5′- RP) and the fragments were gel purified. Corresponding fragments were mixed together and coamplified with external primer pairs (F555 - RP). Products of the second round of PCR were digested with SacI restriction enzyme and cloned in pLAL1.1 Sac- vector.

2.10 Vaccinia virus Expression system

2.10.1 Preparation of Vaccinia virus stocks:

HeLa cells were grown in monolayer in 150 cm^2 tissue culture flasks. When they have just reached confluence, virus stock was thawed from -80°C and sonicated at 10μm amplitude for 10 secs. in a Soniprep 150 MSE apparatus in order to disperse any aggregates of cell debris. Later, 0.1vol. of 2.5 mg/ml trypsin was added to this virus suspension and incubated at 37°C for 30min after which FBS was added to a final concentration of 10%. At this point, the HeLa cell monolayer cultures were washed once with PBS and once with serum free DMEM containing 100 mg/ml of gentamycin. The virus stock

treated with trypsin was diluted in a small volume of DMEM containing 0.1% BSA and added to the cell monolayer in a ratio of virus: cell = 5:1. Incubated at 37^{o}C for 1-2 hrs. with occasional rocking. Subsequently, the virus inoculum was removed and DMEM containing 5% FBS was added. Incubation was continued at 37^{o}C for 36-48 hrs until the majority of cells in the monolayer showed cytopathic effects indicating effective viral infection. The cells were scraped and in the medium and recovered by centrifugation at 1000 rpm in a bench top centrifuge. The cells were resuspended in hypotonic buffer (10mM Tris-HCl, pH 9; 1mM EDTA pH 8) in order to have a concentration of 1x10^7 cells/ml of buffer. Three cycles of freeze-thawing were performed in order to release the virus. Cell debris was pelleted by low speed centrifugation and the supernatant containing the virus was aliquoted and stored at -80^{o}C.

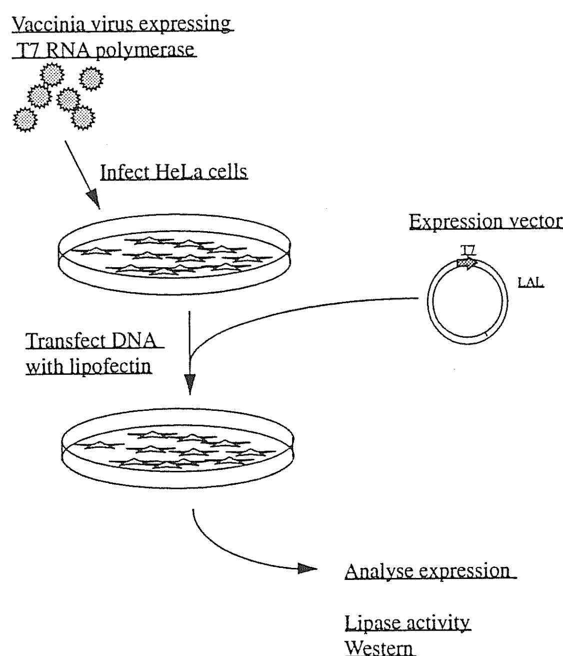


Fig.2.10.1: Diagramatic representation of Vaccinia T7 expression system.

2.10.2 Plaque Assay for titer determination.

MA104 cells were grown to confluence. The virus stock was sonicated and treated with trypsin as described in section 2.10.1. Monolayer cultures of MA104 cells were washed first with PBS and then with serum free DMEM containing 100µg/ml of gentamycin. 10-fold serial dilutions of the trypsin treated virus in a small volume with serum free DMEM containing 0.1% BSA, and 10^{-5} - 10^{-9} dilutions were added to the cell monolayer and incubated at 37oC for 1-2 hrs. with occasional rocking. The virus inoculum was aspirated and the monolayers were overlaid with DMEM containing 1% low melting point agarose and 5% FBS. After incubation at 37^{o}C for 36-48 hrs., the cells were

stained by overlaying the agarose with DMEM containing 1% agarose, 0.1% neutral red and 5% FBS. After further incubation at 37°C for 2-4 hrs, the plaques were counted and the plaque forming units per millilitre of the virus stock (pfu/ml) was calculated.

2.10.3 Vaccinia Virus infection:

HeLa cells were grown to 70% confluence in 25cm^2 tissue culture flasks. 6-8 hrs before transfection, the cells were washed and fed with fresh medium (DMEM + 10% FCS). 30-40 min. before infection, cell lysate containing the virus was thawed, 0.1vol. of Trypsin in PBS (2.5mg/ml) was added and incubated at 37°C for 30 min. Meanwhile, the cells were washed twice with PBS and when the incubation is over, the reaction is stopped and adding 0.1vol of serum (100% FBS) and 10vol. of appropriate vol. of serum free DMEM and 1ml of this cocktail is added to each flask. Virus was used at moi of 30pfu. Incubated at 37°C for 30min. with occasional rocking of the plate. After 30min. of adsorption, the inoculum was aspirated and proceeded with transfection with lipofectin.

2.10.4 Transfection of LAL and CAT constructs with Lipofectin:

5μg of LAL plasmid + 100ng of CAT plasmid was mixed with 20μg of Lipofectin and the mixture was incubated at room temperature for 15min. to allow the formation of DNA-liposome complexes. The mixture was added to the cells in 2ml of serum free DMEM and incubated at 37°C for up to 24hrs. After 24hrs, the medium was collected and the cells were scraped off in PBS and aliquoted. The cells were pelleted and the supernatant was aspirated. These cell pellets were frozen at -80°C and were used later for determination of Lipase activity, CAT activity and western blot analysis.

2.10.5 Measurement of Transfection efficiency:

Phase extraction assay for CAT activity (*Seed et al., 1988*).

The cells were harvested in 6ml of PBS and 1.5ml aliquot was pelleted for measuring CAT activity. The cell pellets were resuspended in 100μl of hypotonic buffer (0.25M Tris pH 7.6; 5mM EDTA) and subjected to 3 cycles of freezing and thawing. The cell debris was precipitated by centrifugation at 10000rpm for 10min. at 4°C and the endogenous acetyl transferases were inactivated by incubation at 65°C for 10min. The tubes were cooled down to room temp. and spun down in an eppendorf centrifuge. The reaction mixture contained 5μl of the cell extract, 0.25M Tris HCl pH 7.6 and 1.5mM of Butyryl CoA (Sigma) and 5μl of ^{14}C labelled Chloramphenicol (57mCi/mmol,

Amersham) in a total volume of 150μl. The reaction mixture was incubated at 37°C for 1hr. Reaction was stopped by adding a 2:1 mixture of Tetra methyl penta decane (TMPD, Sigma) and mixed Xylene (equimolar mixture of Xylene enatiomers, Sigma). The mixture was vortexed well and centrifuged at 14000rpm for 5min. at room temp. in an eppendorf centrifuge. The butyrylated chloramphenicol extracted in the organic upperphase was collected, 5ml. of scintillation fluid for organic solvents (Beckman) was added and counted in a liquid scintillation counter (Model: LS 1801, Beckman). The enzyme activity was calculated as cpm/min/μg of protein and used as a measure of transfection of efficiency.

2.11 Measurement of LAL activity:

2.11.1 Fluorimetric assay

Acid lipase activities in PBMNCs were determined by the fluorimetric method developed by *Kelly & Bakhrau-Kishore (1979)* . Cell pellets containing up to 10^6 cells were resuspended in 0.1 ml of 0.2M Sodium acetate buffer (pH 4.6) and the suspension was sonicated three times for 2sec.each at 10μm amplitude. The reaction was assembled with 0.05ml of sonicated cell homogenate, 0.95ml of 0.2M Sodium acetate (pH 4.6) and 5μl of 20mM 4-methyl umbelliferyl palmitate (4-MUP dissolved in 2-methoxy ethanol) and incubated at 37°C for 15min.The reaction was stopped by adding 0.2ml of 10% TCA followed by incubation at room temperature for 5min. The reaction mixture was neutralised with 2ml of 0.27M Glycine-NaOH , pH7.4. Centrifuged for 5min. at 1800g and the clear supernatant was used for measurement of fluorescence was measured in a Fluorometer (Model: FCT-151, JASCO International) with excitation at 335nm and emission at 445nm.

2.11.2 Radioactive Assay

Acid lipase activities in HeLa cell extracts were determined by using ^{14}C labelled substrates using a method modified from that of *Ameis et al., 1994* . Cell pellets were resuspended in 160μl of Incubation buffer (0.1M Sodium Acetate, pH 4.8) and 1 λ of a cocktail of protease inhibitors (0.25mM PMSF, 15μg/ml Leupeptin, 5μg/ml Pepstatin 15μg/ml aprotinin and 0.7 mM EGTA). Samples were sonicated at an amplitude of 10μm for 10 secs. (Soniprep 150 MSE) and 0.2 % w/v of Triton X-100 was added.

Reaction mixtures contained cell extracts (30-50 μg of protein), 100mM Sodium acetate buffer (pH 4.8), 0.2 % w/v of Triton X-100 and either Cholesteryl- [^{14}C] oleate (0.2 mM, 5 mCi/ml) or Tri-[^{14} C] oleyl-glycerol (0.3 mM, 5mCi/ml) in a total volume of 100μl. Incubations were carried out for

30min. at 37^oC and the reaction was stopped by adding 20μl of 2M NaOH and 600μl of a mixture of Benzene-Methanol-Chloroform (10 : 12 : 5 v/v/v) and 0.3mM oleic acid as carrier, vortexed thoroughly for 30sec., and centrifuged at 14000rpm in an eppendorf centrifuge for 5min. An aliquot of 200μl of the aqueous upper phase containing hydrolysed oleic acid was collected without disturbing the interphase and mixed with 300μl of H_2O and 5ml of Scintillation fluid (Beckman) and counted in a Liquid Scintillation system(Model: LS 1801, Beckman). The values were processed using the Computer programme Microsoft Excel based on the following relations.

$$\text{Enzyme activity(counts/hr/}\mu\text{g protein)} = \frac{(Sc-Bc) \times 4}{P}$$

Where Sc = Sample counts
Bc = Blank counts
P = Protein/50 μl

$$\text{Relative activity} = \frac{\text{Activity in the Sample}}{\text{Activity in pULB control}}$$

Where pULB control is the sample infected and transfected with the CAT plasmid alone. This value accounts for the endogenous LAL activity in the infected cells. Protein concentrations of the extracts were determined using Bradford reagent (Bio Rad).

2.12 Endoglycosidase H treatment:

Cell extracts were prepared by sonication for 10sec at an amplitude of 10μm (Soniprep 150, MSE), and subsequent addition of Triton X-100 (0.2% [w/v] final concentration). The cell extracts were incubated with 50mU of Endoglycosidase H (Boehringer Mannheim) at 37°C for 12 hrs (*Maley et al., 1989*). The samples were then immunoblotted and an aliquot used for the determination of the acid lipase activity.

2.13 Immunoblotting

SDS-PAGE was performed according to the method of *Laemmli et al., (1970)*. Extracts of HeLa cells (~40μg protein) expressing normal or mutant LALs were resuspended in denaturing sample buffer containing 4% SDS and 3 % dithiothreitol. After 5min incubation at 100^oC the samples were loaded on to 12% polyacrylamide gels and subjected to electrophoresis. Western blotting of

the proteins to PVDF membrane was performed for 2hrs. at 200mA. After transfer, the membranes were stained with Ponceau Red S, washed and blocked for 1hr in TBS containing 5% skimmed milk. Tag antigens were detected by incubating with anti-HSVtag monoclonal antibody (Novagen, Madison WI) followed by an alkaline phophatase-conjugated goat anti-mouse IgG.

CHAPTER 3

RESULTS

3.1 Identification of the molecular basis of CESD in Patients:

3.1.1 Patient 1 is a compound heterozygote for Ex.7 skipping and P181L mutation:

RNA was isolated from PBMNCs and fibroblasts of this patient and first strand cDNA was synthesised using a random hexanucleotide primer. The coding region of LALcDNA was amplified using primers F1 and RC1274. The sequence of the primer RC 1274 was designed in such a way that an a HindIII restriction enzyme site will be incorporated in the amplification product. This site was utilised for subsequent cloning. The PCR products size fractionated on agarose gels showed a smaller band in addition to the expected 1274 bp fragment (Fig. 3.1.1.1; CESD1). Both fragments were electroeluted from the gel, digested with XhoI and HindIII restriction enzymes and cloned in pUC19 vector and sequenced. Sequencing of the smaller band revealed an internal deletion of 147 bp region from the LAL cDNA (Fig.3.1.1.2a). Since this region corresponds to Exon 7 of LAL cDNA, it was likely that a splice site mutation was involved. In order to verify this possibility, exon-intron junctions were amplified from the genomic DNA using primers EX.7F and Ex.7R. Direct sequencing of the PCR product with Ex.7F primer revealed heterozygosity for an A->G mutation at the -2 position of the 3' splice acceptor site of Exon 7 (Fig. 3.1.1.2 b&c). The critical location of this mutation explains skipping of Exon 7 in the mRNA transcribed from the allele with this mutation. The predicted consequence of this mutation is that a protein lacking 49 internal aminoacids will be produced from this allele (Δ 205-253).

Sequencing of the clones derived from the normal sized fragment revealed the presence of a C->T mutation at position 645 of LAL mRNA predicting an amino acid change P181L. All 10 clones analysed showed this mutation excluding the possibility of a PCR artefact. For further verification, genomic DNA from the parents of the patient were analysed. The Exon 6 fragment was amplified using primers Ex.6F and Ex.6R and the PCR product

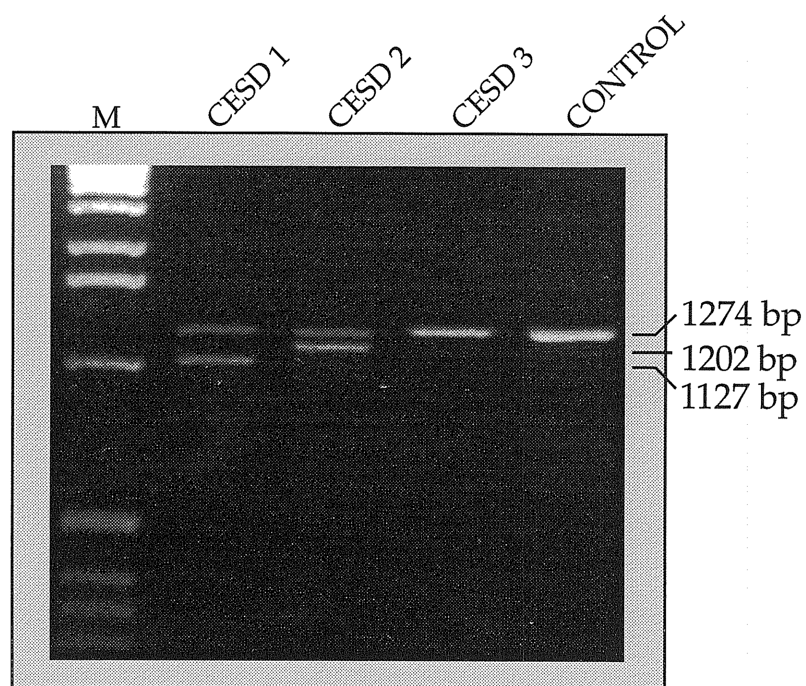


Fig. 3.1.1.1: Agarose gel electrophoresis of LAL cDNA amplification products from CESD patients and normal control. The amplification products were run on 1.8% agarose gel, stained with Ethidium Bromide and the fluorescence was visualised by means of a UV transilluminator. The expected RT PCR product of 1274bp is present in all the samples while CESD patient 1 and 2 showed additional bands of 1202bp and 1127bp respectively. The intensity of the 1274bp fragment in the CESD patient 3 was lower compared to normal control. M refers to DNA molecular weight marker. (1 kb ladder, BRL).

