國立臺灣師範大學生命科學系博士論文

人類遺傳疾病

第一部份：第二型黏多醣儲積症IDS基因的分子遺傳研究

第二部份：家族性高膽固醇血症LDL受體基因突變的記述

Human Genetic Diseases:

Part I: Molecular and genetic studies of the IDS gene associated with mucopolysaccharidosis type II

Part II: Characterization of LDL Receptor Gene Mutations in Familial Hypercholesterolemia

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第一部份：第二型黏多醣儲積症IDS基因的分子遺傳研究

摘 要

第二黏多醣儲積症(又稱 Hunter syndrome)為 X 染色體隱性遺傳性疾病，其起因為缺乏分解 heparan sulphate 及 dermatan sulphate 的溶小體水解酵素 iduronate-2-sulfatase (IDS)。全世界有接近三百種和 MPS II 相關的突變被報導。本研究利用單股核酸構形多型性及 DNA 定序等技術，對臺灣地區 10 位來自不同家庭且無血緣關係的 MPS II 患者進行分子致因研究，結果發現 5 種新穎的和 5 種已報導過的突變。合計先前臺灣 14 位 MPS II 患者的 IDS 基因變異分析所發現的 10 種突變，於 24 位的臺灣 MPS II 患者共發現 20 種突變，顯示 IDS 基因突變的高度異質性變化。R468Q 和 R468W 突變分別發現於 3 位無血緣關係的患者，其發生率共佔 25.0%。利用 IDS 基因鄰近的 DXS1123、DXS1113 二核苷重複多型性標記，建構突變基因的單套型，結果發現無血緣關係的 R468Q 突變為不同的起源，但無血緣關係的 R468W 突變則可能具相同的起源。患者 1150 的 R468Q 突變及患者 710 的 I485R 突變發生於精蟲形成的減數分裂。白血球酵素活性檢測顯示，正常人族群及女性 MPS II 突變基因攜帶者的 IDS 酵素活性範圍分別為 19.2 ~ 70.6、8.4 ~ 26.6 nmol/h/mg cell protein，分佈範圍雖然有顯著差異，且女性攜帶者的平均酵素活性值亦低於正常人平均值的一半，但因為兩樣品群活性範圍的小部分重疊，故單獨檢測白血球酵素活性的數值，並不能用來判斷是否為突變基因攜帶者。此外本研究亦對所發現的 17 種胺基酸置換、缺失及終止密碼突變做進一步記述。突變的 IDS cDNA 轉移入 COS-7 細胞後，各突變的 cDNA 所表現的 IDS 活性，僅為野生型 cDNA 的 0%～3.9%，顯示突變的有害；但僅 231del6 突變造成 mRNA 的不穩定性(降低 57%)。西方吸漬分析及共軛焦顯微鏡分析顯示，所檢測的 11 種單一胺基酸置換突變，僅 K347E 突變酵素成熟蛋白的大小及生成量與野生型酵素相似，其餘 10 種點突變前軀蛋白表面上正常，但成熟蛋白量降低或不具，顯示突變蛋白的正常成熟但無法正常運送至溶小體。所檢測的 6 個缺失、終止密碼突變中，1055del12 和 E521X 的成熟不正常，共軛焦顯微鏡分析顯示截短的 W267X 和 1184delG 滯留在早期的 vacuolar 隔間。231del6 和 1421delAG 則未看到表現的 IDS 蛋白，顯示突變 IDS 蛋白的不穩定性及被分解。在增進 IDS 重組蛋白在酵素替換治療應用的研究上，PEIa 銜接的 IDS 較易被

COS-7 細胞分泌出去，被細胞內噬後亦生成有活性的酵素，但銜接的 PEIa 並沒有預期的結合 LRP 的功能。黏多醣儲積症的分子遺傳學研究，可清楚的確認患者的分子致因並提供出生前及家族檢測。進一步的突變記述來釐清突變對 IDS 酵素活性及成熟的影響，可將此疾病的症狀和基因型相關聯，提供臨床上預後及治療方法的評估。

Part I: Molecular and genetic studies of the IDS gene associated with mucopolysaccharidosis type II

Abstract

Hunter syndrome (mucopolysaccharidosis type II) is an X-linked recessive lysosomal storage disease caused by a defect of the iduronate-2-sulfatase (IDS) gene. Nearly 300 different mutations underlying mucopolysaccharidosis type II (MPS II) have been identified worldwide. To investigate the molecular lesions underlying Taiwanese MPS II, 10 probands and families were identified and screened for iduronate-2-sulfatase (IDS) mutation by single strand conformation polymorphism and DNA sequencing. Five novel and five previously reported mutations were found. Together with the previously reported 10 mutations in 14 probands and families, a total of 20 mutations were identified in 24 Taiwanese MPS II patients, supporting the mutational heterogeneity for MPS II. The identified R468Q and R468W account for 25.0% mutations found in our patients. Haplotype analysis using flanking DXS1113 and DXS1123 revealed that the unrelated R468Q alleles are independent origin whereas the unrelated R468W alleles are probably of the same origin. The R468Q mutation in patient 1150 and I485R mutation in patient 710 occurred *de novo* in male meioses. Leukocyte IDS measurement revealed significantly different range of IDS activity in normal controls and MPS II carriers (19.2 ~ 70.6 vs.8.4 ~ 26.6 nmol/h/mg cell protein). The mean leukocyte IDS activity in female carriers was less than a half of the normal level. However, due to a small overlapping range of normal and carriers, the level of enzyme activity can not be used alone for carrier detection. In addition, a total of 17 identified missense, small deletion, and nonsense mutations were further characterized by transient expression studies. Transfection of COS-7 cells by the mutated cDNA did not yield active enzyme, demonstrating the deleterious nature of the mutations. A 57% decrease in IDS mRNA level was seen with 231del6 mutation. Among the 11 missense mutations examined, K347E substitution showed apparent normal maturation and targeting on immunoblot and confocal fluorescence microscopy examination. The rest 10 missense mutations showed

apparent normal precursor with little or reduced mature forms, indicating normal maturation but incorrect targeting of the mutant enzymes. Among the 6 deletion and nonsense mutations examined, 1055del12 and E521X showed abnormal maturation. The staining pattern of truncated W267X and 1184delG proteins suggested retention within early vacuolar compartments. The mutated 231del6 and 1421delAG proteins were unstable and largely degraded. To improve the uptake of the recombinant enzyme for enzyme replacement therapy, PEIa-fused IDS showed increased secretion profiles, leading to increased uptake compared with the wild-type enzyme. However, the expected LRP-mediated uptake was not observed. Molecular and genetic studies of the IDS gene will clearly identify the patient's cause and allow antenatal and family studies. The further characterization of gene mutations may delineate their functional consequence on IDS activity and processing and may enable future studies of genotype-phenotype correlation to estimate prognosis and to lead to possible therapeutic intervention.

第二部份：家族性高膽固醇血症LDL受體基因突變的記述

摘要

家族性高膽固醇血症(familial hypercholesterolemia，簡稱 FH)為一體染色體顯性遺傳疾病，患者因低密度脂蛋白(LDL)受體基因突變而導致血漿中 LDL 膽固醇值過高，易發展出早發性的冠狀心臟病。至今有超過 840 種分散在 LDL 受體基因上的缺失、插入、點突變、裁接突變被報導。先前本實驗室檢測了 170 位膽固醇濃度大於 240 mg/dl 的高脂血症患者 LDL 受體基因，於患者中發現 10 種可能和疾病相關的變異，包括兩種缺失變異(Del e3-5 和 Del e6-8)及八種點變異(W-18X、D69N、R94H、E207K、C308Y、I402T、A410T、A696G)。本研究延續上述發現，對檢測到的變異作進一步的記敘。結果發現 A696G cDNA 質體轉移細胞 LDL 受體的表現量和活性與野生型受體相近。含 D69N 突變的 cDNA 質體表現時出現異常的中間型蛋白。含 R94H、E207K、C308Y、I402T 及 A410T 突變的 LDL 受體活性為野生型的 20～64%。相對的，Del e3-5 及 Del e6-8 突變的 LDL 受體活性僅為野生型的 0～13%。D69N、R94H、E207K、C308Y 及 I402T 等突變受體大部分停留在內質網，A410T 及 Del e6-8 突變受體則停留在 endosome/lysosome。此 LDL 受體的分生研究能清楚的確定病人的高血脂原因，可應用於出生前、家族分析及提供臨床上治療的評估。

Part II: Characterization of LDL Receptor Gene Mutations in Familial Hypercholesterolemia

Abstract

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by increased levels of plasma LDL cholesterol, which cause cholesterol deposition in tissues in the form of tendon xanthomas and atheroma, leading in turn to premature arteriosclerosis and coronary heart disease. FH is caused by mutations in the LDL receptor gene resulting in defective clearance of plasma LDL. To date, more than 840 mutations including gross deletions, minor deletions, insertions, point mutations, and splice-site mutations scattered over the LDL receptor gene have been reported. Previously DNA screening for LDL receptor mutations was performed in 170 unrelated hyperlipidemic Chinese patients and two clinically diagnosed familial hypercholesterolemia patients. Two deletions (Del e3-5 and Del e6-8) and eight point mutations (W-18X, D69N, R94H, E207K, C308Y, I402T, A410T, and A696G) were identified. The effects of the identified mutations on LDL receptor function were characterized in the present study. The LDL receptor level and activity were close to those of wild type in A696G transfected cells. A novel intermediate protein and reduction of LDL receptor activity were seen in D69N transfected cells. For R94H, E207K, C308Y, I402T and A410T mutations, only 20~64% of normal receptor activities were seen. Conversely, Del e3-5 and Del e6-8 lead to defective proteins with 0~13% activity. Most of the mutant receptors were localized intracellularly, with a staining pattern resembling that of ER (D69N, R94H, E207K, C308Y and I402T) or endosome/lysosome (A410T and Del e6-8). Molecular analysis of the LDL receptor gene will clearly identify the cause of the patient's hyperlipidemia and allow appropriate early treatment as well as antenatal and family studies.

LIST of ABBREVIATIONS

AAV: adeno-associated virus
ACAT: acyl CoA:cholesterol acyltransferase
apoB-100: apolipoprotein B-100
BCIP: 5-bromo-4-chloro-3-indolyl phosphate
CHD: coronary heart disease
CNS: central nervous system
DMEM: Dulbecco's modified Eagle's medium
FDB: familial defective apolipoprotein B-100
FH: Familial hypercholesterolemia
GAGs: glycosaminoglycan(s)
GalNAc: N-acetylgalactosamine
GlcUA: D-glucuronic acid
GluNAc: N-acetylglucosamine
HDL: high density lipoprotein
HMG-CoA: 3-hydroxy-3methylglutaryl-CoA
HSV: herpes simplex virus
IDS: intermediate low density lipoprotein
IDS: iduronate-2-sulfatase
IdUA: iduronic acid
LDL: low density lipoprotein
LRP: low density lipoprotein receptor-related protein
MIM: Mendelian Inheritance in Man
MPS: mucopolysaccharidosis
MPRs: mannose-6-phosphate receptors
M-6-P: mannose-6-phosphate
PCR: Polymerase chain reaction
PE: *Pseudomonas* exotoxin A
PEIa: domain Ia of *Pseudomonas* exotoxin A
NBT: nitroblue tetrazolinm
RER: rough endoplasm reticulum
RT-PCR: reverse transcription polymerase chain reaction
SSCP: Single strand conformation polymorphism
VLDL: very low density lipoprotein

Part I: Molecular and genetic studies of the IDS gene associated with mucopolysaccharidosis type II

第一部份：第二型黏多醣儲積症IDS基因的分子遺傳研究

Introduction

Proteoglycan and glycosaminoglycans

Proteoglycans, one of the major protein components of the extracellular matrix, are found in all connective tissues. They are composed of glycosaminoglycans (GAGs) covalently attached as side chains to a core protein. The GAGs are long repeating linear polymers of specific disaccharides. Usually, one sugar is either D-glucuronic acid (GlcUA) or L-iduronic acid (IdUA) and the other is either N-acetylglucosamine (GluNAc) or N-acetylgalactosamine (GalNAc). One or both of the sugars may contain one or two sulfate residues, either as O-esters or N-sulfates. Thus, each GAG chain would bear many negative charges. There are seven types of GAGs: Chondroitin 4-sulfate, chondroitin 6-sulfate, keratan sulfate, hyaluronic acid, dermatan sulfate, heparan sulfate, and heparin. GAGs differ from one other by the amino sugar and uronic acid composition, the linkages between these residues, and the presence and position of the sulfate group. The chondroitin 4-sulfate and chondroitin 6-sulfate are composed of GlcUA residues in β-1,3 linkage with GalNAc. The GalNAc is sulfated in forth or sixth position. The keratan sulfate is composed of GlcNAc in β-1,3 linkage to galactose. The hyaluronic acid consists of GlcUA in β-1,3 linkage to GlcNAc. The dermatan sulfate is formed from chondroitin sulfate by intracellular epimerization of GlcUA to IdUA. Heparin and heparan sulfate are similar in structure; the repeating disaccharide units could be GlcUA in β-1,4 linkage to GalNAc or IdUA in α-1,4 linkage to GluNAc. Heparan sulfate differs from heparin by having more acetylated glucosamine sugars and fewer sulfate groups (Malmstrom et al., 1980; Paulsson and Heinegard, 1984). The functions of proteoglycans include stabilizing and supporting tissue, lubricating and providing resiliency for joints, maintaining water and salt balance in the body, and signal transduction (Paulsson and Heinegard, 1984; Lin, 2004).

Glycosaminoglycan degradation

The catabolism of proteoglycans begins in the extracellular matrix, releasing smaller

fragments that can be transported into cell through pinocytosis (Sandy et al., 1978; Truppe and Kresse, 1978). The internalized GAGs fragments fuse with the lysosome for further degradation of the polysaccharide chain. The normal process of GAGs is a sequential action of multiple lysosomal exoglycosidases and sulfatases from the non-reducing terminus of the GAGs toward its reducing terminus; and the endoglycosidases act at interior points on GAGs and cleave the molecule into smaller fragments. The GlcUA and IdUA residues are cleaved by β-glucuronidase and α-L-iduronidase, respectively. The monosaccharides, GluNAc and GalNAc, are respectively cleaved by N-acetylglucosaminidase and N-acetylgalactosaminidase. The sulfate groups are removed specifically by sulfatases that are specific for the linkage and position of the sulfate group. After the concerted action of these enzymes, the polysaccharide chains of the GAGs are broken down into free sulfate and monosaccharide residues. Then, the degraded monomers are available for use in GAG biosynthetic pathways. Enzyme deficiency in the degradation pathways usually results in the accumulation of substrate polymers, though some deficiencies have not been found. The step-wise degradation of GAG in human is of particular concerned because of the severe clinical disease presentation in children, who are deficient in any one of these enzymes. The Mucopolysaccharidosis (MPS) are a group of inherited lysosomal storage diseases caused by the deficiency of lysosomal hydrolases, which is required in GAG degradation process (**Figure 1** and **Figure 2**).

Cellular biology of lysosomal enzyme

In higher eukaryotic cells, acid hydrolases are transported into lysosomes via two pathways, and both of them rely on mannose 6-phosphate receptors (MPRs) (**Figure 3**). One pathway selectively transports newly synthesized enzymes after they receive the mannose 6-phosphate (M-6-P) recognition marker in the Golgi complex. The transportation of newly synthesized lysosomal enzymes, from rough endoplasmic reticulum (RER) to their final destination in lysosomes, is a multi-step process requiring a series of posttranslational modification and intra-organelle recognition. At their inception, a newly synthesized enzyme will be destined for secretory vesicles or the plasma

membrane, or it may be directed into lumens of the RER, where co-translational addition of large preformed side chain from oligosaccharides to asparagine residues occurs, by lysosomes possessing a hydrophobic amino-terminal signal peptide. The signal peptide is cleaved in RER lumen and the enzymes are transported to the Golgi system, where lysosomal enzymes are distinguished by acquisition of modified phosphomannosyl moieties via the sequential action of a phosphotransferase and N-acetylglucosaminidase (Spiro, 2004). The resulting phosphomonoester ligands displayed by these enzymes are recognized and bound by Golgi membrane-associated 46 kDa cation-dependent MPR (Nakayama and Wakatsuki, 2003), which targets these complexes to an acidified pre-lysosomal compartment. Within the pre-lysosomal organelle, the enzymes dissociate from the receptor and are subsequently sequestered into functional lysosomes.

The second pathway, present in many cell types, is receptor-mediated endocytosis, by which enzymes are taken up from the extracellular medium by the 275 kDa cation-independent MPR, which is on the plasma membrane, and transported to lysosomes. Both cation-dependent and cation-independent MPRs bind phosphomannosyl residues optimally in the pH range of 6.3~6.5. However, only the cation-independent MPR can bind ligand at neural pH, which may explain why only the cation-independent MPR can internalize ligands at the cell surface (Tong et al., 1989; Munier-Lehmann et al., 1996).

Lysosomal storage diseases

The lysosomal storage diseases include a wide range of more than 40 heterogeneous human genetic disorders, including mucopolysaccharidoses, sphingolipidoses, mucolipidoses, glycoproteinoses, and glycogenoses. They are usually characterized by a common pathophysiology, in which very low or absent activity of a specific acid hydrolase causes a progressive accumulation of uncatabolized macromolecular substrates in the lysosomes of diseased cells (Neufeld, 1991). The lysosomal storage diseases generally follow a characteristic pathway in which gradual lysosomal distension impairs cellular and tissue function, resulting in systemic disease, such as cardiovascular,

muscloskeletal and neurological impairment. Although the incidence from each subtype of the diseases is low (1 in 50,000 to 100,000), on the whole, lysosomal storage diseases have a rather high occurrence rate. A recent study conducted in Australia revealed that 1 in 7,700 live births was being diagnosed with lysosomal storage disease based on enzymatic testing, with 35% being mucopolysaccharidoses patients (Poorthuis et al., 1999). The intra-lysosomal accumulation of undigested substrates can occur through several mechanisms: 1) genetic mutations in lysosomal enzymes, 2) mistakes in processing or trafficking of enzymes, 3) co-factors missing, 4) receptors are non-functional sorting, and 5) substrate undigestable (Walkley, 1998). However, most storage diseases result from losing enzymatic activity due to a genetic mutation or mistakes during post-translational processing of the proteins. Many mutations located along the coding regions of the genes have been identified from carriers and affected individuals, including single base mutations, duplications, and insertions.

Mucopolysaccharidoses (MPS)

The mucopolysaccharidoses are the subgroup of lysosomal storage diseases, resulting from deficiencies in lysosomal enzymes required in the sequential degradation of GAG known as mucopolysaccharides. Depending on the enzyme deficiency, the degradation of particular GAG polymers, such as dermatan sulfate, heparan sulfate, keratan sulfate and occasionally chondroitin-sulfate, can be blocked. The faulty degradation of the particular GAG results in excessive lysosomal storage of partially degraded polymers. The inherited defects are the primary cause for lysosomal accumulation of undegraded GAG in multiple organ systems, including the brain, cells, tissues, and organs, with consequent progressive somatic changes and neurological alterations, but some excess GAGs will be excreted in the urine (Meikle et al., 2004). Until today, the deficiencies of 11 lysosomal enzymes, including five glycosidases, five sulfatases, and one acetyl-CoA transferase, have been identified and linked to each specific MPS variant. A systematic classification of MPS, which designated MPS type I to IX along with subtypes, has been proposed, expanded, and modified according to clinical and biochemical findings (Neufeld, 1991) (**Table 1**). All designated MPS types

and subtypes have also been cataloged into the Mendelian Inheritance in Man (MIM) (Wilkie et al., 1994). The accumulation of GAG in lysosomes disrupts normal cellular processes, leading to cell death and cellular and organ dysfunction, eventually, various mental retardation, which is related to the central nervous system, may be found in most forms of MPS.

Mucopolysaccharidosis type II (MPS II)

Mucopolysaccharidosis type II (MPS II *aka* Hunter syndrome) is an X-linked recessive disorder caused by a deficiency of the lysosomal enzyme iduronate-2-sulfatase (IDS, EC 3.1.6.13). The clinical conditions were first described by Hunter in 1917 and now the disease is also called MPS type II with MIM number 309900. The enzyme deficiency leads to intralysosomal accumulation and urinary excretion of partially degraded GAG, heparan sulfate and dermatan sulfate (**Figure 1** and **Figure 2**). The ubiquitous nature of glycosaminoglycans within the connective tissue of the body results in a broad spectrum of phenotypes. Hunter syndrome can be divided into two subtypes. Type A, the classic form, is the severe one, which can usually be seen in children aged 18-36 months.. The children may survive into the second and third decades of life. Clinically, the severe form has an early onset between ages 2-4 years with coarse facial features, dysostosis, dwarfism, skeletal deformities, joint stiffness, hepatomegaly, deafness, cardiovascular disease, mental retardation, and death before adulthood. The mild type B form is differed from the severe form by the late onset, slower progressive somatic involvement, and with little or no intellectual deterioration. Individuals with type B may live into their 70s (Neufeld and Muenzer, 1995). This phenotype variability is presumed to reflect allelic heterogeneity of IDS mutations.

Diagnosis of MPS II

For Hunter syndrome type A, the visible signs and symptoms, such as enlarged liver and spleen and the ivory-colored skin lesions, in young boys, from about 2 to 6 years of age, are usually the early clues for the diagnosis. The common urine test for GAGs can

also be used to screen for an MPS disorder. However, the children with with normal result from the urine test may still have an MPS disorder. The diagnosis of mild Hunter syndrome type B is more difficult and might only be recognized when one of the maternal relatives is possessed with Hunter syndrome. A definitive diagnosis of Hunter syndrome can be performed by measuring the IDS activity in serum, white blood cells, or skin fibroblasts. In some individuals, the clinical severity can be determined by analyzing the IDS gene . Prenatal diagnosis is usually available by measuring the IDS enzymatic activity in amniotic fluid or chorionic villus tissue.

Iduronate-2-sulfatase (IDS) gene and mRNA

The human IDS gene contains 9 exons and spans approximately 24 kb (Flomem et al., 1993; Wilson et al., 1993). The IDS gene has been fully sequenced and a pseudogene (IDS-2) located 20 kb telomeric to the active gene has been identified, which contains sequences related to exon 2 to intron 3 and intron 7 (**Figure 4**) (Bondeson et al., 1995; Rathmann et al., 1995; Timms et al., 1995). During transcription, different polyadenylation signals would generate 5.7, 5.4 and 2.1 kb IDS transcripts (Cudry et al., 1999). In addition, an alternative form of IDS mRNA with 1.4 kb, containing exons 1 to 7b, was discovered in 1995 by Malmgren et al.. The four transcripts were detected in different tissues, including human skin fibroblasts, with different relative amounts found (Malmgren et al., 1995). Moreover, the IDS has been purified to near homogeneity from human plasma (Wasteson and Neufeld, 1982), urine (Lissens et al., 1984), placenta (Di Natale and Daniele, 1985), and liver (Bielicki et al., 1990). The predicted IDS-like enzyme from the 1.4 kb mRNA is identical to the IDS, except for an absence of the 207-amino-acid COOH-terminal domain, which is replaced by 7 amino acids. The functional implication of the 1.4 kb alternative transcript remains to be determined.

IDS proteins

The full-length of IDS cDNA encodes a 550 amino-acid polypeptide, which is highly homologous to sulfatase protein family (Wilson et al., 1990). Post-translational

modifications include glycosylation, phosphorylation and proteolysis. The N-terminal proteolytic pre-lysosomal cleavage removes a propeptide of 8 amino acids (amino acids 26-33) and occurs after removing a 25-amino-acid N-terminal signal sequence (Wilson et al., 1990). The IDS is glycosylated at 8 potential sites (Asn-X-Ser/Thr, positions 31, 115, 144, 246, 280, 325, 513 and 537). *In intro* expression of site-directed mutation showed that all of the potential N-glycosylation sites were used, but none of them appeared to be essential for lysosomal targeting or IDS activity (Millat et al., 1997). Further processing includes two C-terminal proteolytic cleavages, releasing 18 kDa (amino acids 456-550) (Wilson et al., 1990) and 7-8 kDa polypeptides (Wilson et al., 1990). Both 76 and 90 kDa precursors are converted through a 62 kDa intermediate to 55 kDa and 45 kDa mature forms (Froissart et al., 1995; Millat et al., 1997) (**Figure 5**). The optimum pH for IDS in normal skin fibroblasts, determined in 50 mmol/L sodium acetate buffer, was about 4.0-4.2, the Michaelis-Menten constant K_m was estimated to be 4-8 μmol/L, and the V_{max} was about 170 pmol/min (Petruschka et al., 1994).

IDS mutations

To date, over 300 mutations have been found in human IDS gene by molecular studies, using Southern blot analysis and DNA sequencing including deletions, insertions, point mutations, and splicing site mutations in the human IDS gene (http://www.hgmd.org/) (Stenson et al., 2003). The frequency of large alteration is about 20%, which includes complete or partial gene deletions and large rearrangements. The majority of mutations (80%) identified are small deletions, insertions or single base substitutions (missense mutations, nonsense mutations, and mutations affecting splicing). A haplotype analysis revealed that mutations occur more frequently in the meiosis process of males (Rathmann et al., 1996; Froissart et al., 1998). A study on 14 unrelated Taiwanese MPS II patients identified 10 mutations, including seven missense mutations (N63K, P228L, K347E, R468Q, R468W, I485R, G489D), two deletions (1055del12, 1421delAG), and one splicing mutation (IVS2+1G>C) (Lin et al., 2000). Most of the mutations identified were novel, but the recurrent R468Q (Rathmann et al., 1996; Lissens et al., 1997; Froissart et al., 1998; Isogai et al., 1998; Karsten et al., 1998; Vafiadaki et al.,

1998; Li et al., 1999) was found in 3 unrelated patients. The results provide further evidence of mutational heterogeneity for MPS II.

Although over 300 small mutations have been reported from many laboratories, probably due to technical difficulties, only 23 of the cases have been studied with site-directed mutagenesis and *in vitro* expression. The characterized mutations with mild phenotype include R48P, T118I, R468W, P480L, P480Q, and Q531X; intermediate phenotype, R88H, N265I, W337R, and R443X; severe phenotype, L41P, P86L, P86R, 959delT, R88P, S333L, W345X, K347T, R468Q, R468L, G489A, 473delTCC, and 533delTT (Crotty et al., 1992; Sukegawa et al., 1995; Millat et al., 1998; Cudry et al., 2000; Villani et al., 2000; Bonuccelli et al., 2001; Ricci et al., 2003). The severe R468Q and mild R468W mutations were also seen in patients in our study (Lin et al., 2000). The R468Q mutation did not affect the normal maturation or processing, but the conformation of the protein or the transport to lysosomes (Sukegawa et al., 1995; Villani et al., 2000). Characterization of gene mutations not only clarifies their functional consequence on IDS activity, processing and enzyme localization, but also identifies the genotype-phenotype correlation for prognosis estimation.

Treatment of MPS

When it was discovered that different enzyme deficiencies were the cause of MPS disorders, researchers believed that exogenous enzymatic treatment could be an effective therapy (Kaplan et al., 1977). Current treatment strategies for MPS, and other lysosomal storage diseases, include enzyme replacement therapy, cell and tissue transplantation, and gene therapy.

(1) Enzyme replacement therapy. Enzyme replacement therapy refers to the infusion of enzyme, which is artificially manufactured and pure, into patient who is lack of or deficient from that particular enzyme. Enzymes are taken into lysosomes via mannose-6-phosphate (M-6-P)-receptor-mediated endocytosis (Kornfeld, 1990; Kornfeld, 1992; Hille-Rehfeld, 1995) to reverse the GAG deficiency. Only 1 ~ 5% of normal

cellular activity was required to correct the metabolic defects in enzyme-deficient cells. Enzyme replacement therapy is a regular, life-long form of treatment, which requires the patients to spend hours of time to receive an infusion of enzyme once a week or fortnight. Currently, clinical trials using recombinant human enzymes are being undertaken in MPS type I (Miebach, 2005; Kakkis et al., 2001), type II (Muenzer et al., 2002) and type VI (Harmatz et al., 2005). However, this approach could not be used to treat MPS that are characterized by neuronal pathology, because intravenously administered enzymes cannot go across the blood-brain barrier to correctly store in central nervous system (CNS). The drawbacks from the the long-term therapy and the limited application in CNS restrict the use of enzyme replacement therapy. Still, recombinant enzyme therapy is likely to be valuable in treating the somatic symptoms of the disease.

(2) Cell and tissue transplantation. Allogeneic bone marrow and unrelated umbilical cord blood transplantations have also been used to treat Hunter disease. The principle is to transfer the healthy bone marrow or blood-derived cells to patients and provide functioning enzymes for enzymatically defective cells, which store materials in lysosomes. It might be the variability in symptoms and age, the transplantation has not been shown to ameliorate the symptoms of Hunter disease with an obvious outcome (Bergstrom et al., 1994; Coppa et al., 1995; Li et al., 1996; McKinnis et al., 1996; Vellodi et al., 1999; Mullen et al., 2000; Peters and Krivit, 2000).

(3) Gene therapy. In this treatment, the defective gene presented in a patient will be replaced with a normal gene to activate enzymes preventing the diseases. Many viral vectors are now available for gene delivery, such as herpes simplex virus (HSV), adenoviruses, adeno-associated virus (AAV), retroviruses and lentiviral vectors (Di Natale et al., 2002). Moreover, gene transfer can be achieved by transplantation of virus-transduced cells or direct viral injection. Gene therapies with adenoviral or retroviral transduction has been evaluated for the treatment of MPS II (Braun et al, 1993; Braun et al, 1996; Whitley et al, 1996; Stroncek et al, 1999; Pan et al, 1999). However, so far, many technical difficulties need to be solved to achieve the stable insertion and expression of the normal gene in various gene positions of the diseases.

Pseudomonas exotoxin A (PE) and lipoprotein receptor-related protein (LRP)

Pseudomonas exotoxin A (PE) is a 66 kDa secretory single chain toxin produced by *Pseudomonas aeruginosa*. The three–dimensional structure of PE has been determined by X-ray crystallography (Allured et al., 1986). PE contains three structure domains. Domain I contains amino acid residues 1 to 252 (Domain Ia) and 365 to 404 (Domain Ib). Domain II contains amino residues 253 to 364; and domain III contains amino acid residues 405 to 613. Among the *Pseudomonas aeruginosa* extracellular proteins, PE is the most toxic one (Liu, 1966). This toxin is active on most eukaryotic cells and species, including human. PE exerts its toxicity by irreversibly arresting eukaryotic protein synthesis. The intoxication process was thought to proceed by following steps. First, PE binds to cells through the low density lipoprotein receptor-related protein (LRP) on the cell surface (Kounnas et al., 1992), then the PE-receptor complex is internalized into cell and leads to cell death via the enzymatic attachment of elongation factor-2 (Iglewski et al., 1977). The structural domain Ia of PE (PEIa) is required for cell recognition (Hwang et al., 1987).