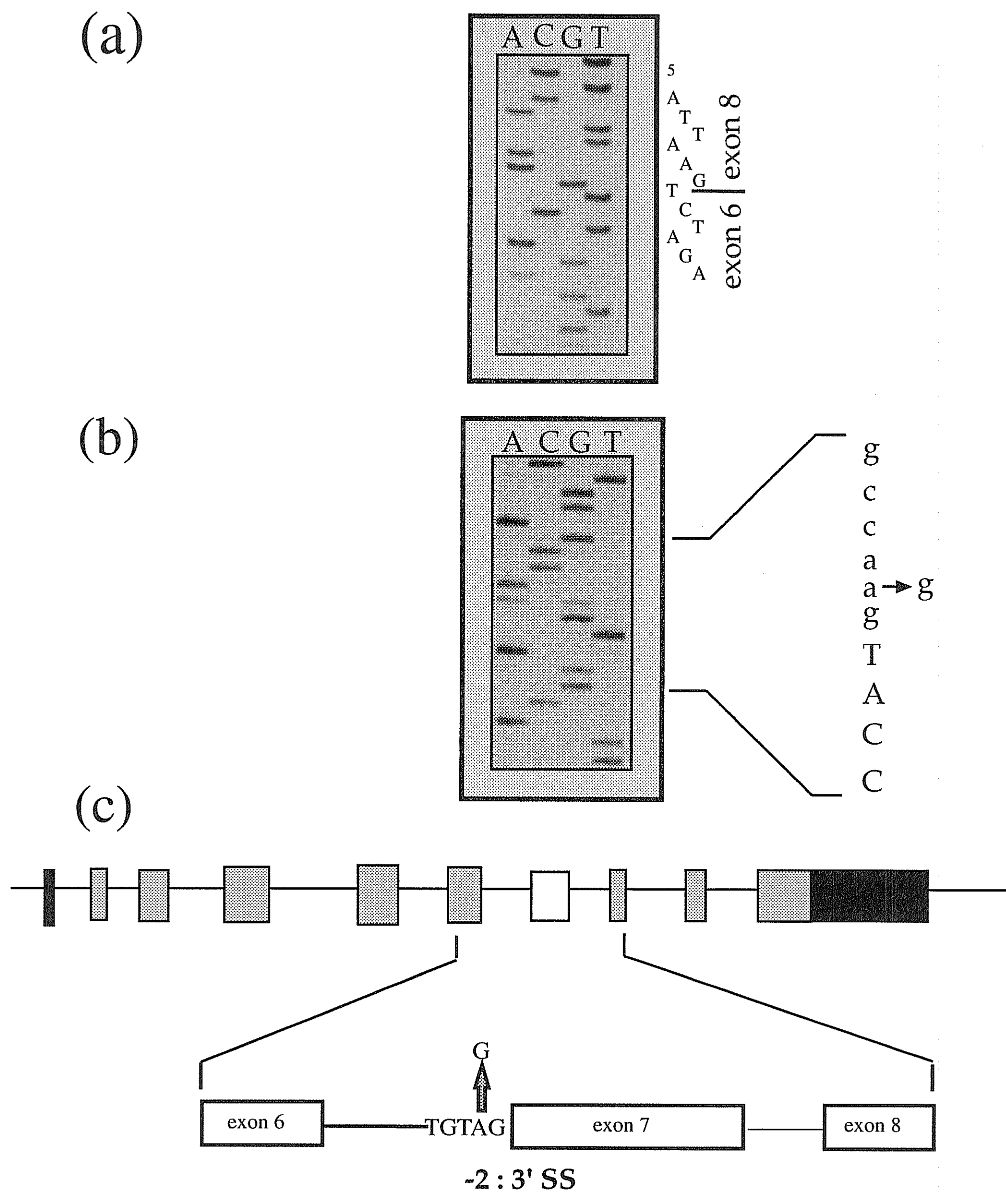


Fig. 3.1.1.2(a): Identification of exon skipping in Patient 1. Sequencing of a LAL cDNA clone from Patient 1 with primer RC912. It can be noted that the last codon of Ex.6 is followed by the first codon of Ex.8. indicating the possibility of skipping of Ex.7. (b): Identification of the point mutation responsible for exon skipping in Patient 1. Direct sequencing of the PCR product representing Ex.7 genomic fragment with primer Ex.7F. Expanded region shows the nucleotide sequence at the intron/exon junction. The intron sequences are shown in small letters while the exon sequences are represented in block letters. Heterozygosity for A->G substitution is indicated. (c): Schematic representation of the splicing defect in Patient.1. Black boxes indicate the noncoding exons while grey boxes represent normal, coding exons. White box indicate Ex.7 that undergoes exon skipping. Enlarged portion shows the 3' splice acceptor site of Ex.7 that is defective in one of the alleles due to the A->G transition at the -2 position of 3' Splice site consensus.

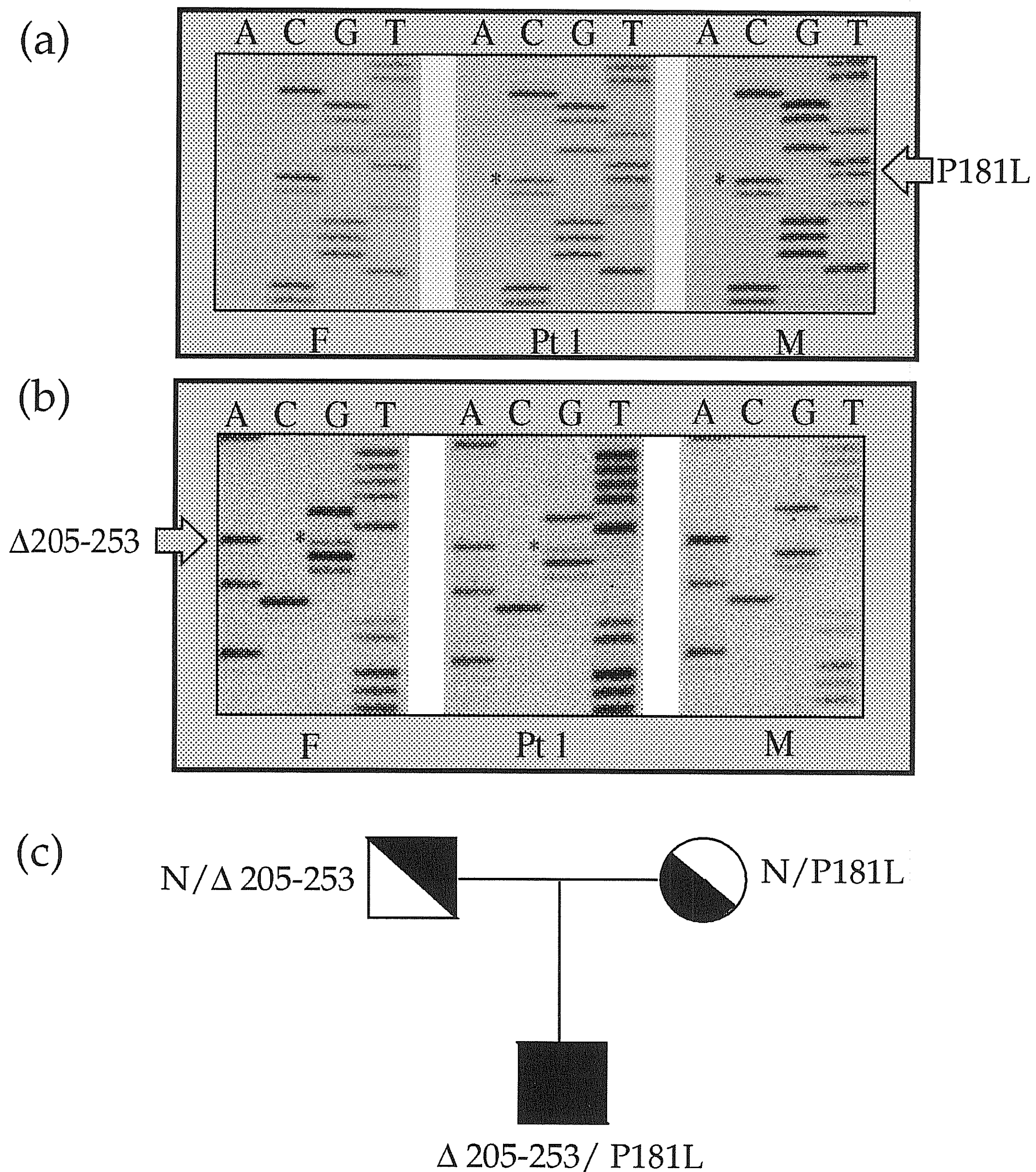


Fig. 3.1.1.3(a): Analysis of the pattern of inheritance of C->T transition in Ex.6 in patient 1 and his parents. Amplification products from genomic DNA were subjected to direct sequencing with primer Ex.6R. Heterozygosity for C->T transition leading to P181L substitution is indicated by the asteriks. F: Father; M: Mother; Pt1 patient 1. (b): Analysis of the pattern of inheritance of A->G splice junction mutation in patient1 and his parents. Amplification products from genomic DNA were analysed by direct sequencing with primer Ex.7F. The asteriks indicate heterozygosity for the A->G transition in patient 1 and his father, that leads to Δ205-253 mutation. (c): Summary of the pedigree analysis. N refers to the normal allele. Blackened sectors represent the mutated alleles.

was subjected to direct sequencing with primer Ex.6R (Fig. 3.1.1.3a). As it is shown in the figure, patient's mother was heterozygous for the C->T mutation while the father was normal (Fig. 3.1.1.3a; F&M). Amplification of Exon.7 with primers Ex.7F and Ex.7R followed by sequencing with primer EX.7 confirmed that the patient's father was heterozygous for the A->G splice junction mutation where as the mother was normal (Fig. 3.1.1.3b; F&M). This data indicate that the P181L allele was derived from the mother and the allele with the splice site mutation was derived from the father (Fig. 3.1.1.3c)

3.1.2 Patient 2 is a compound heterozygote for Ex.8 skipping and G66V missense mutation.

PCR amplification of the cDNA from this patient resulted in a 1274bp normal fragment and a shorter fragment (Fig. 3.1.1; CESD 2). Cloning and sequencing of the shorter fragment showed that a 72bp region was missing from the LAL cDNA (Fig. 3.1.2.1a). This 72bp region corresponds to the Exon 8 of LALcDNA. Mutations resulting in Ex.8 skipping has been already reported. Exon-intron junctions were amplified from the genomic DNA using primers Ex.8F and Ex.8R. Direct sequencing of the PCR product using Ex.8R showed the presence of a G->A mutation at the -1 position of the 5' splice site of Exon 8 in one of the alleles (Fig. 3.1.2.1 b&c) predicting a resultant polypeptide lacking 24 internal amino acids (Δ 254-277). This mutation explains skipping of exon 8 in the mRNA derived from this allele. Unlike the -2 position of 3' splice site, -1 position of 5' splice donor site is not 100% conserved. Attempts to amplify low amount of correctly spliced mRNA derived from this allele have not been successful so far..

All clones from the normal sized band had a missense mutation (G->T) at position 300 of LALcDNA predicting an amino acid change of G66V. Amplification of Genomic DNA with primers Ex.4F and Ex.4R followed by sequencing analysis showed that the patient was heterozygous for this mutation (Fig. 3.1.2.2). While analysing the clones from this patient's cDNA, it was found that an additional XbaI restriction site was present in the exon 2 of all the clones from the normal sized band in addition to the normal XbaI site in Ex.7. The new XbaI site was created by a G->A substitution at position 107 of the cDNA and this transition results in an amino acid change glycine to arginine (G2A). Association of this polymorphism to the point mutation was further verified by XbaI digestion of the PCR product from the cDNA using primers LALF1 and RC1057 and digesting the PCR product by XbaI (data not shown).

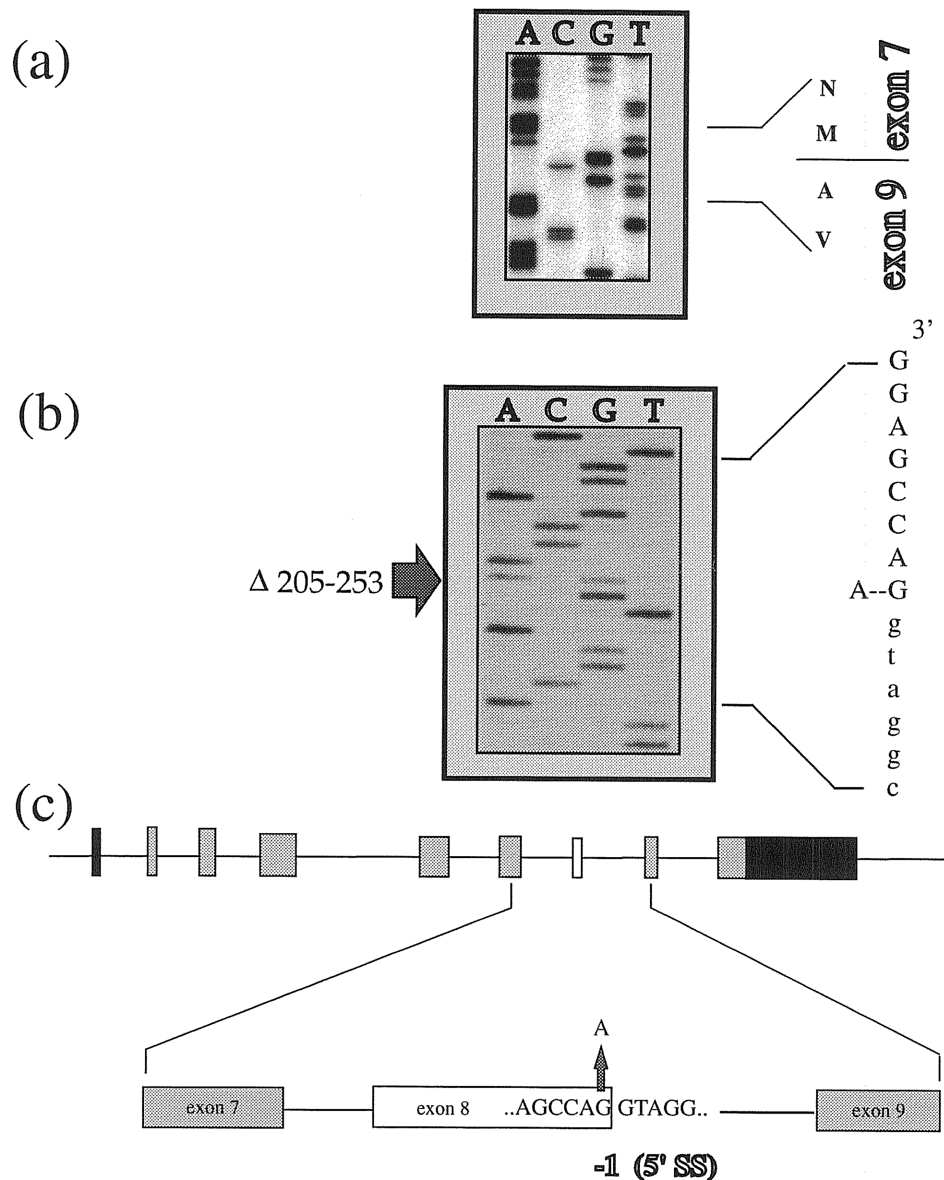


Fig. 3.1.2.1.(a): Identification of Exon skipping in Patient 2. Sequencing of a LAL cDNA clone from patient 2, with primer RC 1057, is shown in the figure. It can be observed that the last codon of Ex.7 is followed by the first codon of Ex.9 indicating skipping of Exon 8. The junction of two exons is marked by a horizontal bar.
(b): Identification of the point mutation responsible for Exon skipping in Patient 2. Direct sequencing of the PCR product from the genomic DNA, using primer Ex.8R. The G->A substitution at the last nucleotide of Ex.8 leading to deletion of amino acids 205-253 of the LAL polypeptide is indicated.
(c): Schematic representation of the splicing defect detected in Patient 2. Black boxes indicate the noncoding exons of the LAL gene. Grey boxes stand for the normal exons and the white box represent Ex.8 that undergoes skipping in the case of this patient. The nucleotide sequence at he 5' splice donor site of Ex.8 is shown in the expanded version indicating the G->A transition at the -1 position of the 5' splice site consensus.