The LRP is a cell surface glycoprotein capable of binding and delivering multiple ligands to lysosomes for their degradation. Known ligands include lipoproteins, proteinases, proteinase-inhibitor complexes, matrix proteins, and bacterial toxins and viruses (Gliemann, 1998). In addition, LRP also cooperates with other cell surface molecules to effect a signal in response to alterations within the cellular environment (Strickland and Ranganathan, 2003). The LRP is synthesized in the endoplasmic reticulum as a 600 kDa glycosylated precursor and is cleaved to 515 and 85 kDa subunits associated non-covalently (Herz et al., 1990). The isolation of LRP-deficient cells that are highly resistant to PE confirmed the role of LRP as a cellular receptor that mediates cytotoxicity (Willnow and Herz, 1994; Fitzgerald et al., 1995). The LRP is found in many tissues and cells and is expressed in high levels in fibroblasts and hepatocytes (Moestrup et al., 1992). Several hormones (insulin, oestrogen) and growth factors (nerve growth factor, epidermal growth factor, platelet-derived growth factor-BB) have been shown to stimulate LRP expression in diverse cell types (Descamps et al., 1993; Weaver et al., 1996; Bu et al., 1998; Li et al., 1998).

Aims

Accurate knowledge of the specific mutations involved in this disease may provide helps on clarifying the relation between genotype and phenotype in an individual patient and identifying the female carriers. To characterize the biochemical and molecular defects in IDS-deficient patients and their families, this study aims to investigate IDS gene mutations in Taiwanese patients with MPS II. The effects of identified mutations on IDS catalytic activity, mRNA stability, and protein maturation and targeting were further analyzed using transient expression systems. In addition, IDS enzyme activities in female carriers were evaluated and haplotyped on the mutant allele and were analyzed using flanking DXS1113 and DXS1123 microsatellite markers to reveal the original parental mutation on X chromosome. Finally, a atoxic PEIa fragment of *Pseudomonas aeruginosa* was in-frame fused with IDS to generate PEIa-IDS and IDS-PEIa recombinant proteins to assess the usage in enzyme replacement therapy.

Materials and Methods

Patients

The patient group consisted of 24 unrelated Taiwanese patients with Hunter syndrome. Among them, 10 mutations were identified previously from 13 patients with severe disease and one patient with a mild phenotype (Lin et al., 2000). The rest ten patients, including 3 with mild and 7 with severe MPS II were screened for IDS mutations. Together, the 20 patients with severe disease had a typical clinical course with an early onset before 5 years of age and characteristic, progressive neurological and somatic involvement. The 4 patients with a mild phenotype have normal intelligence and slow progression of the somatic features. The diagnosis of MPS II was made by demonstrating a large amount of urinary glycosaminoglycans, two-dimensional electrophoresis to identify heparan sulfate and dermatan sulfate in the urine, and undetectable IDS activity in peripheral blood leukocytes (provided by Dr. Shuan-Pei Lin from Mackay Memorial Hospital, Taipei).

Mutation analysis

Genomic DNA was extracted from whole blood according to standard procedures. Polymerase chain reaction (PCR) of IDS exons including adjacent intronic regions was done with primers and conditions listed in **Table 2**. PCR products were mixed with an equal volume of 95% formamide buffer and electrophoresed for single strand conformation polymorphism (SSCP) and heteroduplex analyses as previously described (Lee-Chen et al. 2002). Fragments showing single strand mobility shifts or heteroduplex bands were sequenced directly using the MegaBACE Analyzer. For patients with mutations not readily detected on SSCP screening, all exon-containing fragments were sequenced to identify mutations. Mutations were verified by restriction analysis of PCR amplified products (**Table 3**) for family studies. One hundred control chromosomes were

analyzed for novel missense mutations H138R and W267C using restriction analysis.

Measurement of leukocyte IDS activity

Fifty unrelated Taiwanese individuals without MPS II and high-risk family members from 7 MPS II patients were studied. Leukocytes were isolated from 2 ml blood by means of centrifugation through Ficoll Pague (Pharmacia). Cell lysates were prepared by suspension of leukocyte pellet in 0.2 ml of 0.85% NaCl and disrupted by six cycles of freeze-thawing. Proteins were determined using Coomassie Plus protein assay reagent (Pierce). The assay for IDS activity was carried out using 4-methylumbelliferyl- α-iduronide-2-sulphate as a substrate (Moscerdam Substrates) as previously described (Voznyi et al., 2001; Ricci et al., 2003).

Haplotype Analysis

The microsatellite markers DXS1113 and DXS1123 (**Table 4**) flanking the IDS locus were analysed in MPS II families to establish haplotypes at the IDS locus. One hundred unrelated Taiwanese individuals without MPS II were collected from the general population to serve as controls. The forward primer was fluorescence labeled in each reaction, and the amplified PCR products were analyzed in linear polyacrylamide gel on an automated MegaBACE Analyzer. Genotype and haplotype frequencies were calculated and chi-square tested for significance.

cDNA constructs

Total RNA was isolated from human cultured fibroblasts and reverse transcription-PCR amplification carried out as previously described (Lee-Chen et al.

2002). Sense and antisense primers used for amplification of IDS cDNA were shown in **Table 2**. After the amplified full-length 1.8-kb IDS cDNA were cloned into pGEM-T Easy vector (Promega) and sequenced, the IDS cDNA was then excised with *Eco*RI and subcloned into pcDNA3 (Invitrogen) to produce plasmid pcDNA3-IDS (**Figure 6**). The point mutations in the IDS cDNA were generated by QuickChange^{TM} XL Site-Directed Mutagenesis Kit (Stratagene) (sequences of primers are listed in **Table 5**). The mutated sequence constructs were confirmed by DNA sequencing. pcDNA3-IDS/231del6 was derived from pcDNA3-IDS by replacing a 123-bp *Mae*II-*Fsp*I fragment (cDNA 114~236) with a 117-bp fragment excised from the exon 2 clone of the patient with 231del6. pcDNA3-IDS/1055del12 was derived from pcDNA3-IDS by replacing a 112-bp *Ban*II-*Hin*dIII fragment (cDNA 1015~1126) with a 100-bp fragment from the exon 8 clone of the patient with 1055del12 (Lin et al. 2000). The replaced sequences were confirmed by restriction enzyme digestion and DNA sequencing.

IDS antibody preparation

The 1.0-kb *Sac*I-*Hin*dIII fragment containing IDS codon 18-337 (cDNA 52-1011) was cloned into pET-22b(+) (**Figure 7**) and introduced into BL21(DE3)pLysS (Novagen). After IPTG induction, the 45-kDa IDS antigen was purified by His-Tag resin and used for immunization. Three BALB/c mice were injected subcutaneously with 50 μg antigen, mixed with Freund's complete adjuvant. Four weeks later, the animals were boosted at intervals of 2 weeks with antigen in Freund's incomplete adjuvant. Sera were collected at 7 and 14 days after boosting. The antibody induced was examined by ELISA immunoquantification and western blotting.

Expression studies

COS-7 cells cultivated in DMEM containing 10% FCS were transfected with the various IDS cDNA constructs by lipofection procedure. Forty-eight hours after transfection, cells were harvested and extracts prepared by freeze-thawing. The assay for IDS activity was carried out using 4-methylumbelliferyl-1iduronide-2-sulphate as a

substrate (Moscerdam Substrates) as previously described (Ricci et al. 2003). The total RNA (5 μg) from transfected cells was reversed-transcribed and a 254-bp IDS 3' cDNA fragment was amplified using sense primer 5'-CAAGCCGAGTTTAAAAGATA-3' and the full-length antisense primer. A 450-bp neomycin fragment were co-amplified using primers 5'-TTGGGTGGAGAGGCTATTCG-3' and 5'-CCCTGATGCTCTTCGTCCAG-3' as internal control for transfection efficiency. Total proteins (10 μg) from transfected cells were separated by SDS/PAGE and blotted on to nitrocellulose filters by transverse electrophoresis. After blocking, the membrane was stained with antiserum raised in mouse (1:5000 dilution). The immune complexes were detected with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1:3000 dilution, Boehringer Mannheim).

Immunofluorescence and microscopy

For subcellular localization of wild type and mutant IDS proteins, cells were seeded in coverslips for transfection. The intracellular IDS protein was visualized by staining cells fixed in 0.05% glutaraldehyde for 15 min. Cells were incubated for 60 min at room temperature with primary antibody diluted 1:1000 in 1% BSA in PBS, washed in PBS, and incubated for 60 min at room temperature in CY5-conjugated secondary antibody diluted 1:50 in 1% BSA in PBS, washed in PBS. Lysosomes were detected using lysotracker (Molecular Probes) at 0.05 μmol/L for 60 min. The endoplasmic reticulum was identified using $DiOC_6$ dye (Molecular Probes) for 15 min at a final concentration of 2 μg/ml. The stained cells were analysed using a Leica TCS confocal laser scanning microscope.

Construction of PEIa-IDS and IDS-PEIa recombinant proteins

pET-15b-PEIa plasmid containing a 759-bp PEIa domain (residues 1-252) of *Pseudomonas* exotoxin A in pET-15b was obtained from Dr Jaulang Hwang, Institute of Molecular Biology, Academia Sinica. The PEIa domain in pET-15b-PEIa was amplified

by PCR using primers with *Pst*I (5'-GCGCCTGCAGAGGAAGCCTTCGA-CCTCTGG-3') or *Bam*HI (5'-CGGCGGATCCGCCCTCGGGAAAGTGCAGGCG-3') restriction site on the 5' end. The amplified fragment was cloned into pGEM-T Easy (Promega) and sequenced. A 85-bp *Hin*dIII (in the multiple cloning site of pcDNA3)-*Pst*I fragment containing the N-terminal signal peptide of the LDL receptor (-19 ~ +66, where +1 represents the first nucleotide to be transcribed), the cloned *Pst*I-*Bam*HI PEIa fragment, and the *Bam*HI-*Eco*RI (in the multiple cloning site of pcDNA3) IDS cDNA fragment containing amino acids 34-550 were placed between *Hin*dIII-*Eco*RI sites of pcDNA3 to generate pPEIa-IDS (**Figure 8**). To construct pIDS-PEIa, the termination codon (TGA) of IDS cDNA was removed and *Pst*I restriction site added by PCR using primer 5'-GCCTGCAGGCATCAACAACTGGAAAAG-3'. In addition, a termination codon (TGA) was added to the 3' end of PEIa fragment by PCR using primer 5'-CCTCAGCCCTCGGGAAAGTGCAGGCG-3'. The amplified IDS and PEIa fragments were cloned into pGEM-T Easy and sequenced. The cloned *Eco*RI (in the multiple cloning site of pGEM-T Easy)-*Pst*I IDS cDNA fragment and the *Pst*I-*Not*I (in the multiple cloning site of pGEM-T Easy) PEIa fragment were placed between *Eco*RI-*Not*I sites of pcDNA3 to generate pIDS-PEIa (**Figure 9**).

PEIa-IDS and IDS-PEIa uptake by LRP

COS-7 cells were transfected with pcDNA3-IDS, pcDNA3-PEIa-IDS or pcDNA3-IDS-PEIa construct by lipofection procedure. Forty-eight hours after transfection, supernatant fluids were collected and cell lysates prepared. To examine the uptake of IDS, PEIa-IDS and pIDS-PEIa proteins by COS-7 cells, the collected supernatant fluids were filtered through a 0.2-μm filter and added to cells in the presence or absence of 5 mM M-6-P. M-6-P can selectively block the uptake of IDS, PEIa-IDS and pIDS-PEIa through both cation-dependent and cation-independent MPRs. Forty-eight hours after uptake, cells were collected and cell lysates prepared. The cell lysates and supernatant fluids were examined by western blotting (10 μg cell lysates or 10 μl supernatant fluids) and IDS activity assay (5 μg cell lysates or 5 μl supernatant fluids) as previously described. The expression of LRP in COS-7 cells was verified by RT-PCR

using sense primer 5'-GAGTACCAGGTCCTGTACATCGCTG-3' and antisense primer 5'-CTCGTCAATCATGCCCGAGATGAGC-3'.

Results

Identification of mutations

The IDS exons from the 10 MPS II patients were amplified and screened for mutations. Small deletion 231del6 was readily detected on electrophoresis of amplified exon 2 products. Point changes A85T, R88C, H138R, and G374sp were identified by sequencing of fragments showing mobility shifts of single strands. Other changes, including IVS5+2T>C, W267C, W267X, 1184delG, and E521X were detected by sequencing all exon-containing fragments. As summarized in **Table 3**, a total of 8 different point changes and 2 small deletions were detected. These included 5 novel and 5 previously reported (Stenson et al., 2003) mutations. The novel H138R and W267C were not found on screening 100 control chromosomes. Also shown in **Table 3** are the mutations found previously in Taiwanese MPS II patients (Lin et al. 2000).

IDS activity measurement

Leukocyte IDS activity from 50 unrelated Taiwanese individuals were determined. As shown in **Figure 10**, a wide range of IDS activity (19.2 ~ 70.6 nmol/h/mg cell protein) with an average of 43.2 (SD, 12.6) nmol/h/mg cell protein was observed. The leukocyte IDS activity of confirmed 10 MPS II carriers ranged from 8.4 to 26.6 nmol/h/mg cell protein (mean ± SD = 17.5 ± 5.7), significantly different from that of the normal controls ($P = 2.709 \times 10^{-11}$). For the 14 MPS II patients, 0.2 to 2.5 nmol/h/mg cell protein (mean ± 1SD = 0.9 ± 0.6) of IDS activity were observed.

Haplotype analysis on the mutant allele

Genotypes at two flanking dinucleotide repeat markers were examined to establish haplotypes associated with Taiwanese MPS II mutations. As shown in **Table 6**, a total of 10 DXS1113 alleles (153nt ~ 173nt) were identified, with 167nt allele occurring in the

majority of the normal chromosomes (54.1%). For DXS1123, a total of 7 alleles (167nt ~ 181nt) were identified, with 175nt (34.5%) and 177nt (32.4%) alleles occurring in the majority of the normal chromosomes. There is no frequency difference for these common alleles between MPS II and normal chromosomes ($P > 0.05$). Complete haplotypes were constructed and frequencies were compared between normal and MPS II chromosomes (**Table 7**). Twelve different haplotypes were observed for the identified 24 mutations. Haplotypes 7-d is the common haplotype, each accounting for 20.8% of MPS II chromosomes. The three unrelated R468W alleles were associated with haplotype 7-b (12.5%), which seen on only 2.9% of normal chromosomes ($P = 0.004$).

The family studies of MPS II patients 710, 1150, and 814 are shown in **Figure 11**. The I485R mutation in patient 710 was associated with DXS1113 169nt / DXS1123 171nt (**Table 7**, haplotytpe 8). The mutation occurred *de novo* in male meioses since maternal grandfather did not carry the mutation. The R468Q allele in patient 1150 was associated with DXS1113 167nt / DXS1123 175nt (haplotype 7-c). A *de novo* mutation in male meioses was also suggested since the same maternal grandfather allele in maternal aunt did not carry the mutation. The 1055del12 allele in patient 814 was associated with DXS1113 157nt / DXS1123 175nt (haplotype 3). The parental origin of the X chromosome bearing this mutation was not clear in this family.