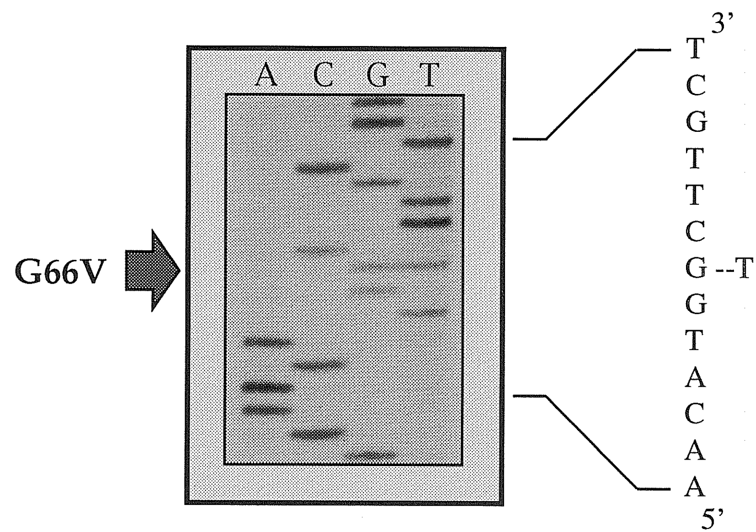
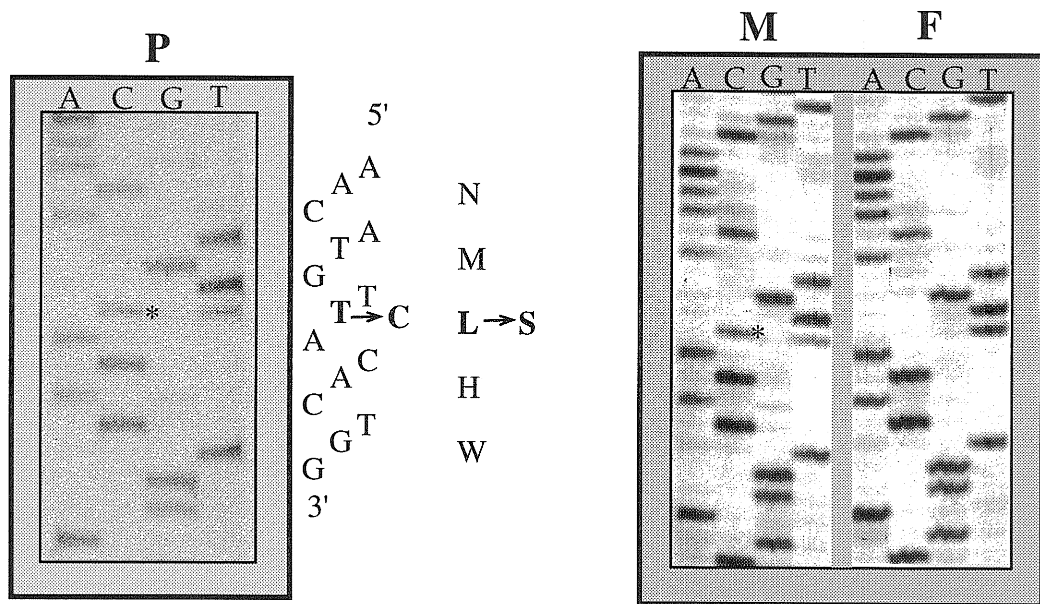


Fig. 3.1.2.2: Identification of G66V substitution in patient 2. Ex. 4 fragment amplified from genomic DNA, subjected to direct sequencing with primer Ex.4F. Heterozygosity for G->T transversion leading to G66V substitution can be observed.

(a)



(b)

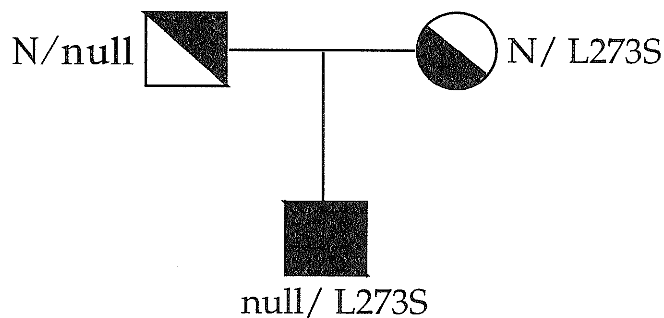
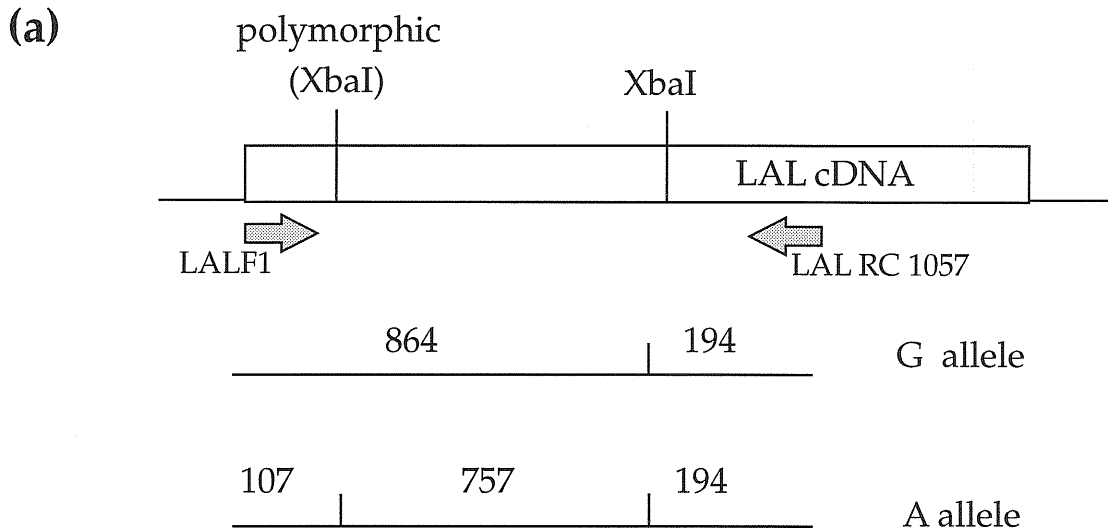


Fig. 3.1.3.1(a): Analysis of the pattern of inheritance of L273S mutation in the family of Patient 3. The Ex.8 fragments amplified from genomic DNA were subjected to direct sequencing with primer Ex.8R. Asteriks indicate the T->C transition in patient 3 and his mother leading to the L273S substitution. It can be noted that both the patient and his mother are heterozygous for this transition while the father is homozygous for the normal T allele.
(b): Summary of the pedigree analysis. N indicates the normal allele while the null allele is represented as 'null'. Blackened sections indicate the mutated alleles.

(a)



(b)

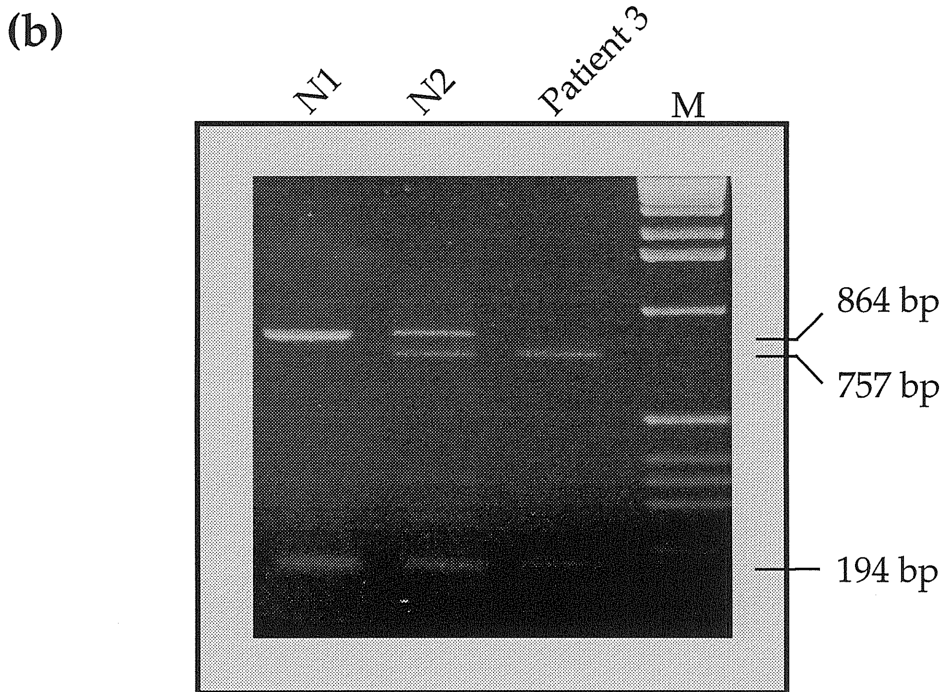


Fig. 3.1.3.3(a): Diagramatic sketch of the strategy used for identification of the null allele in Patient 3. The solid arrows indicate position of the RT-PCR primers.
(b): Agarose gel electrophoresis (1.8%) of the RT-PCR products digested with XbaI restriction enzyme. The 864 bp and 194 fragment represent the product digestion at the constitutive XbaI site in Ex.7 while 757 bp and frgment represent products of digestion at the polymorphic XbaI site in Ex.2. N1: normal control, homozygous for the absence of Xba polymorphism in Ex.2. N2: normal control heterozygous for the presence of the XbaI polymorphism. It can be noted that Patient 3 appears to be homozygous for the presence of XbaI polymorphism in Ex.2 indicating that the paternal allele is not represented at the mRNA level and thus suggests the presence of a null allele.

cloned in pUC 19 vector. 6 clones were sequenced and compared with the published sequences (*Aslanidis et al., 1994a*). Except for the addition of a G nucleotide at position -54, close to an SP1 binding site, no significant sequence changes were observed. Sequencing of the opposite strand showed that this region of the sequence is highly susceptible for compression. Sequencing of clones derived from normal controls showed the same sequence.

3.1.4 Analysis of XbaI polymorphism in the population.

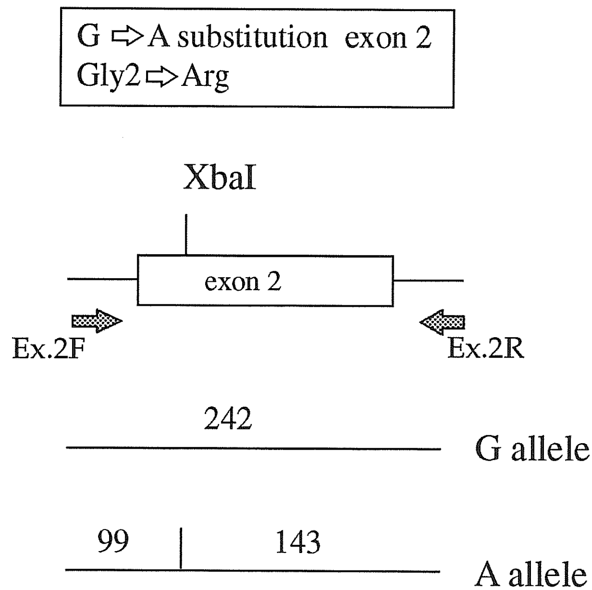
Since the G-2A polymorphism was observed in two CESD alleles associated with G66V mutation in patient 2 and L273S mutation in patient 3, it was interesting to evaluate the frequency of this polymorphism in the common population and its correlation with the acid lipase activity in these individuals. 30 normal subjects were analysed for the presence of this polymorphism. Exon 2 was amplified from the genomic DNA of these subjects using Ex.2F and Ex.2R primers and the PCR products were digested with XbaI and resolved on 2% agarose gel. Subjects heterozygous for the XbaI polymorphism showed 3 distinct bands. The 242 bp fragment corresponds to the undigested PCR product while 143 bp and 99 bp fragments that correspond to the products of digestion by XbaI (Fg.3.1.3.2 a&b). Individuals homozygous for the presence of this polymorphism were not detected in the analysed population. Allelic frequency of this polymorphism was calculated to be 0.19. In order to determine the relation between this polymorphism and the catalytic activity of LAL, two different kind of experiments were performed(see section 3.3.4)

3.2 In vitro Expression and characterisation of lysosomal acid lipase.

3.2.1 Recombinant LAL expressed in bacteria is not catalytically active:

Lysosomal acid lipase is present in very low amount in tissues and in cultured fibroblasts. Therefore, it was hard to study the functional significance of different mutations detected in the CESD patients using native protein from the tissues of the patients or cultured cells. In this context, it was necessary to establish a suitable in vitro system for expression of functional LAL protein. The most straight forward approach in this regard was to try to express LAL in bacteria using high efficiency expression vectors. For this purpose, LAL cDNA was cloned in pGEX vector and transformed in competent *E.coli* BL 21 cells. When induced with IPTG, these cells produced recombinant LAL as a GST fusion protein of ~64 kDa as assessed by SDS PAGE (Fig. 3.2.1). Acid lipase activity of the bacterial cell extracts was measured using 4-methyl

(a)



(b)

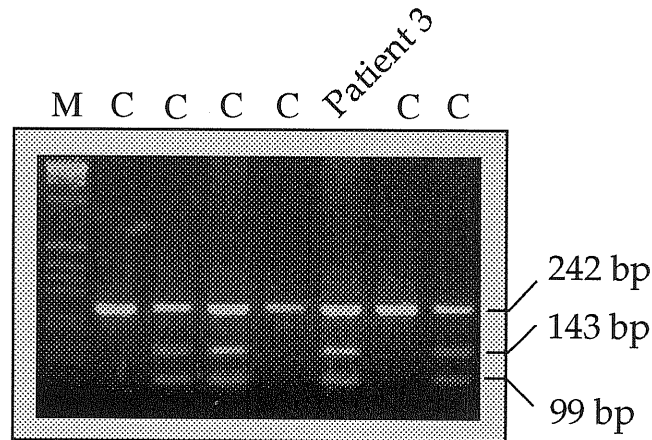


Fig. 3.1.3.2(A): Illustration of the strategy used for identification of XbaI polymorphism in Ex.2 of LAL gene by geomic DNA analysis. The solid arrows indicate the position of PCR primers. (B): Agarose gel electrophoresis of Ex.2 PCR products digested with XbaI restriction enzyme. The 242 bp fragment represents the undigested PCR product while 143 bp and 99 bp fragments represent the products of digestion. Heterozygous and homozygous controls are indicated as C. M stands for DNA molecular weight marker (1Kb ladder, BRL). It can be noted that Patient 3 is heterozygous for the presence of Xba polymorphism at the genomic level.

umbelliferyl palmitate (4-MUP) as substrate and compared with the respective activities in control extracts from cells transfected with wild type pGEX vector. Enzyme activities expressed as nmol of palmitate released/mg of protein/min., showed that the activities in the induced cell extracts were not above the background activity of non induced cell extracts (Data not shown).