Expression of IDS cDNA mutants

The effect of identified 11 missense mutations, 2 nonsense mutations, and 4 small deletions in Taiwanese MPS II patients was investigated by transient expression in COS-7 cells. As shown in **Table 8**, untransfected COS-7 cells showed an IDS activity of 12.2 nmole/h/mg of protein; COS-7 cells transfected with wild-type IDS cDNA had an activity of 530.2 nmole/h/mg, resulting in a 43 fold increase. However, cells expressing mutant cDNA exhibited trace amounts of IDS activity, with 2.8% and 3.9% residual activity, respectively, for mild type A85T and W267C mutations, 2.2% for intermediate type P228L mutation, and 0.0% to 0.9% for other severe type mutations. The level of IDS

mRNA that derived from each mutant was examined by RT-PCR. Results are reported in **Figure 12A**. While other mutations caused a change in steady-state IDS mRNA level from 78% to 115% of wild type, 231del6 mutation reduced the level of IDS mRNA to 43% of wild type. The presence of IDS polypeptides in transfected COS-7 cell extracts was determined by immunoblot analysis, using anti-IDS serum. As shown in **Figure 12B**, cells transfected with the parental pcDNA3 vector did not produce cross-reacting protein. Cells expressing the normal IDS cDNA had major bands of 78 to 75 kDa precursor forms as well as 55 and 48 to 46 kDa mature forms of the enzyme. With the exception of K347E, the missense mutations examined showed a similar or slightly lower molecular mass of precursor with little or reduced mature forms were detected. In cells transfected with W267X, 1055del12, 1184delG, and E521X, the smaller 37, 72, 54, and 65 kDa IDS proteins were detected. Conversely, no IDS protein was seen with 231del6 and 1421delAG.

Fluorescence micrographs of cells expressing the wild type and the 7 mutant IDS proteins are presented in **Figure 13**. The representative images of endoplasmic reticulum are shown in green (**Figure 13A**), the lysosome-specific dye in red (**Figure 13B**), and the IDS-specific dye in blue (**Figure 13C**). When the overlapped images of endoplasmic reticulum and IDS (**Figure 13D**) as well as lysosome and IDS (**Figure 13E**) were compared, the wild-type IDS protein was mostly localized in the lysosome. The intracellular localization of A85T, K347E, 1055del12, and E521X was similar to that of wild-type IDS protein; whereas W267X and 1184delG produce proteins that seem to be localized in ER. The 231del6 mutant did not show IDS protein in any compartment. For the other 9 point mutations examined, lysosomal staining patterns were also observed (data not shown).

Expression and uptake of PEIa-IDS and IDS-PEIa recombinant proteins

The expression of recombinant PEIa-IDS and IDS-PEIa proteins was examined using COS-7 cells. As shown in **Table 9**, cell lysates and supernatant fluids from cells

transfected with pcDNA3 vector showed an IDS activity of 7.5 nmole/h/mg protein and 0.5 nmole/h/ml, respectively. Increased IDS activities were detected in both cell lysates (381.6 ~ 405.0 nmole/h/mg protein) and supernatant fluids (24.6 ~ 55.2 nmole/h/ml) upon transient expression of pcDNA3-IDS, pcDNA3-PEIa-IDS, and pcDNA3-IDS-PEIa. After subtracted the endogenous IDS activity in COS-7 cells, the IDS activities of cell lysates and supernatant fluids from pcDNA3-IDS transfected cells were 374.1 nmole/h/mg protein (a 50 fold increase) and 24.1 nmol/hr/ml (a 48 fold increase), respectively. Upon transfecting the modified pcDNA3-PEIa-IDS or pcDNA3-IDS-PEIa into COS-7 cells, a slightly increase of IDS activity compared with the wild-type (53 and 52 fold vs. 50 fold), was detected in cell lysates. However, a significant increase of IDS activity (109 and 106 fold vs. 48 fold) was detected in supernatant fluids. The expressed IDS polypeptides in cell lysates and supernatant fluids were further examined by immunoblot analysis. As shown in **Figure 14**, cell lysates from cells transfecting with the IDS cDNA had 78 kDa precursor forms as well as 55 and 48 ~ 46 kDa mature forms of the enzyme, whereas the supernatant fluids collected displayed a 90 kDa secreted precursor form. The cell lysates from pcDNA3-PEIa-IDS and pcDNA3-IDS-PEIa transfected cells displayed 110 kDa precursor forms as well as 55 and 48 ~ 46 kDa mature forms of the enzyme, whereas the supernatant fluids collected displayed a 120 kDa secreted precursor form.

The uptake of secreted IDS, PEIa-IDS and IDS-PEIa proteins is displayed in **Table 10 and Figure 15**. The cell lysates prepared from cells feeding with the supernatant fluids containing IDS, PEIa-IDS and IDS-PEIa showed a 17, 23, and 21 fold increase of endogenous IDS activity (**Table 10**, 135.6, 186.6 and 166.2 vs. 7.7 nmole/h/mg protein). Immunoblot analysis revealed 55 and 48 ~ 46 kDa mature forms of the enzyme (**Figure 15**). Upon addition of 5 mM M-6-P, the uptake of IDS was completely blocked (**Table 10**, 6.7 vs. 7.3 nmole/h/mg protein; **Figure 15**, lane IDS+M-6-P). However, the uptake of PEIa-IDS and IDS-PEIa recombinant proteins were also blocked (**Table 10**, 7.2 and 7.0 vs. 7.3 nmole/h/mg protein; **Figure 15**, lanes PEIa-IDS+M-6-P and IDS-PEIa+M-6-P). Since LRP is crucial for PEIa-mediated uptake, the expression of LRP in COS-7 cells was further confirmed by RT-PCR (data not shown).

Discussion

IDS activity in MPS II patients and female carriers

Various IDS gene mutations cause a deficiency of IDS enzyme activity and result in blockade of GAG degradation. The clinical phenotype is related to the residual IDS enzyme activity, the age of disease onset, and the degree of severity. The IDS enzyme assay in 14 patients with confirmed MPS II, the residual enzyme activity in leukocytes was less than 5% of mean normal activity, a result very similar to previously reported data (Voznyi et al., 2001). In female carriers, inactivation or a defect of the IDS gene on one X-chromosome should result in IDS enzyme activity lower than normal but still adequate for effective catabolism of GAG. In our investigation, the mean leukocyte IDS activity in female carriers was less than a half of the normal level (17.5 vs. 43.2 nmol/h/mg cell protein). However, there was an overlap in the ranges of normal and carriers, so the level of enzyme activity can not be used alone for carrier detection.

Mutation identification and characterization

In the present study, mutations in Taiwanese MPS II patients were identified and *in vitro* expression experiments were carried out to verify if the mutations are indeed the pathological lesion causing disease. Four complementary experiments were used to characterize the mutations: IDS activity measurement to verify the enzymatic defect (**Table 8**), RT-PCR study to examine the IDS mRNA stability (**Figure 12A**), immunoblot analysis to visualize the IDS protein maturation (**Figure 12B**), and confocal microscopy examination to reveal the IDS enzyme targeting (**Figure 13**). Upon expressing in COS-7 cells, the precursor and mature forms of wild-type IDS enzyme resemble the previously reported results of IDS processing in transfected COS cells (Millat et al. 1997, 1998; Cudry et al. 2000). The expressed mature enzyme was targeted to lysosomes and resulted in a 43 fold increase of IDS activity. However, the mutant enzymes carrying missense mutations exhibited only trace amounts of IDS activity. Among these, A85T and W267C

mutations showed 2.8% and 3.9% residual activity, respectively. Immunoblot and intracellular localization experiments suggested an apparently normal maturation but only partial targeting of polypeptides to lysosomes, since the amounts of mature IDS produced were reduced on immunoblot. The two substitutions may result in incorrect folding to affect the normal targeting. Patients carrying A85T and W267C mutations displayed a mild phenotype. The P228L substitution retained 2.2% of the normal activity in transfected COS-7 cells. Little mature forms were observed on immunoblot. The trace amount of mutant enzyme might reach the lysosomes to explain the low residual IDS activity. An intermediate phenotype was observed in the patient carrying P228L mutation. The R88C and H138R substitutions showed 0.5% and 0.2% residual IDS activity, respectively. An apparently normal maturation but incorrect targeting was suggested by immunoblot and intracellular localization experiments. The Arg_{88} and His_{138} represent critical residues for the catalytic activity of sulfate ester hydrolysis (Daniele et al. 1993). Patients with R88C and H138R mutations displayed a severe phenotype. The K347E substitution showed 0.9% residual IDS activity. Although apparent normal maturation and targeting, the substitution probably perturbs the molecular interactions and the structural architecture of the active site, hence a severe phenotype was associated with the patients carrying this mutation. For N63K, R468Q, R468W, I485R, and G489D mutations examined, little mature forms was observed on immunoblot. The retention of 0.0% to 0.6% IDS activity was in agreement with the severe phenotype reported in these patients. The drastic effect of R468W and R468Q mutations on IDS processing and/or catalytic activity has been reported previously (Crotty et al. 1992; Sukegawa et al. 1995; Villani et al. 2000). All the 11 missense mutations examined predicted the replacement of evolutionarily conserved amino acids. In comparison to N63K, A85T, R88C, H138R, P228L, K347E, R468Q, R468W, I485R, and G489D, the related N63D, A85P, A85S, R88G, R88H, R88L, R88P, H138D, P228T, K347I, K347Q, K347T, R468G, R468L, I485K, and G489A mutations seen in other patient groups (Stenson et al. 2003) indicate that the loss of Asn_{63}, Ala_{85}, Arg_{88}, His_{138}, Pro_{228}, Lys_{347}, Arg_{468}, Ile_{485}, or Gly_{489} causes disease.

The 4 small deletions and 2 nonsense mutations were also investigated. The novel

231del6 and 1055del12 predict loss of two (F78 and A79) and four (V353 to H356) amino acids, respectively. The novel frameshift mutation 1184delG predicts addition of 44 unrelated amino acids at codon 395 followed by termination, whereas the recurrent 1421delAG predicts addition of 17 unrelated amino acids at codon 474 followed by termination. The novel R267X and the recurrent E521X predict lack of 284 and 30 amino acids, respectively, at the carboxyl terminus of the protein. These six mutations can be assumed to cause disease. In transfected COS-7 cells, all six mutations resulted in no appreciable IDS activity (0.0% of normal activity), correlating with the severe clinical manifestations of the patients. A 57% decrease in IDS mRNA level was seen with the 231del6 mutation. A deletion mutation is likely to lead to instability of the mRNA (Moskowitz et al. 1993). In cells transfected with 1055del12 and E521X, incorrect maturation of 1055del12 and E521X were indicated. The expressed truncated W267X and 1184delG proteins retained largely within early vacuolar compartment. For 231del6 and 1421delAG, no IDS protein was seen on the immunoblot; the mutated proteins were apparently unstable and largely degraded.

Two false splicing mutations were found in this study. The recurrent IVS5+2T>G affects the 5' splice site of intron 5 and predicts addition of 12 new amino acids followed by termination. The largely deleted or truncated IDS proteins are likely unstable and probably result in a severe phenotype of the patient with IVS5+2T>G. The G374sp mutation has repeatedly been observed and represents a frequent recurrent splice mutation in patients with MPS II. The mutation creates a preferentially used new donor splice site and results in deletion of 20 amino acids (Bunge et al. 1992; Rathmann et al. 1996). The patient with a G374sp mutation had a mild phenotype, suggesting the presence of a low amount of normally spliced transcript.

Haplotype analysis on the mutant allele

Together, 17 out of these 19 mutations were present in only one patient, indicating

molecular heterogeneity of MPS II among Taiwanese. Among the mutations affecting highly mutable CpG dinucleotides, the A85T mutation was apparently *de novo* since the mother does not carry the mutation (data not shown). The R468Q and R468W mutations were reported "hot spot" for IDS mutations in several other populations (Froissart et al., 1998; Gort et al., 1998; Isogai et al., 1998; Karsten et al., 1998; Filocamo et al., 2001). Together R468Q and R468W account for 25.0% mutations found in our patients. The three unrelated R468Q alleles are associated with two different haplotypes (7-a and 7-c, **Table 7**). Among them, R468Q allele in patient 1150 occurred *de novo* in male meioses (**Figure 11**). Paternal inherited *de novo* I485R mutation was also proven by haplotype analysis (**Figure 11**), adding to the observation of a high incidence of mutations in male meioses (Rathmann et al., 1996; Froissart et al., 1998). These results support the hypothesis that methylation-mediated deamination of 5-methylcytosine in hypermutable CpG dinucleotide is frequently associated with point mutations of various genes (Cooper and Youssoufian, 1988). Although affecting highly mutable CpG dinucleotides, genotyping results show that the three R468W alleles are probably of the same origin, as indicated by association with the same haplotype 7-b, which is present only in 2.9% normal chromosomes (**Table 7**).

Secretion and uptake of PEIa-IDS and IDS-PEIa

Recombinant PEIa-IDS and IDS-PEIa were generated in an attempt to improve the uptake of the recombinant enzyme for enzyme replacement therapy. Compared to the 90 kDa wild-type precursor form, the 120 kDa modified PEIa-IDS and IDS-PEIa precursor forms were secreted into the supernatant fluids. The modified proteins showed increased secretion profiles (**Table 9** and **Figure 14**), leading to increased uptake compared with the wild-type enzyme (**Table 10** and **Figure 15**). The N-terminal or C-terminal addition of PEIa domain may present a steric hindrance to the optimal interaction between the M-6-P moieties on fully modified proteins and the M-6-P receptor. A similar result was obtained with the tetanus toxin C fragment and β-glucuronidase fusion enzyme (Jiang et al., 2005).

COS-7 uptake and fully blocking with M-6-P (Sun et al., 1999) suggested that uptake of the PEIa and IDS fusion protein took place only via M-6-P receptor pathway. The expected LRP-mediated uptake was not observed. The binding activity of PEIa may be affected by post-translational modification when PEIa domain was synthesized in eukaryotic cells.

Conclusions

In conclusion, 17 mutations in the IDS gene in 24 unrelated Taiwanese patients with MPS II were examined in the present study. The finding of several patients with R468W or R468Q mutations along with similar results in Caucasian patients, indicates that these molecular defects are common and may be hot spots for IDS mutations. The characterization of gene mutations not only delineates their functional consequence on IDS activity and processing but may also enable future studies of genotype-phenotype correlation to estimate prognosis. The fact that the various mutations described here were differentially associated with a mild, intermediate, or severe phenotype may also help to pinpoint amino acids essential for enzyme function.

References

Allured, V. S., Collier, R. J., Carroll, S. F. and McKay, D. B. (1986) Structure of exotoxin A of Pseudomonas aeruginosa at 3.0-Angstrom resolution. *Proc Natl Acad Sci U S A* 83(5):1320-4.

Bergstrom, S. K., Quinn, J. J., Greenstein, R. and Ascensao, J. (1994) Long-term follow-up of a patient transplanted for Hunter's disease type IIB: a case report and literature review. *Bone Marrow Transplant* 14(4):653-8.

Bielicki, J., Freeman, C., Clements, P. R. and Hopwood, J. J. (1990) Human liver iduronate-2-sulphatase. Purification, characterization and catalytic properties. *Biochem J* 271(1):75-86.

Bondeson, M. L., Malmgren, H., Dahl, N., Carlberg, B. M. and Pettersson, U. (1995) Presence of an IDS-related locus (IDS2) in Xq28 complicates the mutational analysis of Hunter syndrome. *Eur J Hum Genet* 3(4):219-27.

Bonuccelli, G., Di Natale, P., Corsolini, F., Villani, G. and Filocamo, M. (2001) The effect of four mutations on the expression of iduronate-2-sulfatase in Mucopolysaccharidosis type II. *Biochim Biophys Acta* 1537(3):233-8.

Braun, S. E., Aronovich, E. L., Anderson, R. A., Crotty, P. L., McIvor, R. S. and Whitley, C. B. (1993) Metabolic correction and cross-correction of mucopolysaccharidosis type II (Hunter syndrome) by retroviral-mediated gene transfer and expression of human iduronate-2-sulfatase. *Proc Natl Acad Sci U S A* 90(24):11830-4.