3.2.2 Expression of recombinant LAL in mammalian cells:

Lysosomal enzymes are known to be heavily glycosylated proteins and it has been demonstrated that cotranslational glycosylation could be important for the catalytic activity of these proteins. Therefore, the possibility of expressing recombinant LAL in mammalian cells was considered.. The normal LALcDNA was cloned downstream of T7 promoter in a pBluescript vector and transfected in HeLa cells previously infected with a recombinant Vaccinia virus strain carrying the gene for bacteriophage T7 RNA polymerase. The cDNA construct was designed in such a way that the LAL coding region was followed by a glycine rich hinge region (GSGGG) and an epitope tag derived from the Herpes Simplex Virus Glycoprotein D (QPELAPEDPED).. Thus the expressed protein could be immunologically distinguished from the endogenous LAL using a commercially available monoclonal antibody against the HSV peptide. The cells were cotransfected with another construct (pULB) containing Chloramphenicol Acetyl Transferase (CAT) cDNA downstream of T7 promoter as a standard internal control for transfection efficiency.

3.2.2.1 Tagged and non-tagged LAL were equally active:

In order to evaluate the possibility that the addition of the tag epitope may interfere with the catalytic activity of the enzyme, pLAL1.1 and pLALtag constructs were transfected in HeLa cells previously infected with recombinant Vaccinia virus and the cell extracts were assayed for acid lipase activity towards triglycerides and cholesteryl esters using ^{14}C labelled tri-oleyl glycerol and cholesteryl oleate as substrates. These two constructs were identical in all respects except for additional 16 amino acids at the carboxy terminus in the case of pLALtag (Fig.3.2.2.1a). Acid hydrolase activities were calculated as cpm/μg protein/hr and the relative activities in the samples were determined by comparing with the respective activities in control extracts from cells mock infected and transfected with pULB plasmid alone taken as 1 (see section 2.11.2).

Transfection efficiencies in different samples were normalised by Chloramphenicol Acetyl transferase (CAT) assay using Butyryl CoA as cofactor and ^{14}C labelled chloramphenicol as substrate (see section 2.10.5). Graphic

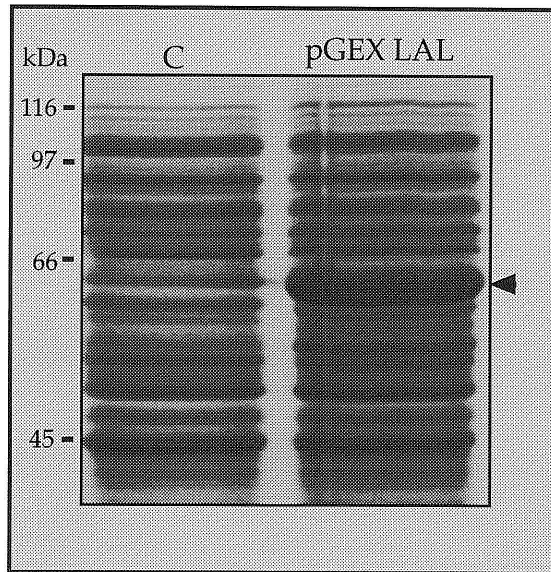


Fig.3.2.1: Expression of recombinant LAL in bacteria. Extracts of E.coli BL21 cells were analysed on 11% SDS polyacrylamide gel and stained with coomassie blue. pGEX LAL refers to the extract of cells transformed with pGEX LAL plasmid and induced with IPTG while C refers to control extracts where the cells were transformed with pGEXLAL plasmid but not induced with IPTG. pGEX LAL lane showed a strong band of ~64 kDa, corresponding to the the size expected for LAL-GST fusion protein. M refers to molecular weight standards.

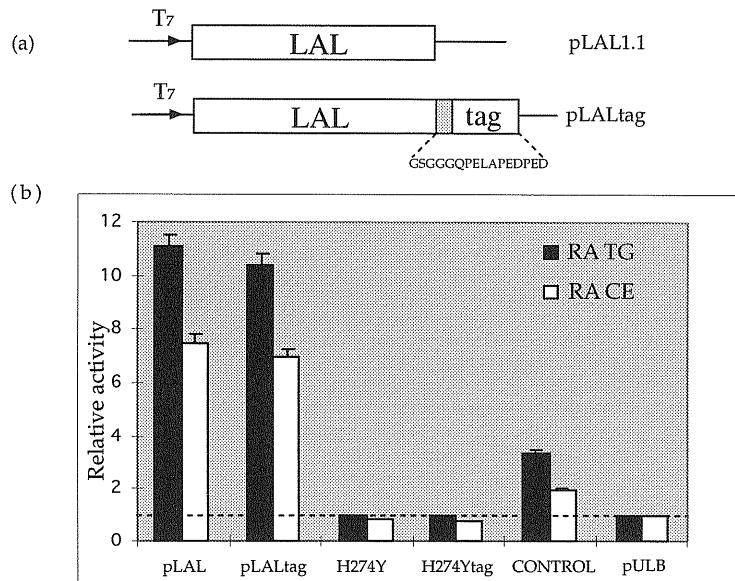


Fig.3.2.2.1(a): Schematic representation of the constructs pLAL1.1 and pLALtag. The shaded box indicates the glycine rich hinge region. T7: Bacteriophage T7 promoter. (b): Comparison of acid hydrolase activities in the extracts of cells transfected with pLAL and pLAL and pLALtag constructs. The values on the Y axis indicate the acid hydrolase activities in the respective extracts expressed as relative activities compared to pULB control. CONTROL refers to extracts of HeLa cells without Vaccinia virus infection.

representation of the data from 10 different experiments summarised in Fig.3.2.2.1b. shows that pLAL transfected cells had 10-12 fold tri-oleyl glycerol lipase activities compared to control extracts while the cholesteryl oleate hydrolase activities were up to 7-8 fold higher than the controls. As it is evident from the figure, cell extracts transfected with pLALtag constructs showed both hydrolase activities in the same range of normal LAL suggesting that the addition of tag epitope doesn't interfere with the catalytic properties of the enzymes. As for the endogenous LAL, the rate of hydrolysis of triglycerides was higher than that of cholesteryl esters. H274Y construct was used as a negative control in these experiments. Even in this case, it can be noted that tagged and non-tagged constructs behave the same way as far as the tri-oleyl glycerol lipase and cholesteryl oleate hydrolase activities are concerned.

3.2.2.2 Recombinant LAL expressed in HeLa cells showed 4 different molecular weight forms.

Extracts of cells infected and transfected with pLALtag plasmid or pULB alone were size fractionated on 11% SDS polyacrylamide gel, blotted on to PVDF membrane and probed with a monoclonal antibody against the tag epitope in order to detect the recombinant protein (Fig. 3.2.2.2). Four different molecular weight forms of LAL protein were observed. Two lower bands of 42 and 43 kDa and two higher bands of 54 kDa and 51 kDa were visible. The proportion between the higher bands to the lower bands was roughly 1:1 as assessed by quantification of the bands using a Bio Rad Imaging densitometer, model GS670.

3.2.2.3 Different molecular weight forms of LAL originate from N-linked glycosylation:

The extracts from cells transfected with pLALtag were treated with Endoglycosidase H and part of these extracts were size fractionated on 11% SDS-polyacrylamide gel along with untreated extracts and transferred to PVDF membrane. Immunoblotting with anti HSV tag antibody showed that only the lower molecular weight forms of 42 and 43 kDa were present in the endo H treated extracts while all the four different molecular weight forms were present in the lane of untreated extracts (Fig. 3.2.2.2). Endo H- extracts were also incubated the same way as the Endo+ extracts except for the presence of endoglycosidase H as a control for non-specific degradation of the protein during incubation.

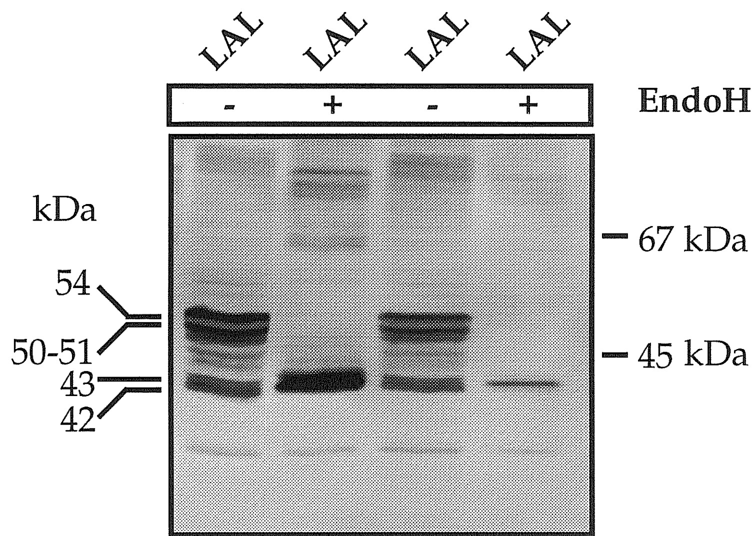


Fig. 3.2.2.2 : Deglycosylation of recombinant LAL expressed in HeLa cells. Cell extracts were incubated overnight at 37°C with and without endoglycosidase H and fractionated on 11% SDS polyacrylamide gels. Higher molecular weight forms of LAL found in untreated extracts were reduced to 42 kDa form in the treated extracts.

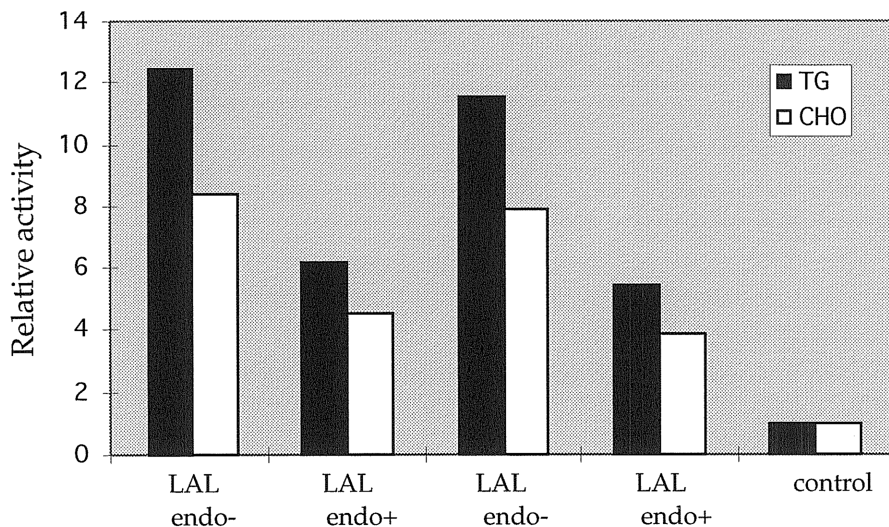


Fig. 3.2.2.4: Effect of deglycosylation on the enzymatic activity of LAL. Aliquotes of the cell extracts treated with endoglycosidase were analysed for acid hydrolase activity using both tri-oleyl glycerol and cholesteryl oleate as substrates. Enzymatic activities calculated as cpm/µg protein/hr were converted in to relative activites considering the respective hydrolase activity in mock infected and transfected cell extracts as 1. It can be observed that both hydrolase activities were reduced to almost one half in deglycosylated extracts. Data obtained for two independent sample, corresponding to the ones used for the immunoblot analysis, is shown here.

3.2.2.4 Effect of deglycosylation on the enzymatic activity of LAL:

Part of the extracts treated with Endoglycosidase H were analysed for acid hydrolase activity towards triglyceride and cholesteryl esters and the data obtained for two independent samples are shown in Fig. 3.2.2.4. Enzymatic activities towards tri-oleyl glycerol substrate in the end-extracts were up to 12 fold higher than the controls whereas in the Endo+ extracts this activity was reduced to 6 fold higher than of the control. The same pattern was observed also for cholesteryl oleate hydrolase activity. As it is shown in the figure, the cholesteryl esterase activity in the Endo- extracts were up to 8 fold higher than the control while the Endo+ extracts showed about 4 fold higher activity than the control. Both Endo+ and Endo- extracts were treated in the same way for the LAL activity measurement. The two samples for which the activity data are shown in Fig. 3.2.2.4 correspond to the samples verified by immunoblotting in Fig. 3.2.2.2. Control refers cells infected and transfected with pULB plasmid alone.

3.3. In vitro expression and characterisation of CESD mutants and polymorphic variants.

3.3.1 CESD mutants expressed in HeLa cells produced inactive LAL protein:

The functional significance of the different CESD mutations described in Italian patients was tested by means of the Vaccinia T7 expression system. The mutant LAL cDNAs were subcloned in pBluescript vector under the control of T7 promoter and transfected in HeLa cells previously infected with recombinant Vaccinia virus. The cell extracts were assayed for LAL activity towards triglycerides and cholesteryl esters and the results were expressed as relative activity compared to the respective activities in control extracts transfected with pULB plasmid alone (Fig. 3.3.1.1). The transfection efficiencies were normalised by CAT assay. All the four missense mutants (P181L, G66V, L273S & H274Y) as well as the two deletion mutants (Δ205-253 & Δ 254-277) showed relative tri-oleyl glycerol lipase and cholesteryl oleate hydrolase activities not significantly different from the negative controls, while the positive control (pLAL) showed tri-oleyl glycerol lipase cholesteryl esterase activities in the normal range (11-12 fold higher than the pULB control for tri-oleyl glycerol lipase and 6.5-8 fold higher than the control for cholesteryl esterase activities). The presence of recombinant protein in these samples was verified by immunoblotting with anti-HSV tag antibody (Fig. 3.3.2).

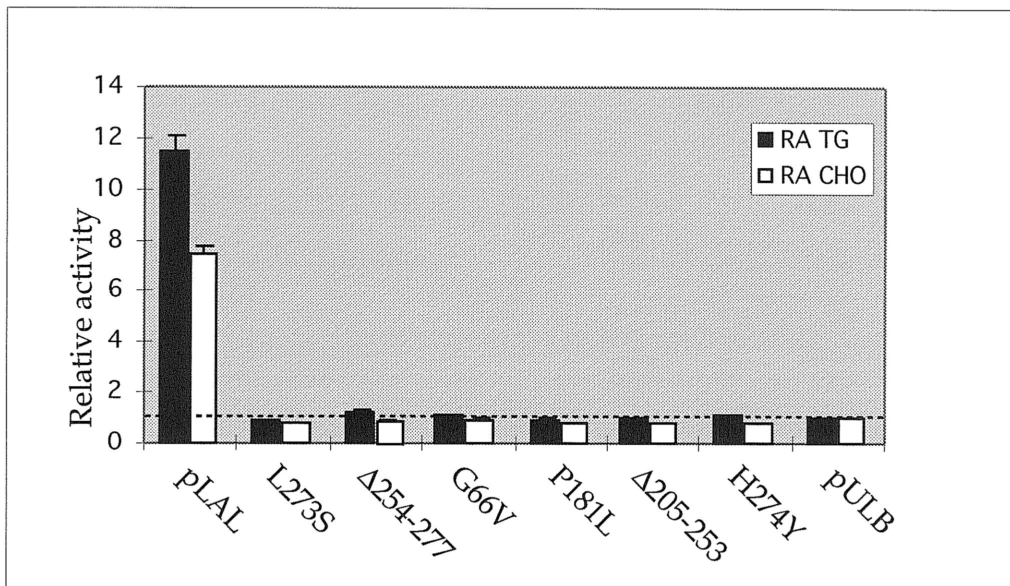


Fig. 3.3.1: Measurement of LAL activity in the extracts of HeLa cells infected and transfected with CESD mutants. Tri acyl glycerol hydrolase and cholestryl ester hydrolase activities are expressed as relative activity in comparison with the respective activities in the control extracts infected and transfected with pULB plasmid alone. It can be noted that all CESD mutants showed activites below the level of significance of the data indicated by the dotted line while the transfection with normal LAL construct showed activities in the expected range.

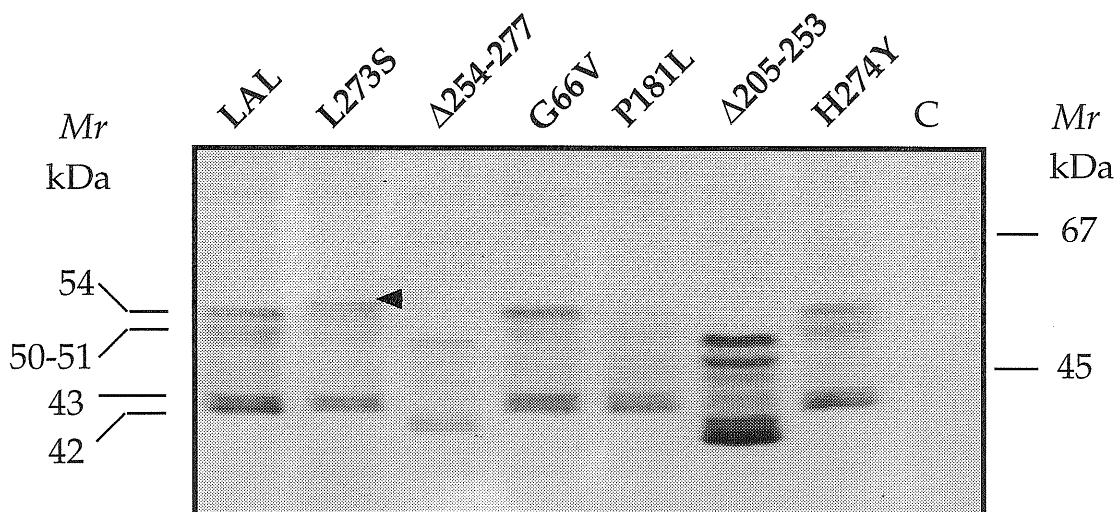


Fig. 3.3.2: Immunoblotting of extracts of cells transfected with CESD mutants. *Mr* indicates apparent molecular mass. LAL refers to normal LAL. Four major molecular weight forms are indicated. Arrow head points to the 56 kDa band unique to L273S mutant.

3.3.2 Some of the CESD mutants showed altered glycosylation pattern:

Careful examination of Fig. 3.3.2 reveals some interesting points. First of all, G66V and H274Y mutants expressed recombinant LAL proteins with the same glycosylation pattern as for the normal LAL construct expressed in HeLa cells using the same Vaccinia T7 expression system. In the case of these mutations, the ratio between the non-glycosylated and heavily glycosylated forms were identical to that of the normal LAL(~1:1). In the case of P181L mutant, the ratio between the non-glycosylated and glycosylated forms was altered and this change was reproducible. The splice junction mutant, Δ 205-253 expressed lower molecular weight forms of the protein in agreement with expected reduction in size due to deletion of 49 amino acids (~5 kDa). The highest molecular weight form observed in this case was 49 kDa and the lowest was 38-40 kDa. In the case of Δ 254-277 mutation, the expected reduction in molecular weight due to deletion of 24 amino acids was about 2 kDa but expression of this mutant resulted in much lower molecular weight forms such as 50 kDa and 40-41 kDa.

L273S mutant expressed a protein with a higher molecular weight form of about 56 kDa, higher than the normal LAL. In fact, from the sequence it is clear that this mutation may create a new putative N- glycosylation site at position 271(Fig.4.1.1). The normal sequence, N-M-L is changed to N-M-S satisfying the requirement of the consensus for N-glycosylation site (N-X-S/T). The possibility of glycosylation at this site was further explored by site directed mutagenesis.

3.3.3 L273S mutation introduces a new glycosylation site at N_{271}:

In order to counter check the possibility that the 56 kDa form of the protein was derived from additional glycosylation at position N_{271}, two constructs were prepared by site directed mutagenesis. One of the constructs had glutamine at position 271 and leucine at position 273 (QML) while the other one had glutamine at position 271 and serine at position 273 (QMS). These constructs were expressed in HeLa cells by means of the Vaccinia T7 expression system as mentioned before. The cell extracts were fractionated on 11% polyacrylamide gel and immunoblotted with anti HSV tag antibody. As shown in Fig. 3.3.3.1, QMS and QML mutants expressed high molecular weight forms equivalent to normal LAL whereas L273 mutant expressed the 56 kDa form. The cell extracts were treated with Endoglycosidase H and it was observed that after incubation with endoglycosidase H, the 56 kDa form was reduced in size to yield lower molecular weight forms of 43 kDa and 42 kDa as it is true for the 54 kDa form of normal LAL, QMS and QML mutants.

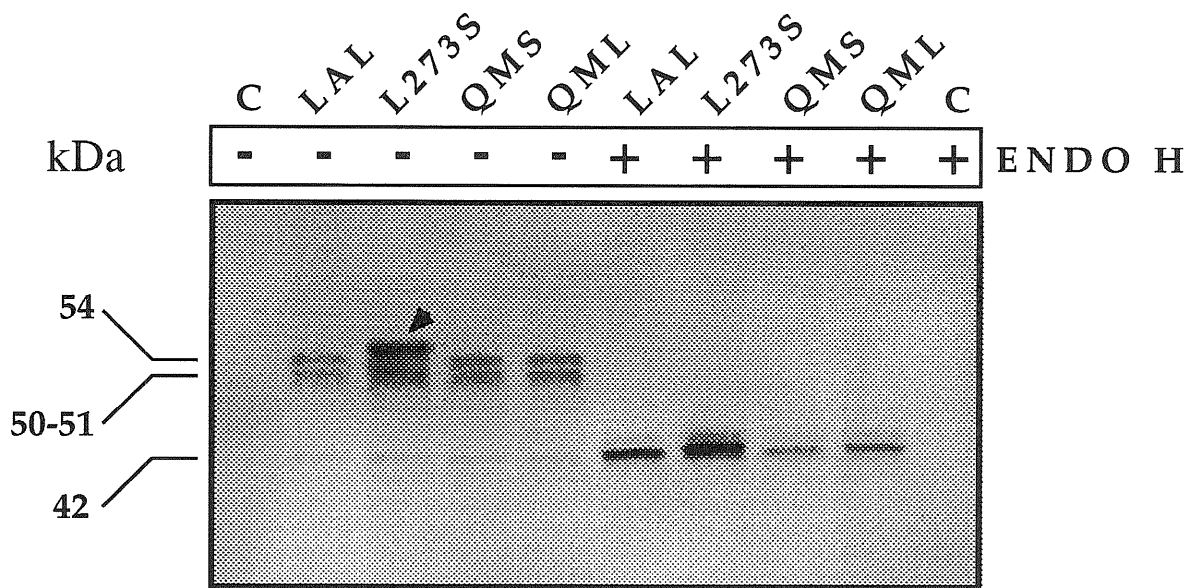


Fig. 3.3.3.1: Immunoblotting of the extracts of cells transfected with site directed mutants of N271 and L273 with and with out EndoH treatment. The arrow head indicates the 56 kDa band unique to L273S mutant. QMS: N271Q/N273S; QML:N271Q.

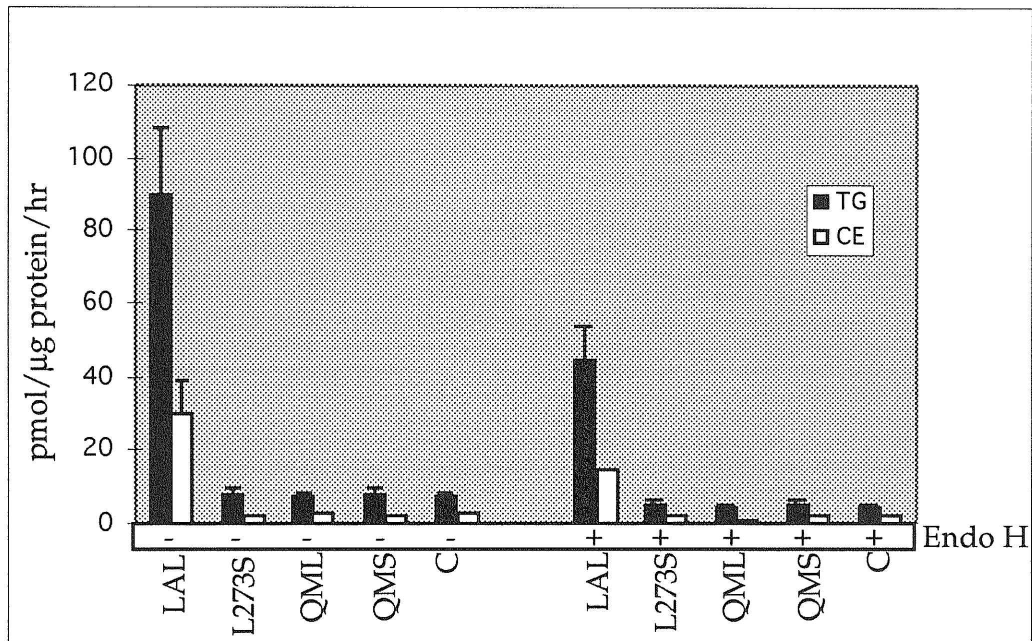


Fig. 3.3.3.2: Comparison of Acid hydrolase activities in extracts of cells transfected with site directed mutants of N271 and L273. Activities are expressed in pmol/µg protein/hr. Both triglycerol lipase and cholesteryl esterase activities were reduced to the level of pULB controls in these mutatns while normal LAL showed up to 10 fold activity than the control (C). Effect of endoglycosidase treatment on the catalytic activity was also verified.

Acid hydrolase activities were also determined in the cell extracts with and without Endo H treatment (Fig.3.3.3.2). The enzyme activities are expressed as pmol of ^{14}C oleate released/μg of protein/hr. The normal LAL showed 70-90 pmol/μg/hr of tri-oleyl glycerol lipase activity and 20-30 pmol/mg/hr of cholesteryl oleate hydrolase activity. In the corresponding EndoH+ extracts, these activities were reduced to 40-50 pmol/μg/hr and 10-15 pmol/μg/hr respectively. In the case of L273S, QML and and QMS mutants, these activities were below 5 pmol/μg/hr and 1.5 pmol/μg/hr respectively in EndoH- extracts while in the EndoH+ extracts, these values were further reduced to half of the activity present in the corresponding EndoH- extracts.

3.3.4 Functional characterisation of the G2A polymorphic variant (XbaI Polymorphism).

3.3.4.1 Measurement of Acid lipase activity in the PBMNCs of subjects:

Acid lipase activity in the PBMNCs of 23 subjects was measured using 4-methyl umbelliferyl palmitate as substrate (4MUP). The activities were expressed as nmol of palmitate released/min/mg of protein and the results are represented as a scatter diagram (Fig.3.3.4.1). It can be noted that a wide variation in the LAL activity exists among individuals of the normal population. The maximum activity observed was 3.9nmol/min/mg protein while the minimum value observed was 0.4nmol/min/mg protein. Individuals with LAL activities above or below the average are present in both groups.

3.3.4.2 In vitro expression of G2A mutants:

LAL construct carrying the G2A mutation was expressed in HeLa cells coupled with Vaccinia T7 expression system. We have tested previously reported T6P(*Anderson et al., 1994*) polymorphism also in this system. The cell extracts were assayed for acid lipase activity towards triglycerides and cholesteryl oleate (Fig. 3.3.4.2). As shown in the figure, these two activities were in the range of the relative activities observed for normal LAL ie., 10-12 higher than the controls for both G2A and T6P polymorphic variants. Transfection efficiencies were normalised by CAT assay and the presence of intact recombinant protein was verified by immunoblotting (data not shown).