Braun, S. E., Pan, D., Aronovich, E. L., Jonsson, J. J., McIvor, R. S. and Whitley, C. B. (1996) Preclinical studies of lymphocyte gene therapy for mild Hunter syndrome (mucopolysaccharidosis type II). *Hum Gene Ther* 7(3):283-90.

Bu, G., Sun, Y., Schwartz, A. L. and Holtzman, D. M. (1998) Nerve growth factor induces rapid increases in functional cell surface low density lipoprotein receptor-related protein. *J Biol Chem* 273(21):13359-65.

Bunge, S., Steglich, C., Beck, M., Rosenkranz, W., Schwinger, E., Hopwood, J. J. and Gal, A. (1992) Mutation analysis of the iduronate-2-sulfatase gene in patients with mucopolysaccharidosis type II (Hunter syndrome). *Hum Mol Genet* 1(5):335-9.

Coppa, G. V., Gabrielli, O., Zampini, L., Jetzequel, A. M., Miniero, R., Busca, A., De Luca, T. and Di Natale, P. (1995) Bone marrow transplantation in Hunter syndrome. *J Inherit Metab Dis* 18(1):91-2.

Cooper, D. N. and Youssoufian, H. (1988) The CpG dinucleotide and human genetic disease. *Hum Genet* 78(2):151-5.

Crotty, P. L., Braun, S. E., Anderson, R. A. and Whitley, C. B. (1992) Mutation R468W of the iduronate-2-sulfatase gene in mild Hunter syndrome (mucopolysaccharidosis type II) confirmed by in vitro mutagenesis and expression. *Hum Mol Genet* 1(9):755-7.

Cudry, S., Froissart, R., Bouton, O., Maire, I. and Bozon, D. (1999) The 2.1-, 5.4- and 5.7-kb transcripts of the IDS gene are generated by different polyadenylation signals. *Biochim Biophys Acta* 1447(1):35-42.

Cudry, S., Tigaud, I., Froissart, R., Bonnet, V., Maire, I. and Bozon, D. (2000) MPS II in females: molecular basis of two different cases. *J Med Genet* 37(10):E29.

Daniele, A., Faust, C. J., Herman, G. E., Di Natale, P. and Ballabio, A. (1993) Cloning and characterization of the cDNA for the murine iduronate sulfatase gene. *Genomics* 16(3):755-7.

Descamps, O., Bilheimer, D. and Herz, J. (1993) Insulin stimulates receptor-mediated uptake of apoE-enriched lipoproteins and activated alpha 2-macroglobulin in adipocytes. *J Biol Chem* 268(2):974-81.

Di Natale, P. and Daniele, A. (1985) Iduronate sulfatase from human placenta. *Biochim Biophys Acta* 839(3):258-61.

Di Natale, P., Di Domenico, C., Villani, G. R., Lombardo, A., Follenzi, A. and Naldini, L. (2002) In vitro gene therapy of mucopolysaccharidosis type I by lentiviral vectors. *Eur J Biochem* 269(11):2764-71.

Filocamo, M., Bonuccelli, G., Corsolini, F., Mazzotti, R., Cusano, R. and Gatti, R. (2001) Molecular analysis of 40 Italian patients with mucopolysaccharidosis type II: New mutations in the iduronate-2-sulfatase (IDS) gene. *Hum Mutat* 18(2):164-5.

Fitzgerald, D. J., Fryling, C. M., Zdanovsky, A., Saelinger, C. B., Kounnas, M., Strickland, D. K. and Leppla, S. (1994) Selection of Pseudomonas exotoxin-resistant cells with altered expression of alpha 2MR/LRP. *Ann N Y Acad Sci* 737:138-44.

Froissart, R., Millat, G., Mathieu, M., Bozon, D. and Maire, I. (1995) Processing of iduronate 2-sulphatase in human fibroblasts. *Biochem J* 309:425-30.

Froissart, R., Maire, I., Millat, G., Cudry, S., Birot, A. M., Bonnet, V., Bouton, O. and Bozon, D. (1998) Identification of iduronate sulfatase gene alterations in 70 unrelated Hunter patients. *Clin Genet* 53(5):362-8.

Flomen, R. H., Green, E. P., Green, P. M., Bentley, D. R. and Giannelli, F. (1993) Determination of the organisation of coding sequences within the iduronate sulphate sulphatase (IDS) gene. *Hum Mol Genet* 2(1):5-10.

Gliemann, J. (1998) Receptors of the low density lipoprotein (LDL) receptor family in

man. Multiple functions of the large family members via interaction with complex ligands. *Biol Chem* 379(8-9):951-64.

Gort, L., Chabas, A. and Coll, M. J. (1998) Hunter disease in the Spanish population: molecular analysis in 31 families. *J Inherit Metab Dis* 21(6):655-61.

Harmatz, P., Kramer, W. G., Hopwood, J. J., Simon, J., Butensky, E. and Swiedler, S. J. (2005) Pharmacokinetic profile of recombinant human N-acetylgalactosamine 4-sulphatase enzyme replacement therapy in patients with mucopolysaccharidosis VI (Maroteaux-Lamy syndrome): a phase I/II study. *Acta Paediatr Suppl* 94(447):61-8; discussion 57.

Herz, J., Kowal, R. C., Goldstein, J. L. and Brown, M. S. (1990) Proteolytic processing of the 600 kd low density lipoprotein receptor-related protein (LRP) occurs in a trans-Golgi compartment. *Embo J* 9(6):1769-76.

Hille-Rehfeld, A. (1995) Mannose 6-phosphate receptors in sorting and transport of lysosomal enzymes. *Biochim Biophys Acta* 1241(2):177-94.

Hwang, J., Fitzgerald, D. J., Adhya, S. and Pastan, I. (1987) Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in E. coli. *Cell* 48(1):129-36.

Iglewski, B.H., Liu, P.V., Kabat, D. (1977) Mechanism of action of Pseudomonas aeruginosa exotoxin A: adenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. *Infection and Immunity* 15:138-144.

Isogai, K., Sukegawa, K., Tomatsu, S., Fukao, T., Song, X. Q., Yamada, Y., Fukuda, S., Orii, T. and Kondo, N. (1998) Mutation analysis in the iduronate-2-sulphatase gene in 43 Japanese patients with mucopolysaccharidosis type II (Hunter disease). *J Inherit Metab Dis* 21(1):60-70.

Jiang, K., Watson D.J., and Wolfe, J.H. (2005) A genetic fusion construct between the tetanus toxin C fragment and the lysosomal acid hydrolase b-glucuronidase expresses a bifunctional protein with enhanced secretion and neuronal uptake. *J Neurochem* 93:1334-1344.

Kakkis, E. D., Muenzer, J., Tiller, G. E., Waber, L., Belmont, J., Passage, M., Izykowski, B., Phillips, J., Doroshow, R., Walot, I., Hoft, R. and Neufeld, E. F. (2001) Enzyme-replacement therapy in mucopolysaccharidosis I. *N Engl J Med* 344(3):182-8.

Kaplan, A., Achord, D. T. and Sly, W. S. (1977) Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. *Proc Natl Acad Sci USA* 74(5):2026-30.

Karsten, S., Voskoboeva, E., Tishkanina, S., Pettersson, U., Krasnopolskaja, X. and Bondeson, M. L. (1998) Mutational spectrum of the iduronate-2-sulfatase (IDS) gene in 36 unrelated Russian MPS II patients. *Hum Genet* 103(6):732-5.

Kornfeld, S. (1986) Trafficking of the lysosomal enzyme in normal and disease states. *J Clin Invest* 77(1):1-6.

Kornfeld, S. (1990) Lysosomal enzyme targeting. *Biochem Soc Trans* 18(3):367-74.

Kornfeld, S. (1992) Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu Rev Biochem* 61:307-30.

Kounnas, M. Z., Morris, R. E., Thompson, M. R., FitzGerald, D. J., Strickland, D. K. and Saelinger, C. B. (1992) The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J Biol Chem* 267(18):12420-3.

Lee-Chen, G. J., Lin, S. P., Lin, S. Z., Chuang, C.K., Hsiao, K. T., Huang, C. F. and Lien, W. C. (2002) Identification and characterization of mutations underlying Sanfilippo syndrome type B (mucopolysaccharidosis type IIIB). *J Med Genet* 39:E3.

Li, P., Thompson, J. N., Hug, G., Huffman, P. and Chuck, G. (1996) Biochemical and molecular analysis in a patient with the severe form of Hunter syndrome after bone marrow transplantation. *Am J Med Genet* 64(4):531-5.

Li, P., Bellows, A. and Thompson, J. N. (1999) Molecular basis of iduronate-2-sulphatase gene mutations in patients with mucopolysaccharidosis type II (Hunter syndrome). *J Med Genet* 36(1):21-7.

Lin, S. P., Chuang, C. K., Tsai, P. J., Chang, J. G. and Wraith, J. E. (2000) Molecular basis of mucopolysaccharidosis type II in Taiwan Chinese. *Euro J Hum Genet* Poster A: P-183.

Li, Y., Wood, N., Donnelly, P. and Yellowlees, D. (1998) Cell density and oestrogen both stimulate alpha 2-macroglobulin receptor expression in breast cancer cell T-47D. *Anticancer Res* 18(2A):1197-202.

Lin, X. (2004) Functions of heparan sulfate proteoglycans in cell signaling during development. *Development* 131(24):6009-21.

Lissens, W., Zenati, A. and Liebaers, I. (1984) Polyclonal antibodies against iduronate 2-sulphate sulphatase from human urine. *Biochim Biophys Acta* 801(3):365-71.

Lissens, W., Seneca, S. and Liebaers, I. (1997) Molecular analysis in 23 Hunter disease families. *J Inher Metab Dis* 20(3):453-6.

Liu, P.V. (1966) The roles of various fractions of *Pseudomonas aeruginosa* in its

pathogenesis. III. Identity of the lethal toxins produced in vitro and in vivo. J Infect Dis 116:481-489.

Malmgren, H., Carlberg, B. M., Pettersson, U. and Bondeson, M. L. (1995) Identification of an alternative transcript from the human iduronate-2-sulfatase (IDS) gene. *Genomics* 29(1):291-3.

Malmstrom, A., Roden, L., Feingold, D. S., Jacobsson, I., Backstrom, G. and Lindahl, U. (1980) Biosynthesis of heparin. Partial purification of the uronosyl C-5 epimerase. *J Biol Chem* 255(9):3878-83.

McKinnis, E. J., Sulzbacher, S., Rutledge, J. C., Sanders, J. and Scott, C. R. (1996) Bone marrow transplantation in Hunter syndrome. *J Pediatr* 129(1):145-8.

Meikle, P. J., Fietz, M. J. and Hopwood, J. J. (2004) Diagnosis of lysosomal storage disorders: current techniques and future directions. *Expert Rev Mol Diagn* 4(5):677-91.

Miebach, E. (2005) Enzyme replacement therapy in mucopolysaccharidosis type I. *Acta Paediatr Suppl* 94(447):58-60; discussion 57.

Millat, G., Froissart, R., Maire, I. and Bozon, D. (1997) Characterization of iduronate sulphatase mutants affecting N-glycosylation sites and the cysteine-84 residue. *Biochem J* 326:43-7.

Millat, G., Froissart, R., Maire, I. and Bozon, D. (1997) IDS transfer from overexpressing cells to IDS-deficient cells. *Exp Cell Res* 230(2):362-7.

Millat, G., Froissart, R., Cudry, S., Bonnet, V., Maire, I. and Bozon, D. (1998) COS cell expression studies of P86L, P86R, P480L and P480Q Hunter's disease-causing mutations. *Biochim Biophys Acta* 1406(2):214-8.

Moestrup, S. K., Gliemann, J. and Pallesen, G. (1992) Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res* 269(3):375-82.

Moskowitz, S. M., Tieu, P. T. and Neufeld, E. F. (1993) A deletion/insertion mutation in the IDUA gene in a Libyan Jewish patient with Hurler syndrome (mucopolysaccharidosis IH). *Hum Mutat* 2(1):71-3.

Muenzer, J., Lamsa, J. C., Garcia, A., Dacosta, J., Garcia, J. and Treco, D. A. (2002) Enzyme replacement therapy in mucopolysaccharidosis type II (Hunter syndrome): a preliminary report. *Acta Paediatr Suppl* 91(439):98-9.

Mullen, C. A., Thompson, J. N., Richard, L. A. and Chan, K. W. (2000) Unrelated umbilical cord blood transplantation in infancy for mucopolysaccharidosis type IIB (Hunter syndrome) complicated by autoimmune hemolytic anemia. *Bone Marrow*

Transplant 25(10):1093-7.

Munier-Lehmann, H., Mauxion, F. and Hoflack, B. (1996) Function of the two mannose 6-phosphate receptors in lysosomal enzyme transport. *Biochem Soc Trans* 24(1):133-6.

Neufeld, E. F. (1991) Lysosomal storage diseases. *Annu Rev Biochem* 60:257-80.

Neufeld, E. F. and Muenzer, J. (1995) The mucopolysaccharidoses. In: Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D. eds. The metabolic basis of inherited diseases. 7th ed. New York: McGraw-Hill, 2465-94.

Nakayama, K. and Wakatsuki, S. (2003) The structure and function of GGAs, the traffic controllers at the TGN sorting crossroads. *Cell Struct Funct* 28(5):431-42.

Pan, D., Shankar, R., Stroncek, D. F. and Whitley, C. B. (1999) Combined ultrafiltration-transduction in a hollow-fiber bioreactor facilitates retrovirus-mediated gene transfer into peripheral blood lymphocytes from patients with mucopolysaccharidosis type II. *Hum Gene Ther* 10(17):2799-810.

Paulsson, M. and Heinegard, D. (1984) Noncollagenous cartilage proteins current status of an emerging research field. *Coll Relat Res* 4(3):219-29.

Peters, C. and Krivit, W. (2000) Hematopoietic cell transplantation for mucopolysaccharidosis IIB (Hunter syndrome). *Bone Marrow Transplant* 25(10):1097-9.

Petruschka, L., Zschiesche, M., Bielicki, J., Seidlitz, G., Machill, G., Hopwood, J. J. and Herrmann, F. H. (1994) Mucopolysaccharidosis type II (Hunter syndrome): characterization of the iduronate-2-sulphatase in MPS II skin fibroblasts. *J Inherit Metab Dis* 17(1):89-92.

Poorthuis, B. J., Wevers, R. A., Kleijer, W. J., Groener, J. E., de Jong, J. G., van Weely, S., Niezen-Koning, K. E. and van Diggelen, O. P. (1999) The frequency of lysosomal storage diseases in The Netherlands. *Hum Genet* 105(1-2):151-6.

Rathmann, M., Bunge, S., Steglich, C., Schwinger, E. and Gal, A. (1995) Evidence for an iduronate-sulfatase pseudogene near the functional Hunter syndrome gene in Xq27.3-q28. *Hum Genet* 95(1):34-8.

Rathmann, M., Bunge, S., Beck, M., Kresse, H., Tylki-Szymanska, A. and Gal, A. (1996) Mucopolysaccharidosis type II (Hunter syndrome): mutation "hot spots" in the iduronate-2-sulfatase gene. *Am J Hum Genet* 59(6):1202-9.

Ricci, V., Filocamo, M., Regis, S., Corsolini, F., Stroppiano, M., Duca, M. D. and Gatti, R. (2003) Expression studies of two novel in CIS-mutations identified in an intermediate case of Hunter syndrome. *Am J Med Genet* 120(1):84-7.

Sandy, J. D., Brown, H. L. and Lowther, D. A. (1978) Degradation of proteoglycan in articular cartilage. *Biochim Biophys Acta* 543(4):536-44.

Spiro, R. G. (2004) Role of N-linked polymannose oligosaccharides in targeting glycoproteins for endoplasmic reticulum-associated degradation. *Cell Mol Life Sci* 61(9):1025-41.

Stenson, P. D., Ball, E. V., Mort, M., Phillips, A. D., Shiel, J. A., Thomas, N. S., Abeysinghe, S., Krawczak, M. and Cooper, D. N. (2003) Human Gene Mutation Database (HGMD): 2003 update. *Hum Mutat* 21(6):577-81.

Strickland, D. K. and Ranganathan, S. (2003) Diverse role of LDL receptor-related protein in the clearance of proteases and in signaling. *J Thromb Haemost* 1(7):1663-70.

Stroncek, D. F., Hubel, A., Shankar, R. A., Burger, S. R., Pan, D., McCullough, J. and Whitley, C. B. (1999) Retroviral transduction and expansion of peripheral blood lymphocytes for the treatment of mucopolysaccharidosis type II, Hunter's syndrome. *Transfusion* 39(4):343-50.

Sukegawa, K., Tomatsu, S., Fukao, T., Iwata, H., Song, X. Q., Yamada, Y., Fukuda, S., Isogai, K. and Orii, T. (1995) Mucopolysaccharidosis type II (Hunter disease): identification and characterization of eight point mutations in the iduronate-2-sulfatase gene in Japanese patients. *Hum Mutat* 6(2):136-43.

Sun H., Yang M., Haskins M. et al. (1999) Retrovirus vector-mediated correction and cross-correction of lysosomal alpha-mannosidase deficiency in human and feline fibroblasts. *Hum. Gene Ther*. 10, 1311–1319.

Timms, K. M., Lu, F., Shen, Y., Pierson, C. A., Muzny, D. M., Gu, Y., Nelson, D. L. and Gibbs, R. A. (1995) 130 kb of DNA sequence reveals two new genes and a regional duplication distal to the human iduronate-2-sulfate sulfatase locus. *Genome Res* 5(1):71-8.

Tong, P. Y. and Kornfeld, S. (1989) Ligand interactions of the cation-dependent mannose 6-phosphate receptor. Comparison with the cation-independent mannose 6-phosphate receptor. *J Biol Chem* 264(14):7970-5.

Truppe, W. and Kresse, H. (1978) Uptake of proteoglycans and sulfated glycosaminoglycans by cultured skin fibroblasts. *Eur J Biochem* 85(2):351-6.

Vafiadaki, E., Cooper, A., Heptinstall, L. E., Hatton, C. E., Thornley, M. and Wraith, J. E. (1998) Mutation analysis in 57 unrelated patients with MPS II (Hunter's disease). *Arch Dis Child* 79(3):237-41.

Vellodi, A., Young, E., Cooper, A., Lidchi, V., Winchester, B. and Wraith, J. E. (1999) Long-term follow-up following bone marrow transplantation for Hunter disease. *J Inherit*

Metab Dis 22(5):638-48.

Villani, G. R., Daniele, A., Balzano, N. and Di Natale, P. (2000) Expression of five iduronate-2-sulfatase site-directed mutations. *Biochim Biophys Acta* 1501(2-3):71-80.

Voznyi, Y. V., Keulemans, J. L. and van Diggelen, O. P. (2001) A fluorimetric enzyme assay for the diagnosis of MPS II (Hunter disease). *J Inherit Metab Dis* 24(6):675-80.

Walkley, S. U. (1998) Cellular pathology of lysosomal storage disorders. *Brain Pathol* 8(1):175-93.

Wasteson, A. and Neufeld, E. F. (1982) Iduronate sulfatase from human plasma. *Methods Enzymol* 83:573-8.

Weaver, A. M., McCabe, M., Kim, I., Allietta, M. M. and Gonias, S. L. (1996) Epidermal growth factor and platelet-derived growth factor-BB induce a stable increase in the activity of low density lipoprotein receptor-related protein in vascular smooth muscle cells by altering receptor distribution and recycling. *J Biol Chem* 271(40):24894-900.

Whitley, C. B., McIvor, R. S., Aronovich, E. L., Berry, S. A., Blazar, B. R., Burger, S. R., Kersey, J. H., King, R. A., Faras, A. J., Latchaw, R. E., McCullough, J., Pan, D., Ramsay, N. K. and Stroncek, D. F. (1996) Retroviral-mediated transfer of the iduronate-2-sulfatase gene into lymphocytes for treatment of mild Hunter syndrome (mucopolysaccharidosis type II). *Hum Gene Ther* 7(4):537-49.

Wilkie, A. O., Amberger, J. S. and McKusick, V. A. (1994) A gene map of congenital malformations. *J Med Genet* 31(7):507-17.

Willnow, T. E. and Herz, J. (1994) Genetic deficiency in low density lipoprotein receptor-related protein confers cellular resistance to Pseudomonas exotoxin A. Evidence that this protein is required for uptake and degradation of multiple ligands. *J Cell Sci* 107:719-26.

Wilson, P. J., Meaney, C. A., Hopwood, J. J. and Morris, C. P. (1993) Sequence of the human iduronate 2-sulfatase (IDS) gene. *Genomics* 17(3):773-5.

Wilson, P. J., Morris, C. P., Anson, D. S., Occhiodoro, T., Bielicki, J., Clements, P. R. and Hopwood, J. J. (1990) Hunter syndrome: isolation of an iduronate-2-sulfatase cDNA clone and analysis of patient DNA. *Proc Natl Acad Sci USA* 87(21):8531-5.

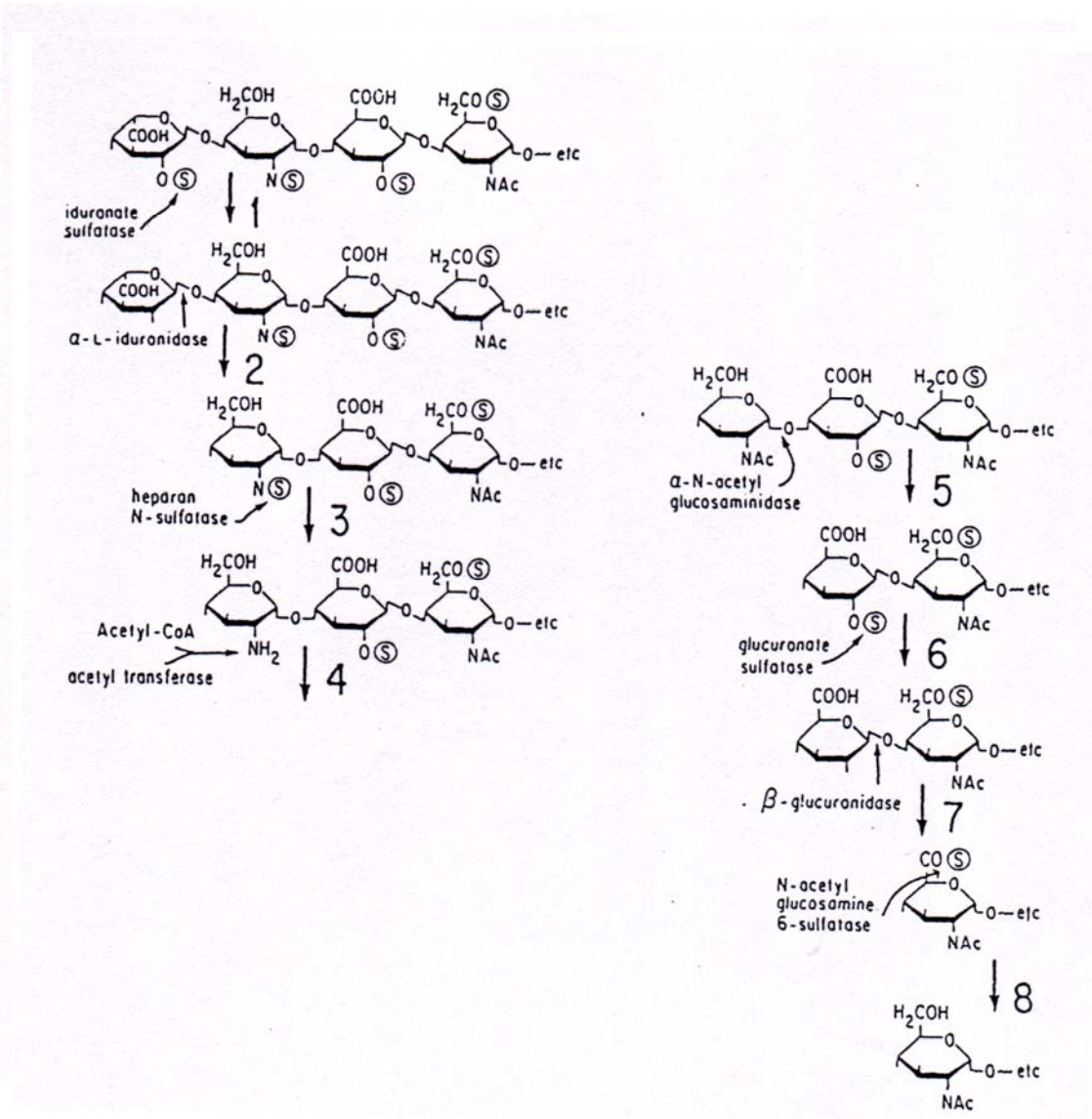


Figure 1. The catabolic process of heparan sulfate. The number indicated the associated MPS type, 1 = MPS II, Hunter syndrome; 2 = MPS I, Hurler, Scheie, and Hurler-Scheie syndrome; 3 = MPS IIIA, Sanfilippo A syndrome; 4 = MPS IIIC, Sanfilippo C syndrome; 5 = MPS IIIB, Sanfilippo B syndrome; 6 = has not been found; 7 = MPS VII, Sly syndrome; 8 = MPS IIID, Sanfilippo D syndrome. (Reprint from Neufeld and Muenzer, 1995)

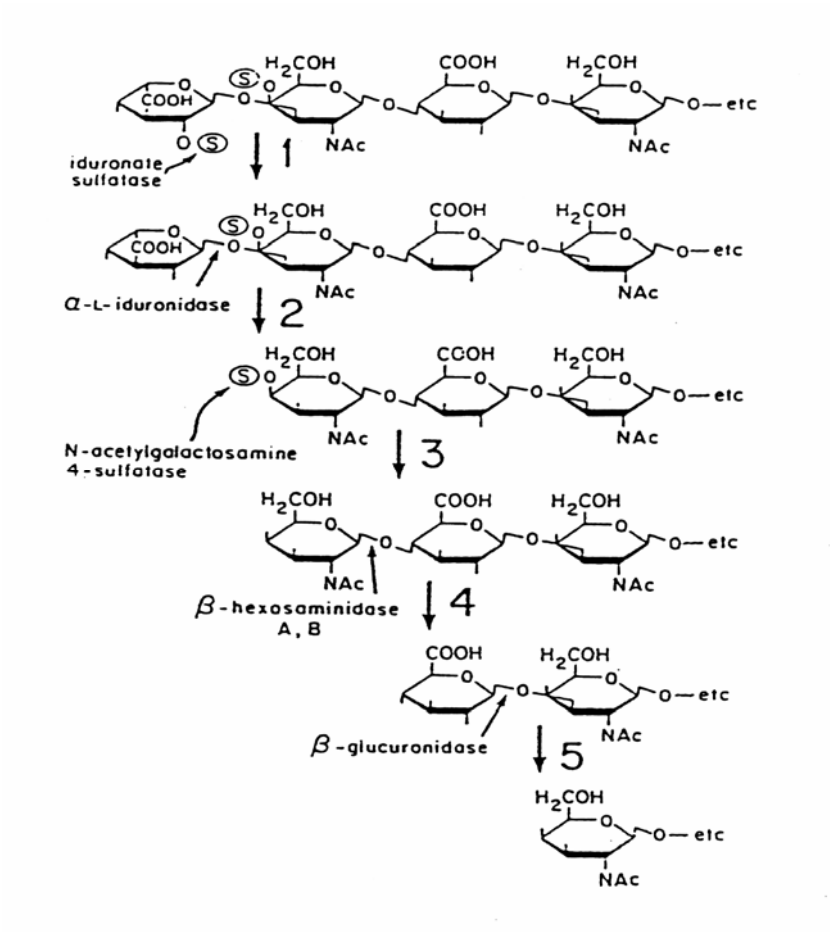


Figure 2. The catabolic process of dermatan sulfate. The number indicated the associated MPS type, 1 = MPS II, Hunter syndrome; 2 = MPS I, Hurler, Scheie and Hurler/Scheie syndrome; 3 = MPS VI, Maroteaux-Lamy syndrome; 4 = Sandhoff disease; 5 = MPS VII, Sly syndrome. (Reprint from Neufeld and Muenzer, 1995)

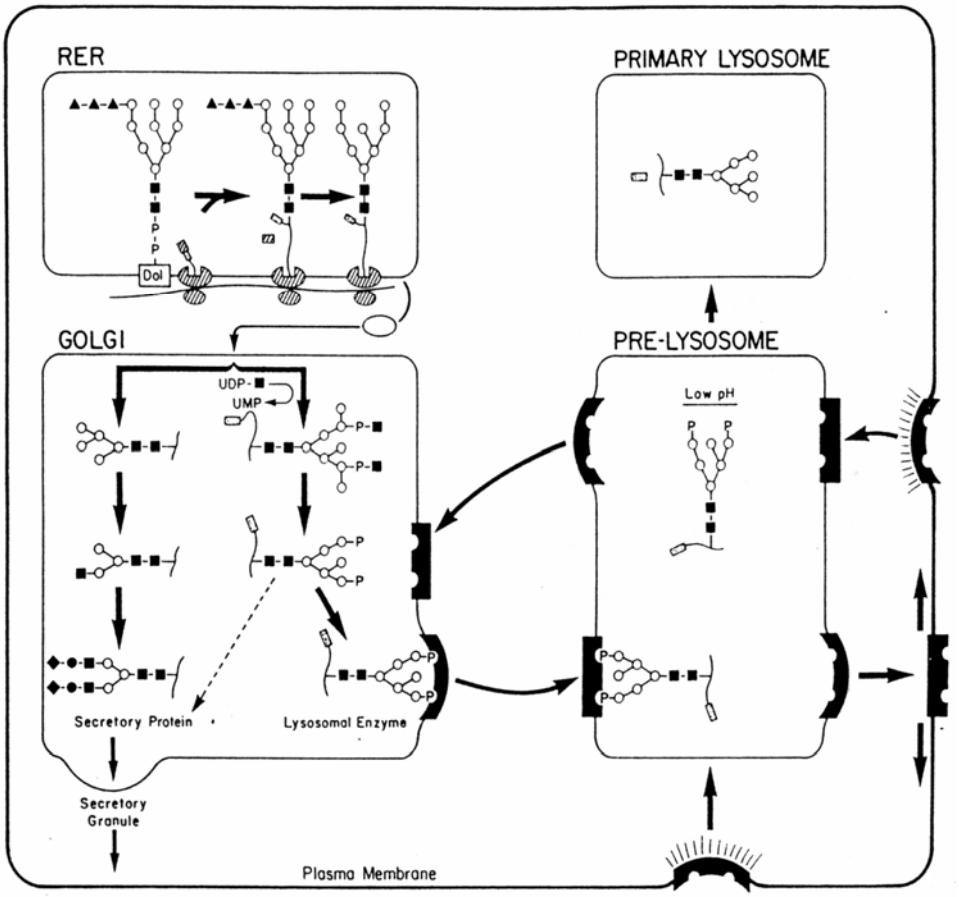


Figure 3. Transport of acid hydrolases to lysosomes. The cleavage of signal peptide and co-translational addition of large preformed oligosaccharide side chains onto certain asparagine residues occur in the lumen of the RER. Then the enzymes are transported to the Golgi system where acquisition of M-6-P occurs. The resulting enzymes are targeted to lysosomes by MPR. A second pathway is receptor-mediated endocytosis, by which enzymes are taken up from the extracellular medium by the MPR on the plasma membrane and transported to lysosomes. (Reprint from Kornfeld, 1986)

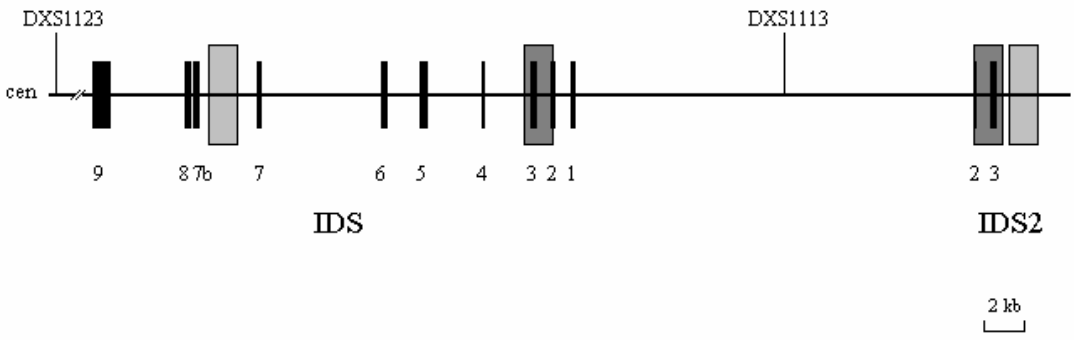


Figure 4. Structure of the IDS gene and the IDS2 locus. Exons are illustrated by small black boxes and the homologous regions by large gray boxes. Above the gene the location of the dinucleotide repeat polymorphisms DXS1123 and DXS1113 are shown.