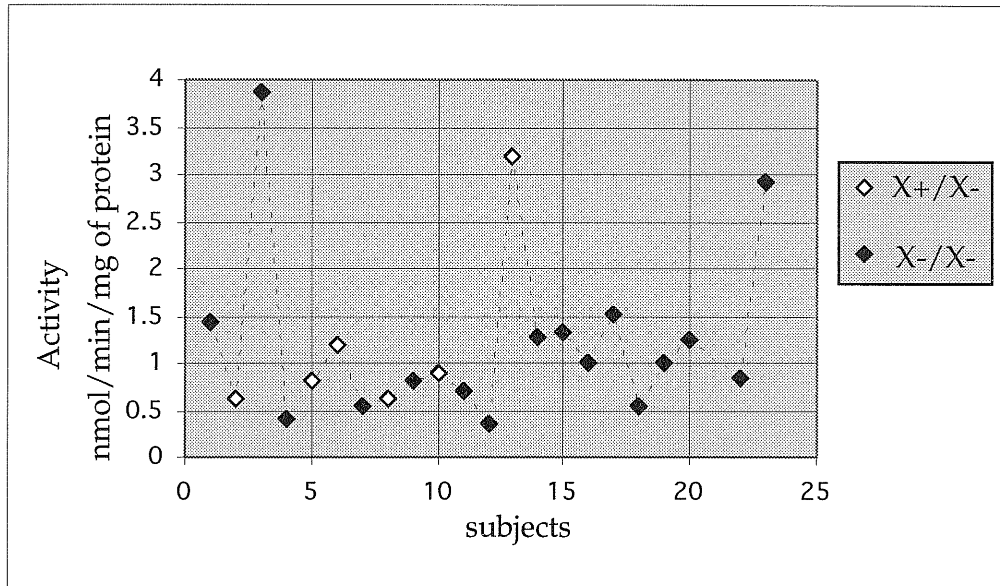


Fig. 3.3.4.1: Scatter diagram showing the Acid lipase activities in 23 different subjects from normal poulation. Acid hydrolase activities in peripheral blood mononucleocytes were measured using 4-methyl umbelliferyl palmitate(4-MUP) as substrate coupled with a fluorimetric assay. Black points indicate the activities in subjects homozygous for the absence of XbaI polymorphism and white points indicate the activities in subjects heterozygous for the presence of XbaI polymorphism.

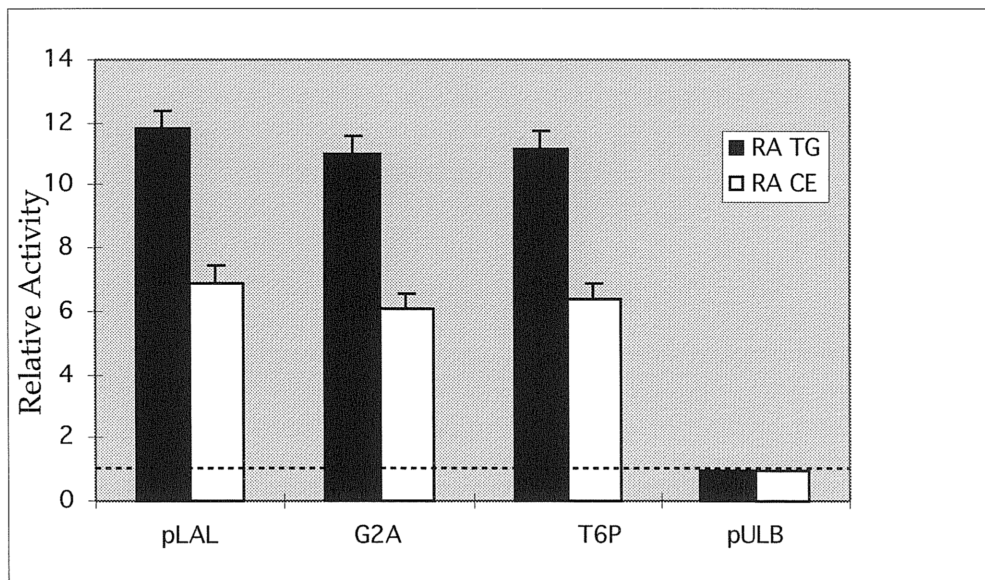


Fig. 3.3.4.2: Comparison of acid hydrolase activities in the extracts of cells transfected with LAL constructs with G2A and T6P polymorphisms. Activities were measured using C14 labelled tri-oleyl glycerol and cholesteryl ester hydrolase as substrates. Values are expressed as relative activities in comparison to pULB controls.

3.4 Characterisation of putative catalytic triad (Ser-His-Asp) residues of LAL:

As mentioned in the introduction, lipases are functionally related to serine proteases in their catalytic mechanism. Sequence homology studies have indicated that the primary aminoacid sequence of LAL harbours two putative serines fulfilling the GXSXG consensus (S^{99} & S^{153}). Two candidate Aspartates (D^{124} & D^{130}) were identified based on their degree of conservation in the Acid lipase family. In vitro expression studies using the CESD mutants H274Y and L273S resulted in catalytically inactive proteins indicating that H274 may be critical for the enzyme activity. We have prepared site directed mutants of these residues and expressed them in HeLa cells by means of the Vaccinia T7 expression system. The amino acid substitutions made at each position are indicated in Table 3.4.1. The cell extracts were assayed for catalytic activity towards ^{14}C glycerol tri-oleate and ^{14}C cholesteryl oleate substrates and for the presence of immunoreactive material as determined by a dot blot assay using anti-HSVtag antibody.

Serine	S99	S99T
		S99G
	S153	S153T
		S153G
Aspartate	D124	D124N
		D124E
	D130	D130N
		D130E
Histidine	H274N	
	H274Y*	
Leucine	L273W	
	L273I	

Table. 3.4.1: Summary of the amino acid substitutions made in the case of each site directed mutant. *indicate that this mutant was cloned from the CESD patient 3.

3.4.1 S^{153} is of critical importance for the catalytic activity:

Fig. 3.4.1.1 shows the relative activity of site directed mutants compared to mock infected extracts. S99G and S99T mutants retained about 8-9 fold activity towards tri-oleyl glycerol and about 4 fold activity towards cholesteryl oleate. On the other hand, S153G and S153T mutants showed relative activities below 1 for both triglyceride and cholesteryl ester substrates. All samples were normalised for transfection efficiency by CAT assay. The

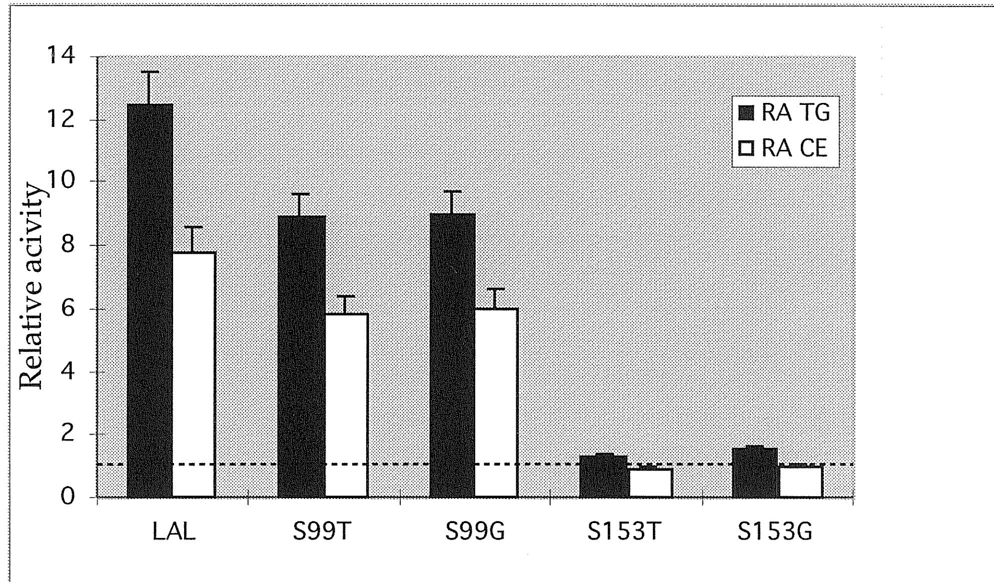


Fig. 3.4.1.1: Comparison of acid hydrolase activities in S153 and S99 muants. Extracts were prepared from cells infected and transfected with the respective plasmids and the hydrolase activities were measured using C 14 labelled tri-oleyl glycerol or cholesteryl oleate as substrates. The results are expressed as relative activities compared to pULB controls.

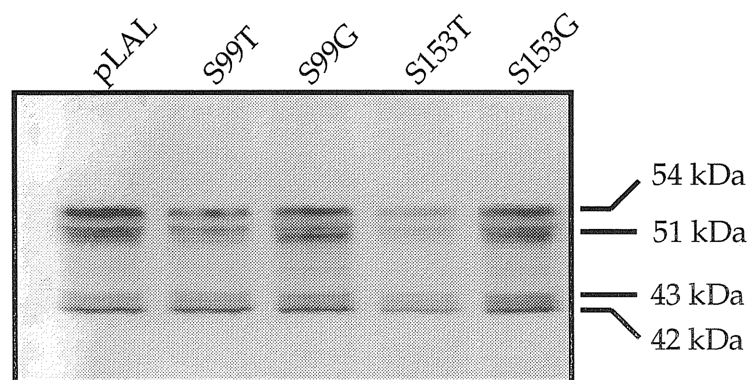


Fig. 3.4.1.2: Immunoblotting of cell extracts in order to verify the production of recombinant LAL proteins in the case of different mutants. Immunoblots were probed with anti-HSV tag monoclonal antibody. All the four major molecular weight forms were present in the case of all mutants.

enzyme activities in the samples were adjusted for the total amount of immunoreactive material present in the cell extracts as determined by the quantification of a dot blot assay using anti-HSVtag antibody (quantified in a Bio Rad Imaging densitometer and analysed by the Mol-analyst software).

The presence of intact recombinant protein in these cell extracts was verified by immunoblotting with anti-HSV tag antibody (Fig.3.4.1.2). It can be noted that all these mutants expressed recombinant LAL proteins of similar size and glycosylation pattern comparable to that of normal LAL expressed in the same system..

3.4.2 D^{130} may form part of the catalytic triad:

Comparison of the relative acid hydrolase activities in HeLa cell extracts transfected with D124E and D124N mutants showed that these mutants retain up to 6 fold activity for triglyceride substrates and about 4 fold activity for the cholesteryl ester substrates (Fig.3.4.2.1). Meanwhile, D130E and D130N mutants showed hardly 1.5-1.8 fold activity for triglyceride substrates while cholesteryl ester hydrolase activities were close to the level of negative controls. All samples were normalised for transfection efficiency and amount of immunoreactive material. Immunoblotting of cell extracts visualised by anti-HSV antibody assured the presence of normal sized protein with apparently normal glycosylation pattern (Fig. 3.4.2.2).

3.4.3 H^{274} and neighbouring residues have a critical role in LAL catalysis:

Comparison of acid lipase activities in the cell extracts transfected with H274W and H274Y (Fig.3.4.3.1) with that of controls showed that these mutants expressed recombinant LAL protein with both catalytic activities below the level of background. On the other hand, L273 mutants showed a peculiar behaviour. Acid lipase activities in L273W mutant were below the background activities while L273I mutant showed relative tri-oleyl glycerol activity up to 18-20 times that of controls and cholesteryl oleate hydrolase activity up to 12-14 times that of controls. Both these activities were more than the respective activities observed for normal LAL. All samples were normalised for the transfection efficiencies by CAT assay and presence of immuno reactive material as determined by dot blot assay with anti-HSV tag antibody. The pattern of expression of the recombinant proteins in the case of these mutants were verified by immunoblotting (3.4.3.2).

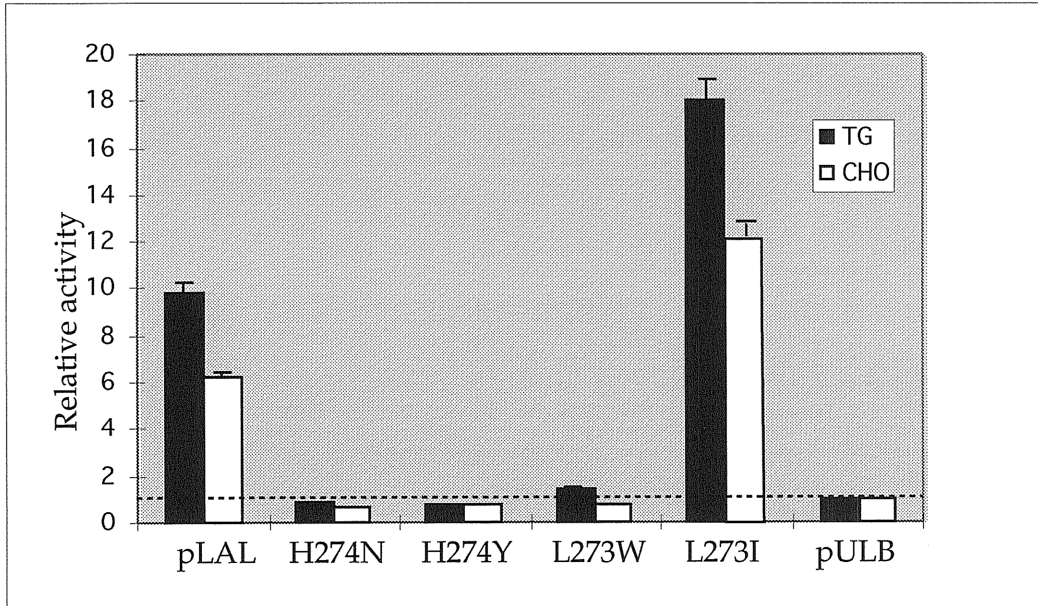


Fig. 3.4.2.1: Acid lipase activities in extracts of HeLa cells transfected with H274 and L273 mutants. Relative activities were calculated considering both hydrolase activities in pULB control as 1. The dotted line indicate the level of significance of the data.

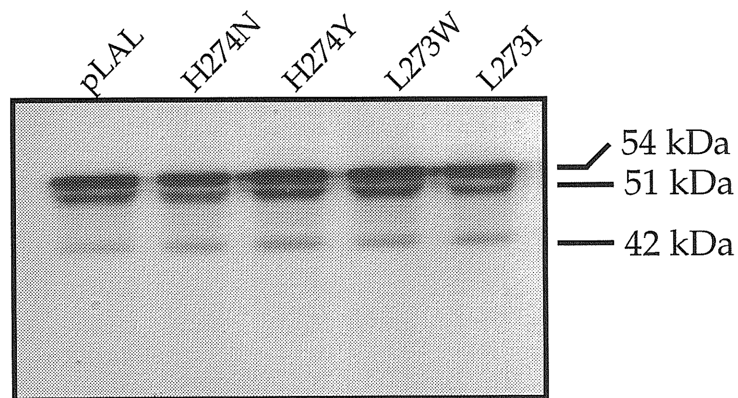


Fig. 3.4.2.2: Immunoblotting of the extracts of cells transfected with H274 and L273 mutants. The recombinant LAL proteins showed the same pattern as in the case of normal LAL inspite of the wide variations in the catalytic activites.