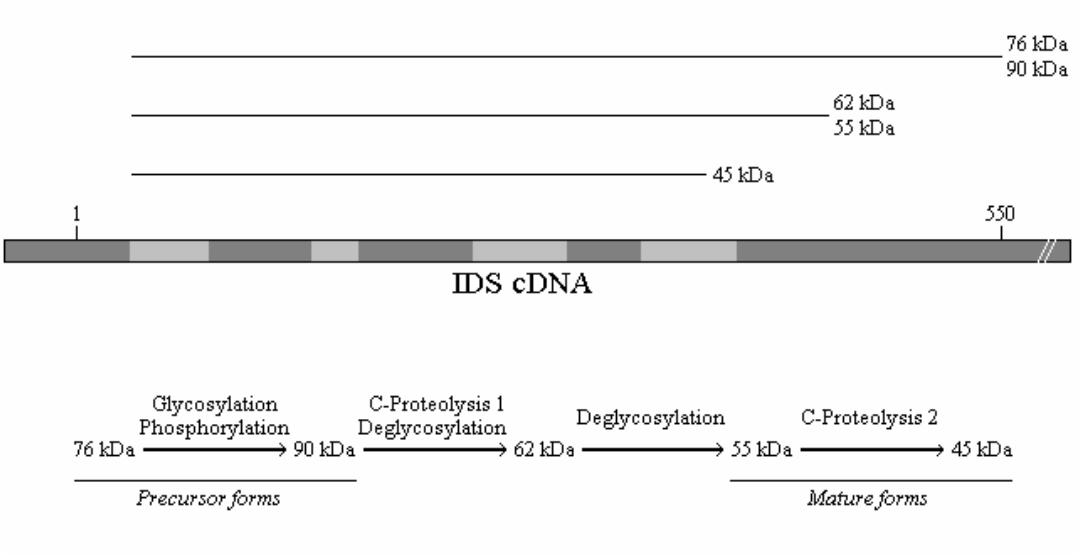


Figure 5. IDS processing. IDS coding sequence is represented by gray box. Amino acid 1 and 550 represent the amino and carboxyl termini, respectively. The 76 kDa and 90 kDa precursors (amino acids 34-550) is converted, through a 62 kDa intermediate (amino acids 34-455), to 55 kDa (amino acids 34-455) and 45 kDa (amino acids 34-?) mature forms.

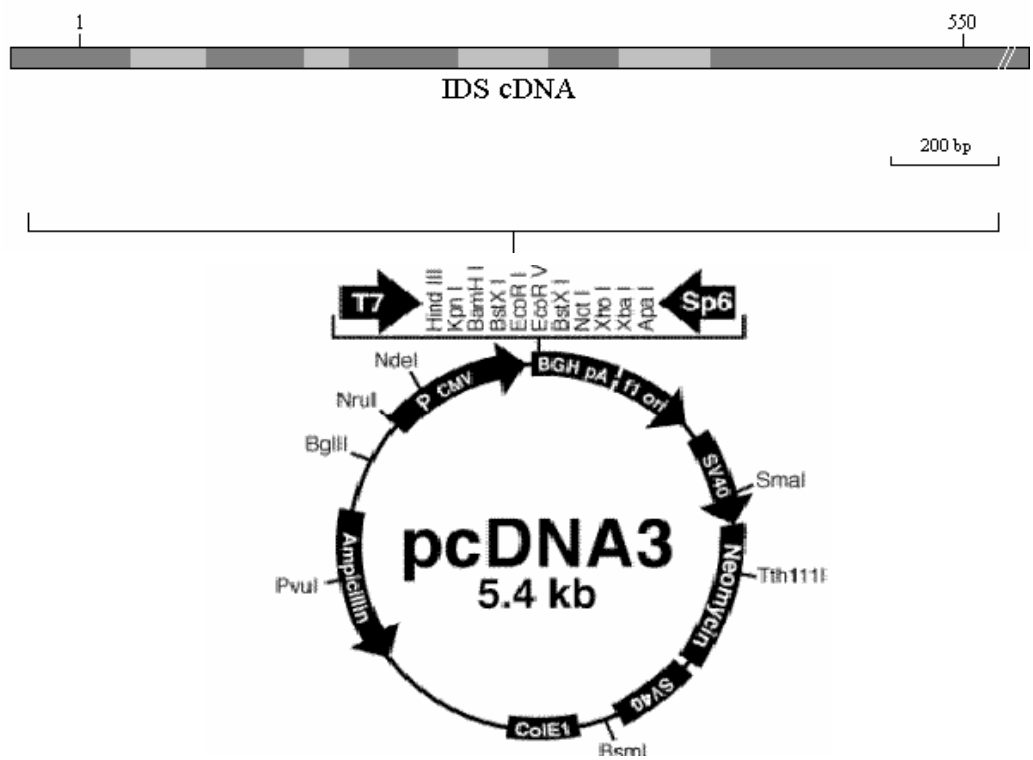
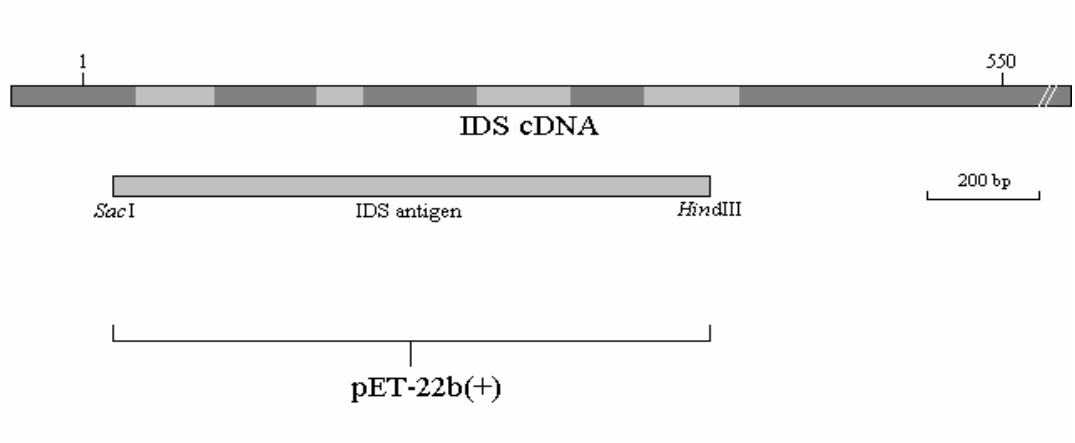


Figure 6. pcDNA3-IDS. IDS coding sequence is represented by gray box. Amino acid 1 and 550 represent the amino and carboxyl termini, respectively. The full-length cDNA is cloned into the *Eco*RI site of eukaryotic expressing vector pcDNA3. The vector utilizes the strong cytomegalovirus (CMV) enhancer-promoter for high level expression, bovine growth hormone (BGH) polyadenylation signal and transcription termination sequence to enhance mRNA stability, and SV40 origin for episomal replication in transfected COS-7 cells expressing large T antigen. The neomycin selectable marker can be used to select stablely transfected cells.



*Bgl*II T7 promoter lac operator

AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCC

*Xba*I rbs *Nde*I

CCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**AAATACCTGCTGCCGACC

MetLysTyrLeuLeuProThr

*Msc*I

GCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGATATCGGAATTAATTCG

AlaAlaAlaGlyLeuLeuLeuLeuAlaAlaGlnProAlaMetAlaMetAspIleGlyIleAsnSer

*Bam*HI *Eco*RI ***Sac*I** *Sal*I ***Hin*dIII** *Eag*I *Xho*I His-Tag

GATCCGAATTC**GAGCTC**CGTCGAC**AAGCTT**GCGGCCGCACTCGAG**CACCACCACCACCACCAC**TGA

AspProAsnSerSerSerValAspLysLeuAlaAlaAlaLeuGlu**HisHisHisHisHisHis**End

GATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCT

Figure 7. Strategy to generate IDS antigen. IDS coding sequence is represented by gray box. Amino acid 1 and 550 represent the amino and carboxyl termini, respectively. The cDNA the *Sac*I-*Hind*III fragment for IDS antigen production is shown. The MCS of pET-22b(+) cloning/expression region is shown in the bottom panel. The cloned *Sac*I and *Hin*dIII restriction sites are indicated by bold symbols.

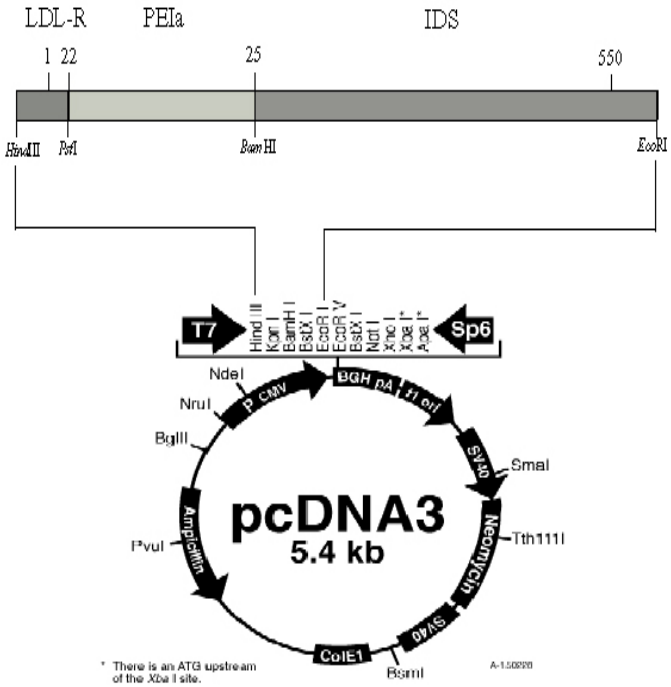


Figure 8. pcDNA3-PEIa-IDS. The PEIa fragment (light-gray box) was made by PCR to contain flanking *Pst*I and *Bam*HI restriction sites. The N-terminal signal peptide from LDL receptor gene (residues 1-22, 5' deep-gray box), the PEIa fragment, and IDS cDNA fragment (residues 25-550, 3' deep-gray box) are cloned into the *Hin*dIII and *Eco*RI sites of pcDNA3.

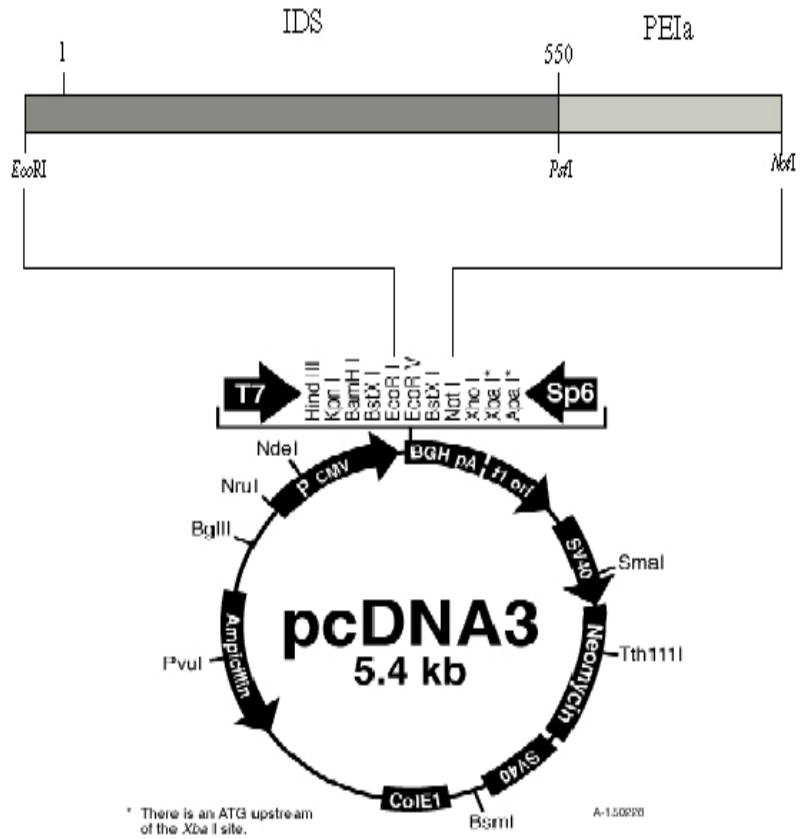


Figure 9. pcDNA3-IDS-PEIa. IDS coding sequence is represented by deep-gray box. Amino acids 1 and 550 represent the amino and carboxyl termini, respectively. The termination codon of IDS cDNA was removed and *Pst*I restriction site added by PCR. A termination codon was added to the 3' end of PEIa fragment (light-gray box) by PCR. The modified IDS cDNA and PEIa fragments are cloned into the *Eco*RI and *Not*I sites of pcDNA3.