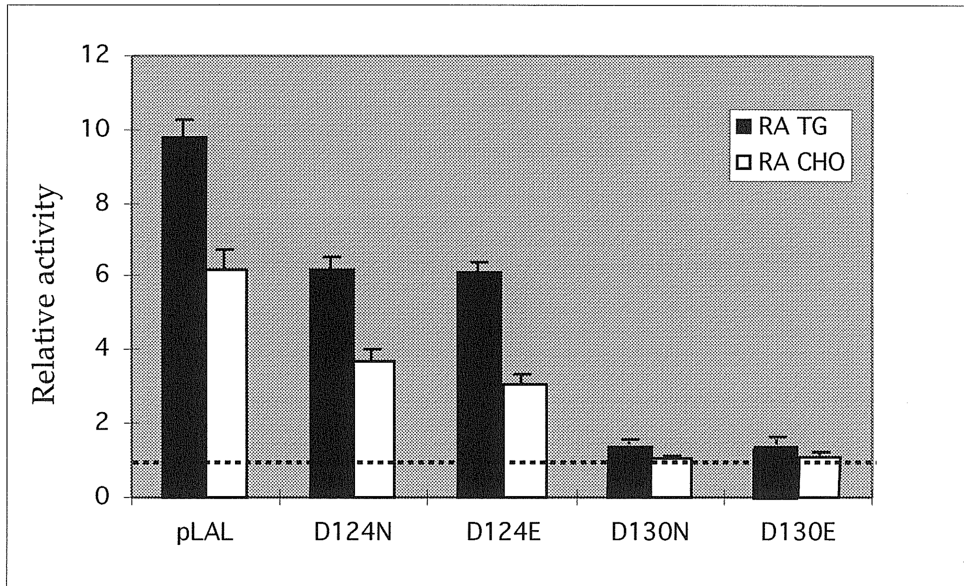


Fig. 3.4.3.1: Relative Acid hydrolase activities in the cell extracts transfected with D124 and D130 mutants. The tri- oleyl glycerol and Cholesteryl ester hydrolase activities in cell extracts were determined as cpm/μg protein/hr. Relative activities were calculated taking acid hydrolase acitvity in pULB control as 1. Black bars indicate tri-oleyl glycerol hydrolase activity while white bars indicate cholesteryl oleate hydrolase activity. Dotted line indicates the level of significance of the data.

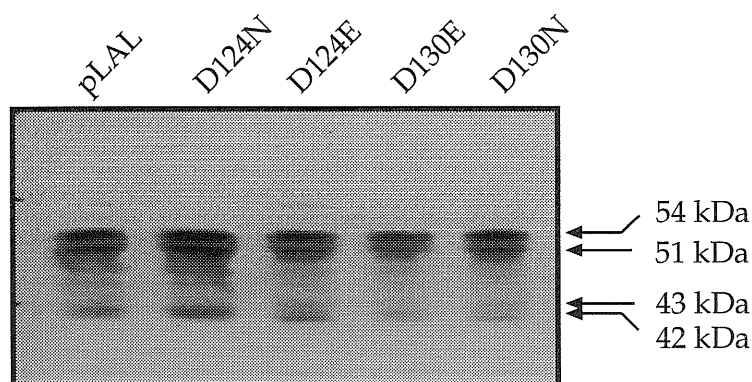


Fig.3.4.3.2: Immunoblotting of cell extracts transfected with D124 and D130 mutants probed with anti-HSV tag monoclonal antibody. All four major molecular weight forms of LAL were present in the case of all mutants.

CHAPTER 4

DISCUSSION

4.1 Identification of LAL mutations in CESD patients and predicted consequences of these mutations:

Molecular basis of cholesteryl ester storage disease in three patients from different parts of Italy has been analysed by reverse transcription polymerase chain reaction (RT PCR) and sequencing techniques. These studies lead to the identification of three missense substitutions, two splicing defects and a new polymorphism in the LAL gene.

A novel 3′ splice acceptor site mutation leading to skipping of Ex.7 is reported in Patient 1. This exon skipping is caused by an A->G mutation at the -2 position of the 3′ splice acceptor site of exon 7. As a consequence 49 amino acids corresponding to Ex.7 will be missing from the protein derived from this transcript. Interestingly, three of the cysteine residues conserved among different members of the acid lipase family and C240 that is unique to hLAL will be missing in the case of this Δ 205-253 mutant (Fig 4.1.1). This exon skipping eliminates a conserved N-glycosylation site located at the junction of Ex.7 and Ex.8 as well.

The AG consensus at the 3′ splice acceptor site is 100% conserved among the protein coding genes of eukaryotic organisms (*Shapiro et al., 1987*). This consensus is disrupted by the A ->G mutation in this patient, suggesting that even a low percentage of correctly spliced LAL mRNA can not be derived from this allele unless compensating mutations occur elsewhere in the intron. This fact is particularly interesting since recent reports have suggested that residual enzymatic activity derived from a low amount of correctly spliced mRNA in a patient with Δ254-277 mutation and a null allele may be the reason for the less severe CESD phenotype in that patient (*Asnalidis et al., 1996*). The nucleotide substitution leading to Ex.8 skipping in that patient was a G->A mutation at the -1 position of the 5′ splice donor site which is only 77% conserved.

```
(MKMRFLGLVVCLVLWPLHSEG)SGGKLTAVDPETNMNVSEIISYWGFPSEE
YLVETEDGYILCLNRIPHGRKNHSDKGPKPVVFLQHGLLADSSNWVTNLANS
SLGFILADAGFDVWMGNSRGNTWSRKHKTLSVSQDEFWAFSYDEMAKYDLPA
SINFILNKTGQEQVYYVGHSQGTTIGFIAFSQIPELAKRIKMFFALGPVASV
AFCTSPMAKLGRLPDHLIKDLFGDKEFLPQSAFLKWLGTHVCTHVILKELCG
NLCFLLCGFNERNLNMSRVDVYTTHSPAGTSVQNMLHWSQAVKFQKFQAFDW
GSSAKNYFHYNQSYPPTYNVKDMLVPTAVWSGGHDWLADVYDVNILLTQITN
LVFHESIPEWEHLDFIWGLDAPWRLYNKIINLMRKYQ
```

Fig. 4.1.1: Amino Acid sequence of LAL indicating the positions of new mutations. Putative signal peptide is shown in brackets. Amino Acid substitutions are indicated above the respective residues.Two GXSXG motifs are shown in boxes. Two Aspartate residues indicated in boxes represent the ones tested for their role in catalysis. Asteriks indicate the cysteine residues. Skipped exons are underlined. Putative N-glycosylation sites are underlined with dotted lines.

On the other hand, P181L substitution reported in the other allele of Patient 1, is located in a highly conserved region of the protein (Fig. 1.5.1). In vitro expression studies have shown that this mutant produces a protein that has less than 10% of relative activity compared to the positive controls. It cannot be excluded that the P181L may have a low level of residual activity that cannot be detected in this in vitro system but can still have functional significance in the in vivo context.

By analysing patient 2, we have identified yet another case of Ex.8 splice site mutation in the LAL gene. The point mutation leading to Ex.8 skipping was a G->A transition at the -1 position of the 5′ splice donor site of Ex.8 as reported in other cases of Ex.8 skipping in CESD patients. High frequency of this mutation (Table.1.9.1) among the European population suggests the possibility of a founder effect. Since the -1 position of 5′ splice donor site is not absolutely conserved, it can be assumed that a 5-8% of correctly spliced mRNA can be produced from this allele as in the case of previous reports. Attempts to amplify this low amount of correctly spliced mRNA by means of allele specific amplification using oligonucleotides that may distinguish the two alleles have failed so far. H_{274}, that seems to be critical for catalytic activity, will be missing from this Δ254-277 mutant. As expected, in vitro expression of this mutant produced catalytically inactive protein (Fig. 3.3.1). Moreover, immunoblotting analysis of the cell extracts indicated that the glycosylation pattern of this protein was altered(Fig.3.3.2). The highest molecular weight form observed in the case of this mutant was 40-41 kDa which is 3-4 kDa less than expected for a deletion of 24 amino acids. The glycosylation site at N_{253} is lost in this case too, accounting for a reduction of 2 kDa in size.

A new XbaI polymorphism (G2A polymorphism) detected in Ex.2 of LAL cDNA was used to follow the fate of parental alleles in Patient 2 and 3 (Fig. 3.1.3.2 & 3.1.3.3). Screening of this allele in the normal population revealed that this polymorphism is represented in the population with an allele frequency of 0.19. Measurement of LAL activity in this population showed that there is no correlation between this polymorphism and the level of LAL activity (3.3.4.1). In vitro expression of the G2A mutant (Fig.3.3.4.2) confirmed this conclusion since this mutant retained relative activities comparable to those of normal LAL. We have tested the T6P polymorphism also in the in vitro expression system and the results confirmed the conclusions of *Muntoni et al., (1996)* based on the biochemical studies carried out in a large population inorder to verify the effect of this polymorphism on serum lipoprotein concentrations. Their studies have shown that T6P polymorphism could not be

correlated to neither abnormal LAL function nor dislipidemia in the general population.

As for a previously described VNTR polymorphism, XbaI polymorphism in Ex.2, also served as an efficient marker to follow individual alleles in heterozygous subjects. In the case of patient 3, this polymorphism was particularly useful to identify the null allele segregating in this family. First of all, it was observed that all the LAL cDNA clones derived from this patient had the T->C substitution leading to L273S mutation. Direct sequencing of the amplification product from genomic DNA representing LAL Ex.8 revealed that the the patient was not heterozygous for this mutation. At this point, LAL Ex.2 was amplified from the genomic DNA and the pattern of XbaI polymorphism was analysed by restriction enzyme digestion (3.1.3.2). The results showed that the patient was heterozygous for the XbaI polymorphism in Ex.2. Meanwhile, the amplification product from the cDNA indicated homozygosity for XbaI polymorphism (3.1.3.3).

Further sequencing analysis showed that the patient's father was homozygous for the absence of XbaI polymorphism while mother was heterozygous for the presence of this polymorphism. This results confirms heterozygosity for XbaI polymorphism in the in the patient. Taken together, these data suggest that the allele without XbaI polymorphism is not represented in the mRNA of the patient, at least not at levels comparable to the mRNA transcribed from the allele with XbaI polymorphism and L273S mutation.

There are many possibilities regarding the cause of this null allele. One of these possibilities is that mutations in the promoter or enhancer region may interfere with initiation or regulation of transcription. Sequencing analysis of the proximal promoter region of LAL from this patient didn't show any mutations that could explain the existence of this null allele. Alternatively, premature stop codons or mutations at the 3' untranslated region of the mRNA may lead to abnormalities in mRNA processing. Further characterisation of this allele was limited by the shortage DNA and RNA from this patient.

4.2 Expression of recombinant lysosomal acid lipase in mammalian system:

Extensive biochemical and functional characterisation of LAL protein has been limited by the availability of sufficient amount of normally processed, catalytically active protein from tissues or cultured cells. Furthermore, as pointed out in the introduction, different molecular weight forms of the protein have been reported by different authors (see section 1.4). It has been presumed that these differences may be due to specific post

translational modifications such as selective proteolysis and glycosylation or due to purification methods biased for the selection of particular molecular weight forms of the protein.

In order to address these questions, it was necessary to characterise recombinant LAL protein expressed in a suitable system. Initially, we tried a simple method by expressing human LALcDNA in *E.coli* BL21 cells as a GST fusion protein. Gel electrophoresis of bacterial cell extracts revealed a prominent 64 kDa band as expected for LAL-GST fusion protein (Fig.3.2.1), but significant acid lipase activity was not detected in the cell extracts. It is possible that complex glycoproteins expressed in bacteria may not be folded properly because of the absence of proper glycosylation in this system. However, we have not tried to refold the protein expressed in bacteria. Our results disagree with preliminary studies reported by *Seedorf et al., (1993).* They have shown that recombinant LAL expressed in *E.coli* was catalytically active.

However, the data of *Sheriff et al., (1995)* supports the results we have obtained. These authors couldn't achieve expression of catalytically active LAL in bacteria using the pET expression system. As a consequence, they have used Baculovirus expression system for expression in Sf9 cells. However, this system has its own limitations as far as the expression of lysosomal enzymes are concerned. It has been shown that Sf9 cells lack the Man6 phosphorylation pathway (*Aeed et al., 1994*) that is commonly used by mammalian cells for labelling nascent lysosomal enzymes by Man6-Phosphate attached to an N-linked carbohydrate chain in order to facilitate recognition by Man6-Phosphate specific receptors (*Kornfeld , 1992*). In the absence of this system, trimming and elongation of the N-linked carbohydrate chains may follow a pattern different from that the normal pathway followed for lysosomal enzymes in mammalian cells.

In the light of these facts, we have attempted expression of LAL in mammalian cells taking advantage of the Vaccinia T7 expression system developed by *Fuerst et al., (1987).* It has been shown that this system permits normal post translational processing and targeting of recombinant glycoproteins (*Elango et al., 1986; Stephens et al., 1986*) expressed in mammalian cells. The recombinant Vaccinia virus Strain, vTF7-3 used in this study produces bacteriophage T7 RNA polymerase in the cytoplasm following infection of mammalian cells. Transfection of a simple recombinant plasmid carrying the gene of interest under the control of T7 promoter permits the transcription and subsequent translation of the recombinant protein in the cytoplasm. Thus an additional step of sub cloning the cDNAs in eukaryotic expression vectors can be skipped. Another advantage of this system is that

high level of expression of the recombinant protein can be achieved. At the same time, the translation of the endogenous gene is considerably reduced so that the activity of the recombinant protein can be well distinguished from that of the constitutively expressed endogenous protein (Fig.3.2.2.1b). Moreover, epitope tagging of the recombinant LAL protein using a 11aa peptide derived from Herpes Simplex Glycoprotein D facilitated immunodetection of the recombinant protein in the cell extracts. Epitope tagging has been successfully employed in different expression systems in order to distinguish the expressed protein from its endogenous counter part in many cases (*Pati et al., 1992*).

Insertion of glycine rich spacer region (GSGGG) has been successfully employed for engineering of recombinant single chain antibodies, (ScFv) in order to minimise steric interferences between two functional domains (*Bird et al., 1988*). We have inserted this type of a spacer region between LAL coding region and the HSV tag epitope (Fig. 3.2.2.1a) in order to avoid probable steric interferences that may lead to alteration of the catalytic properties of LAL. Recombinant LAL produced in HeLa cells using both tagged and non-tagged constructs were equally active towards both triglycerides and cholesteryl esters (Fig.3.2.2.1b) demonstrating that the addition of the tag peptide did not interfere with the catalytic activity of the enzyme.

The presence of 6 putative glycosylation sites (N-X-S/T) in the predicted primary amino acid sequence of LAL suggest that LAL protein is heavily glycosylated as in the case of other lysosomal hydrolases (*Kornfeld & Mellman, 1989*). As discussed in the introduction, there are discrepancies in the literature regarding the molecular weight of human LAL protein. Two human hepatic acid lipases with molecular masses of 29 and 58 kDa or 41 and 56 kDa have been described. In contrast, two molecular weight forms of 41 and 49 kDa have been purified from fibroblasts. The recombinant LAL expressed in Sf9 cells using a Baculovirus expression system showed two major molecular forms of 41 and 46 kDa.

Here, in the present study, we report that recombinant LAL protein expressed in HeLa cells showed 4 different molecular weight forms (Fig. 3.2.2.2). Two higher molecular weight forms of 54 and 52 kDa representing the heavily glycosylated form of the protein. Two lower molecular weight forms of 43 and 42 kDa may account for poorly glycosylated and non-glycosylated protein respectively. Endoglycosidase H treatment reduced the molecular weight of the recombinant protein by 10-12 kDa (Fig. 3.2.2.2) resulting in a single band corresponding to 42 kDa. This size is in agreement with the estimated molecular weight for a polypeptide chain of ~372 amino acids. This data strongly suggest that the lower molecular weight form

represents the precursor protein to which N-linked carbohydrate chains are added during the post translational processing event.

This finding contradicts the conclusions of *Ameis et al., (1994)* who suggest that the lower molecular weight form of hepatic LAL observed by them (41 kDa) originate from N-terminal proteolysis of the 56 kDa precursor. According to the results of micro sequencing of the 41 kDa form, 49 amino acids corresponding to the N-terminus of the LAL polypeptide were missing from this 41 kDa form. Therefore, they have suggested that this form originate from N-terminal proteolysis of the 56 kDa form. We have checked this hypothesis by preparing a construct lacking these 49 N-terminal amino acids and expressed it in HeLa cells. This construct produced inactive LAL protein migrating as a single band of ~37 kDa on immunoblots of the cell extracts suggesting that the lower molecular weight forms of normal LAL (41-42 kDa) must be the non-glycosylated form rather than a proteolytic product. However, it cannot be excluded that the 41 kDa form sequenced by *Ameis et al.,* might be the product of a proteolytic processing event specific to hepatic tissue which does not occur in the HeLa cells. In any case, it should be noted that these authors have performed the Endoglycosidase H treatment on the 42 kDa form only and they didn't check the effect of deglycosylation on the 56 kDa form.

The role of N- glycosylation and presence of Man6-phosphate in lysosomal targeting is well established in the case of lysosomal hydrolases (*Dahms et al., 1989*). Further site directed mutagenesis studies on LAL may help to identify the glycosylation sites that are critical for recognition by phosphotransferase and thus for the addition of Man-6 phosphate marker. Further studies are in progress in this direction. Referring to section 1.6, it is interesting to note that lysosomal acid lipase may serve as a model system for the identification of new sequence elements critical for the sorting of soluble hydrolases to lysosomes.

Studies on the effect of glycosylation on the catalytic activity of lysosomal acid lipase yielded conflicting results. *Ameis etal., (1994)* have reported that endoglycosidase H treatment had no effect on the catalytic activity of lysosomal acid lipase. But these authors have not provided relevant data and details of the reaction conditions used in these experiments. Recombinant LAL expressed in SF9 cells treated with tunicamycin was not catalytically active (*Sheriff et al., 1995*) suggesting that cotranslational glycosylation may be important for the catalytic activity, as it has been shown in the case of human Acid-β glucosidase.

Site directed mutagenesis of Acid-β glucosidase putative N-glycosylation sites have suggested that N- glycosylation site occupancy at N19

could have an affect on the catalytic conformation of this enzyme (*Berg-Fussman et al., 1993*) while occupancy at N59, N146 and N270 was not critical for the catalytic activity of the enzyme. It should be noted that these authors have mutated N19 to different amino acids (N19Q, N19E, N19G, N19D) and observed that the reduction in enzymatic activity was relative to the substitutions made. N19D showed nearly normal activity while the others showed 3-60 fold decrease in the enzymatic activity. The authors attribute these differences to steric effects caused by different substitutions.

As it is evident from Fig. 3.2.2.4, our results show that Endo-H treated cell extracts had 50% activity compared to untreated extracts transfected with the same construct. Endoglycosidase H treatment was performed in non-denaturing conditions. The treated samples were divided into aliquotes and one aliquote was assayed for LAL activity while another aliquote was used for immunoblotting inorder to verify that the protein was not degraded during the treatment. The reduction in the acid hydrolase activity, observed for both triglyceride and cholesteryl ester substrates, was reproducible. These results indicate that N-glycosylation is not crucial for the catalytic activity but could be essential for the proper conformation of the active site or substrate binding site and thus for an optimal rate of catalysis. It is possible that recombinant LAL expressed in tunicamycin treated Sf9 cells was not active because of abnormal folding in insect cells in the absence of glycosylation. In vitro expression of site directed mutants of N-glycosylation sites in HeLa cells will be useful to verify the relation between N-glycosylation and catalytic activity of LAL.

In vitro expression of the mutant CESD alleles identified in this study, showed that all these mutations adversely affect both catalytic activities of the enzyme. In all these mutants relative activities were below the level of negative controls even though recombinant protein could be detected in the cell extracts in equivalent amounts compared to the positive controls. As mentioned earlier, Ex.7 skipping eliminates 4 cysteine residues from the resulting polypeptide and therefore may alter the conformation of the catalytic site. As it is suggested by the site directed mutagenesis studies, H274 is critical for the catalytic activity and in this context, it can be assumed that mutants with Ex.8 skipping and L273S substitution (Fig. 4.1.1) will be catalytically inactive. The location of the P181L mutation in a highly conserved region of the protein, close to a cysteine residue suggests that this residue may some how affect the overall folding of the catalytic conformer.

The L273S mutation identified in Patient 3 was of particular interest because of the location of this residue next to H^{274} that was the site of mutation in a previously described CESD patient (*Pagani et al., 1994*). This

histidine is highly conserved among the members of Acid lipase family (Fig. 1.5.1). Moreover, L273S substitution creates a new N-glycosylation site at position N^{271}. Expression of L273S mutant in HeLa cells showed a high molecular weight band of 56 kDa that was higher than that of the normal LAL. We have verified the possibility that this higher molecular weight form originate from N-linked glycosylation at position N^{271}, by mutagenising N271 to Glutamine. As expected, we observed that neither the N271Q (QML) mutant nor the double mutant N271Q/ L273S (QMS) showed the 56 kDa form when expressed in HeLa cells (Fig. 3.2.8.1). Meanwhile, L273S mutant consistently showed this higher band of 56 kDa and this form was reduced to the lower molecular weight forms of 43 kDa and 42 kDa after endoglycosidase H treatment. This data suggest that the 56 kDa form originate from additional N-glycosylation at position N^{271} rather than a simple alteration in the rate of migration due to any sequence constrains imposed by L273S mutation by itself. Presistence of a small amount of the 43 kDa band in the endoglycosidase H treated extracts suggests that this form may represent the protein retaining the core N-acetyl glucosamine residues.

Measurement of LAL activity in the cell extracts have shown that both H274Y and L273S mutants had catalytic activities below the background suggesting that this part of the protein is particularly important for the catalytic activity of the enzyme. This idea was further supported by the fact that N271Q mutants also expressed proteins with catalytic activity in the range of that of negative controls (Fig.3.2.8.2).

4.3 Putative catalytic triad of LAL: D^{130} - S^{153} - H^{274}

Though different families of lipases share very little sequence homology, an esterase associated pentapeptide motif (GXSXG) surrounded by a stretch of predominantly hydrophobic amino acids, can be identified in the primary amino acid sequence of all known lipases. Two such motifs are present in the primary amino acid sequence of the different members of Acid Lipase family (Fig.1.5.1). It has been demonstrated in the case of gastric lipase that the enzyme is sensitive to active site serine directed inhibitors such as DNP indicating the role of an activated serine as the catalytic nucleophile (*Moreau et al., 1991*). These findings, as well as the functional analogy with other lipases indicate that members of acid lipase family may employ the same catalytic mechanism based on a Ser-Asp-His catalytic triad as in the case of other lipase families. This presumption was further strengthened by experimental evidence from unrelated families of lipases. Site directed mutagenesis studies combined

with three dimensional structures derived from X-ray crystallographic data or modelling studies on 5 different fungal lipases and two mammalian lipases confirmed the existence of a functional Ser-Asp-His catalytic triad (*Derewenda et al., 1994*).

Catalytic features of lysosomal acid lipase were particularly interesting in the sense that this enzyme possess triglyceride hydrolase and cholesteryl esterase activity combined in the same protein. This feature is not present in the members of lipase super family including pancreatic, lipoprotein and hepatic lipases. This type of a dual nature of catalysis is reported for human hormone sensitive lipase (HSL) which shares no sequence homology with human LAL (*Langin et al., 1993*). Another mammalian lipase capable of hydrolysing cholesteryl esters and phospholipids, pancreatic cholesterol esterase or bile salt activated lipase (BAL) (*DiPersio et al., 1990*) is also very different from lysosomal acid lipase as it is suggested from sequence comparison studies.

Therefore, we decided to carry out extensive structure - function studies on lysosomal acid lipase taking advantage of a PCR based site directed mutagenesis strategy (*Higuchi et al., 1988*) coupled with a Vaccinia T7 expression system that helps us to study the functional aspects of the mutant proteins expressed in a mammalian cells.

Two candidate serines (S^{99} & S^{153}) were individually replaced with either glycine or threonine and the constructs were tested for their catalytic activity when transfected in vTF7-3 infected HeLa cells. The S99G and S99T mutants showed 2-3 fold reduction in triglyceride and cholesteryl ester hydrolase activities (Fig. 3.4.1.1) while S153G and S153T mutants showed catalytic activities in the range of that of the negative controls suggesting that S^{153} is essential for catalysis. Similar results were observed for S^{152} of human pancreatic lipase (*Lowe, 1992*), S^{132} of human lipoprotein lipase (*Faustinella et al., 1991*) S^{147} of rat hepatic lipase (*Davis et al., 1990*) from site directed mutagenesis studies confirming the predictions based on the three dimensional structure or sequence homology analysis.

Immunoblotting of the cell extracts (Fig. 3.4.1.2) showed that the recombinant LAL protein was present in the cell extracts in comparable amounts and the proportion of the glycosylated to non-glycosylated protein was similar to that of normal LAL. Thus the possibility that the recombinant proteins expressed from S^{153} mutants were unstable or abnormally glycosylated could be excluded.

Three dimensional structures are not available for any of the proteins belonging to the family of acid lipases, making it difficult to predict

putative active site residues. However, from the data based on the functional assays presented here, we propose that S^{153} of lysosomal acid lipase might form part of the catalytic triad. At the same time, our studies cannot exclude the possibility that S^{99} might be important for the proper conformation of the substrate binding site or some how involved in the process of interfacial activation that is peculiar to enzymes acting at an oil-water interface (*Derewenda et al., 1995*)

When the studies described in this thesis were in progress, *Sheriff et al., (1995)* have reported the same conclusions regarding the role of S^{99} and S^{153} in the catalytic activity of LAL based on their studies with human LAL expressed in Sf9 cells by means of a Baculovirus expression system. These authors have also reported that the catalytic activity of the recombinant enzyme was highly sensitive to active site serine directed inhibitors such as Diethyl p-nitro phenylphosphate (DNP) and Tetrahydro lipstatin (THL) providing chemical evidence that activated serines are necessary for the catalytic activity of LAL.

We have extended these studies in search of the putative partners of serine in the catalytic triad. H^{274} was an ideal candidate in this regard, since we have previously observed a H274Y substitution in homozygous condition, in a CESD patient associated with severe deficiency of LAL activity in PBMNCs. We have expressed this mutant in Vaccinia T7 expression system and observed that this mutation abolished the catalytic activity of the enzyme while there were no significant alterations in the glycosylation pattern (Fig.3.3.1). Site directed mutagenesis of this residue to asparagine (H274N) produced a protein that had both catalytic activities in the range of the negative controls (Fig.3.4.2.1), further supporting the importance of this residue in LAL catalysis.

Another interesting observation was made from this experiment. As in the case of L273S mutation, From the primary aminoacid sequence of LAl, it can be noted that H274N mutation also results putative N-glycosylation consensus (HWS ->NWS). On the contrary, immunoblotting analysis of the cell extracts transfected with H274N construct showed that the high molecular weight form of the protein produced in this case was not higher than that of normal LAL (Fig. 3.4.2.2) suggesting that there is no additional glycosylation. This observation indicates that all putative glycosylation sites are not occupied in vivo. Furthermore, the results obtained for L273S mutant turn out to be more interesting in the context of this data.

Site directed mutants of L^{273}, L273W and L273I had dramatic effects on the catalytic activity of the enzyme. L273W mutant had very little activity towards both substrates while L273I mutant showed relative activity

higher than that of the normal LAL. These results, together with the results obtained for the N271Q mutants suggest that this region of the protein is crucial for the proper folding of the catalytic conformer.

However, this data needs further support from three dimensional structure in order to confirm that H^{274} is situated within hydrogen bonding distance from S^{153} in the three dimensional structure as it is shown in the case of S^{152} and H^{274} of Pancreatic lipase (*Winkler et al., 1990*) as well as S^{132} and H^{241} of lipoprotein lipase (*Tilbeurg et al., 1991*).

Two conserved Aspartate residues (D^{124} and D^{130}) in the primary amino acid sequence of LAL (Fig.1.5.1) were tested for their effect on the catalytic activity of the enzyme. According to the results from transfection assays, D124N and D124E mutants retained substantial hydrolase activity towards both triglycerides and cholesteryl esters (Fig.3.4.3.1). These data suggests that D^{124} may not be very important for the catalytic activity. On the other hand, D130N and D130E mutants showed a significant reduction in both hydrolase activities suggesting that D130 is crucial for the catalytic activity of the enzyme.

4.4 Conclusions.

The data discussed in this thesis leads to some important conclusions regarding the role of lysosomal acid lipase in the pathogenesis of cholesteryl ester storage disease and general catalytic features of this lysosomal enzyme.

First part of the results describe the identification of new mutations observed in the LAL gene in association with cholesteryl ester storage disease. Subsequent in vitro expression studies in mammalian cells have clearly demonstrated that LAL protein derived from these CESD alleles were not catalytically active confirming that the LAL mutations are directly involved in the pathogenesis of this lysosomal storage disease. However, we cannot exclude the possibility that the LAL proteins from these alleles may have a low level of catalytic activity in vivo which cannot be detected in our in vitro system but still having a functional significance in vivo.

A new polymorphism was identified in the LAL gene during these studies. This polymorphism will be highly useful for elaborate molecular genetic studies involving this locus.

In vitro expression and characterisation of recombinant LAL in mammalian cells facilitated detailed analysis of the effect of glycosylation on the catalytic activity of LAL. It can be concluded from these studies that N-glycosylation is necessary for optimal catalytic activity towards both

triglyceride and cholesteryl ester substrates. Epitope tagged LAL constructs prepared for this study can be of use for further characterisation of the role of N-glycosylation in processing and lysosomal targeting of LAL.

Based on the functional analysis of a series of site directed mutants, S^{153}, H^{274} and D^{130} have been identified as the catalytic residues essential for both triacyl glycerol lipase activity and cholesteryl ester hydrolase activity of LAL. These studies can be extended to identify functional domains of the LAL protein specific for either tri-acyl glycerol lipase or cholesteryl ester hydrolase activities.

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