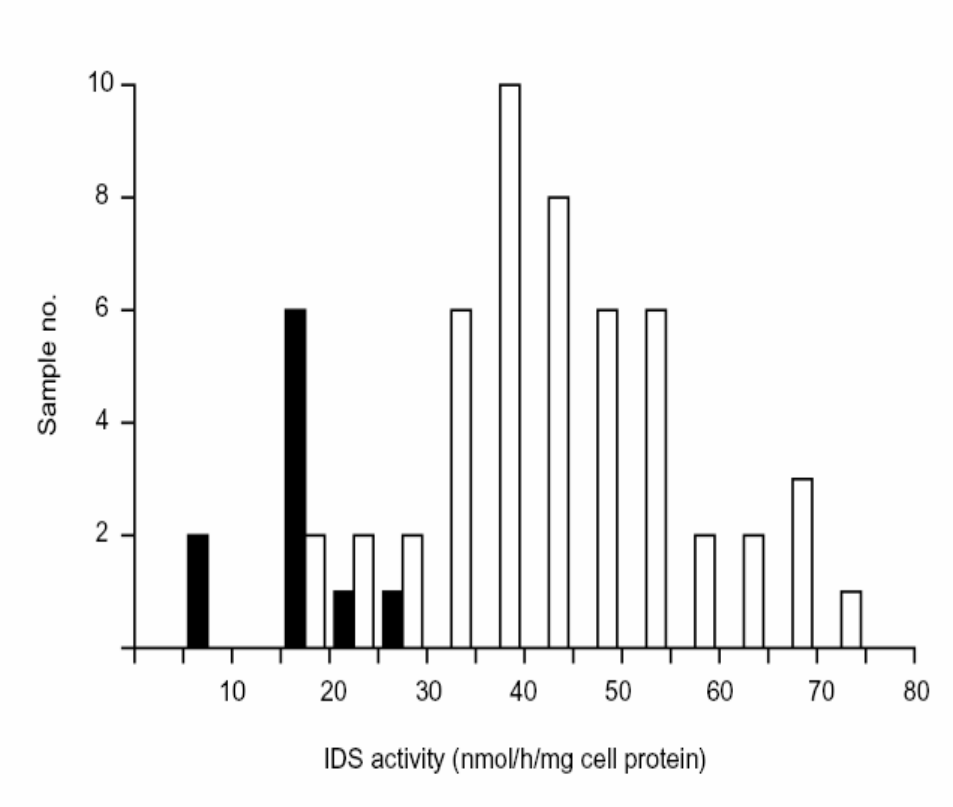


Figure 10. Distribution of leukocyte IDS activity from 50 unrelated Taiwanese individuals without MPS II (open bar) and 10 confirmed MPS II carriers (black bar).

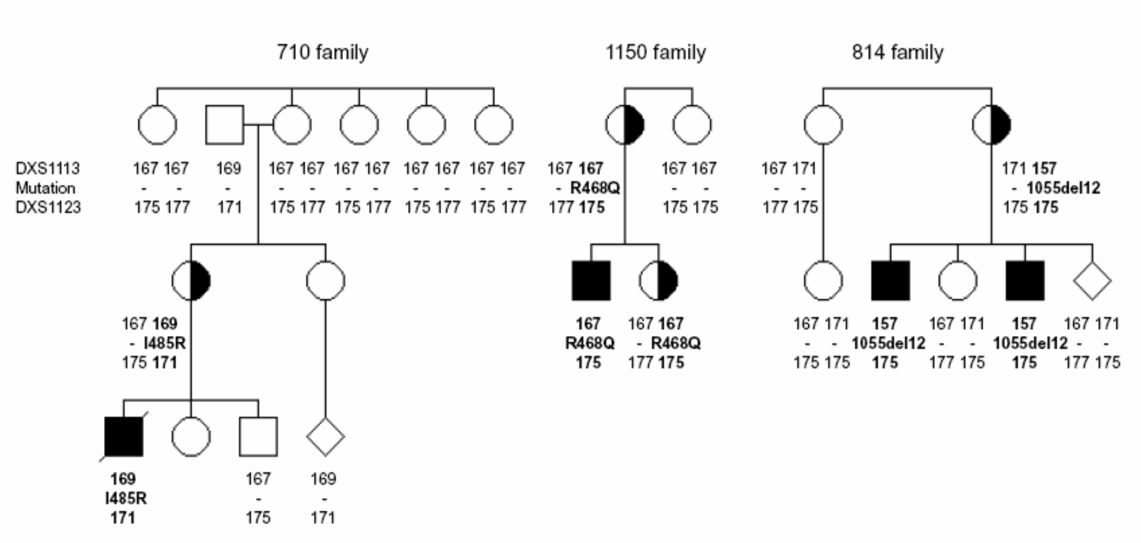


Figure 11. Family pedigrees and haplotype analysis of MPS II patients 710, 1150, and 814. Unaffected individuals are indicated with open symbols; those affected are shown with filled symbols. The half-filled symbols denote heterozygote carriers. The mutation and allele for microsatellite markers DXS1113 and DXS1123 (in nt) are shown under each person. Haplotypes cosegregating with the disease phenotype are indicated by bold numbers.

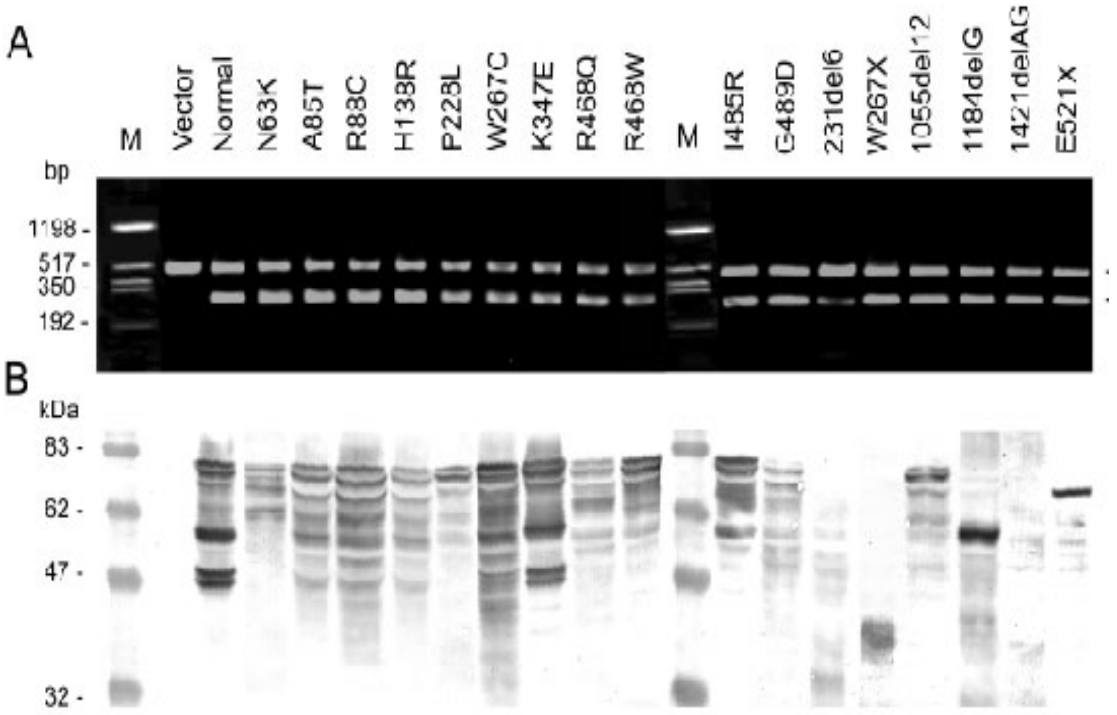


Figure 12. RT-PCR and Western analyses of IDS cDNA mutants. (**A**) The amplified IDS (254 bp) and neomycin (450 bp) fragments were fractionated on 2% agarose gels. (**B**) Total proteins were separated on 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and stained with IDS polyclonal antibody. Weights of the molecular mass markers in lanes M (in bp for *Hin*fI digest of pGEM4 DNA and in kDa for standard proteins from New England BioLabs) are shown on the left.

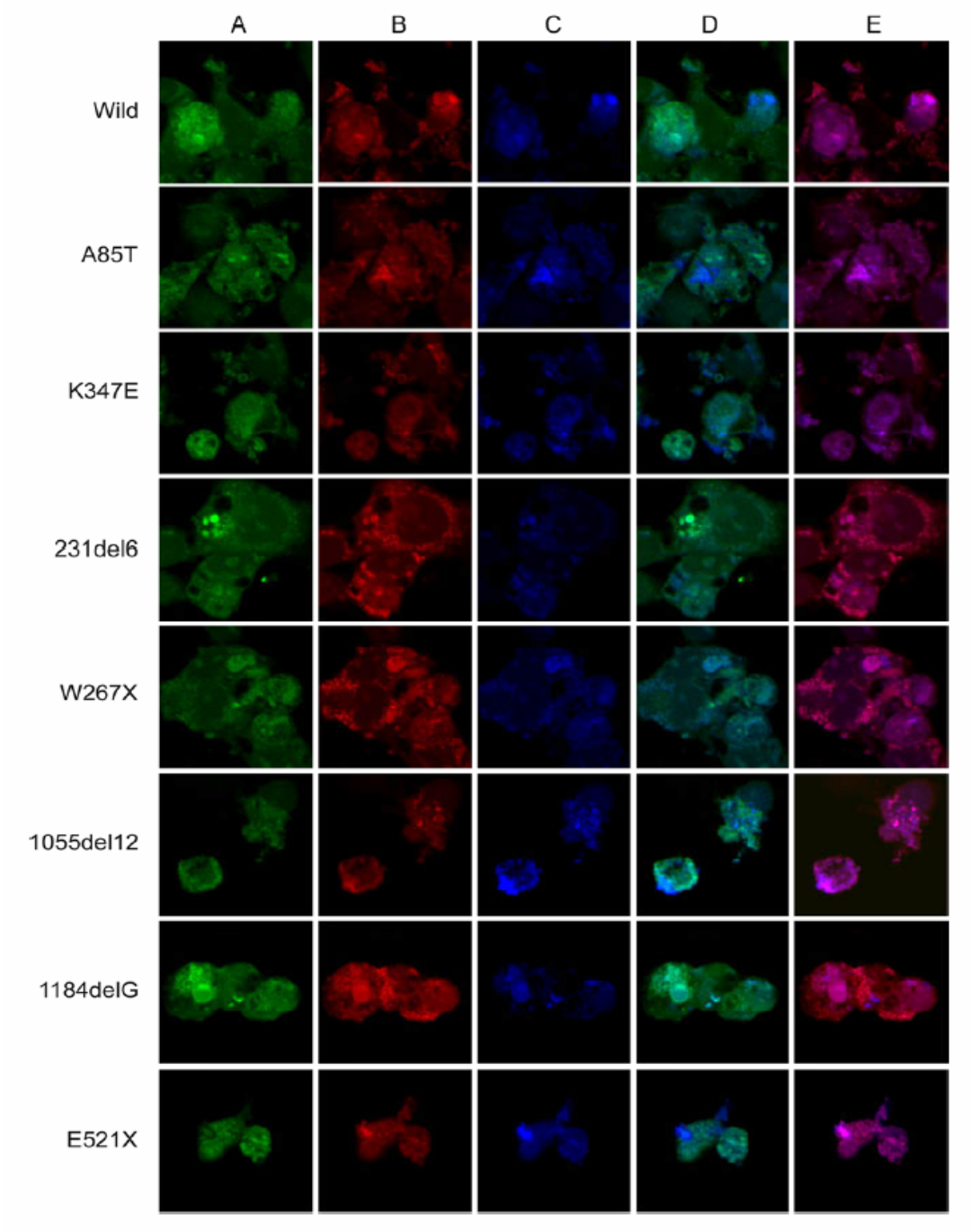


Figure 13. Intracellular localization of IDS in transfected COS-7 cells. Confocal fluorescence microscopy analysis of transfected COS-7 cells triple stained with probes specific for endoplasmic reticulum (green, **A**), lysosome (red, **B**), IDS (blue, **C**), and the combined images of endoplasmic reticulum and IDS staining (**D**) or lysosome and IDS staining (**E**).

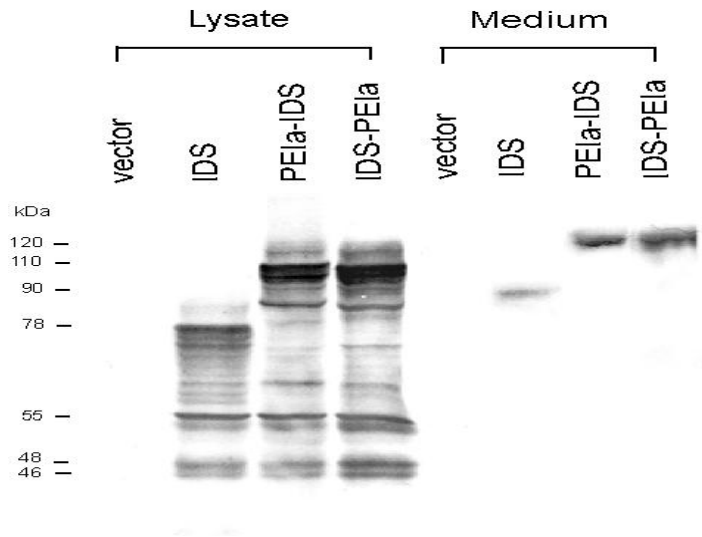


Figure 14. Western analysis of PEIa-IDS and IDS-PEIa recombinant protein expression. Total proteins from pcDNA3 vector, pcDNA3-IDS, pcDNA3-PEIa-IDS and pcDNA3-IDS-PEIa transfected cells (lysate, 10 μg) or supernatant fluids (medium, 10 μl) were separated on 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and stained with IDS polyclonal antibody.

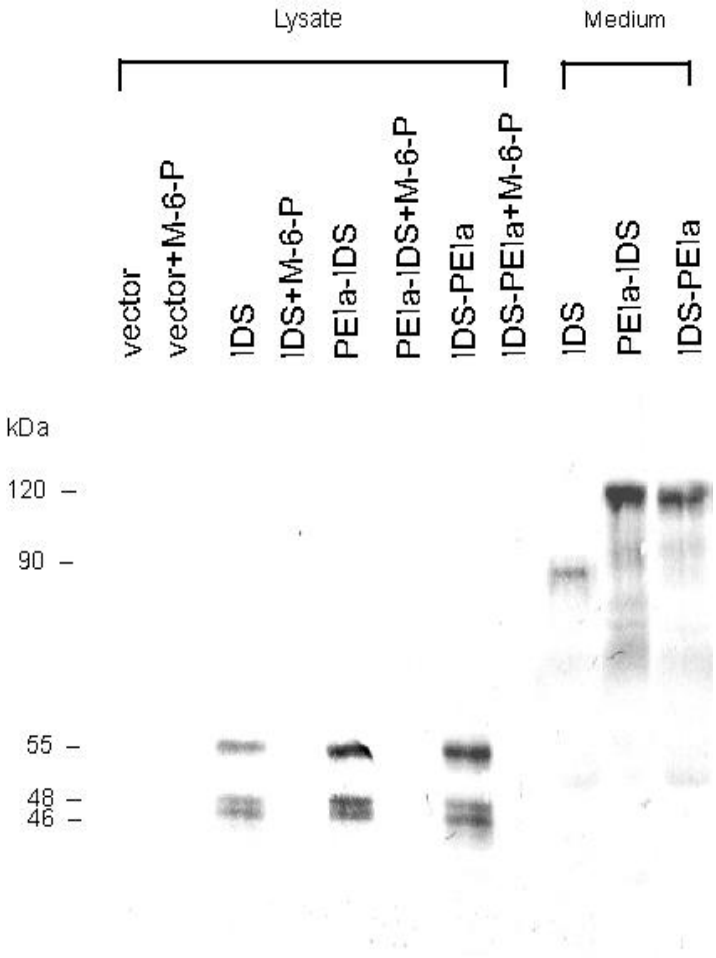


Figure 15. Western analysis of IDS, PEIa-IDS and IDS-PEIa protein uptake. Supernatant fluids from pcDNA3 vector, pcDNA3-IDS, pcDNA3-PEIa-IDS and pcDNA3-IDS-PEIa transfected cells were added to cells in the presence or absence of M-6-P. Forty-eight hours later total proteins from fed cells (lysate, 10 μg) or supernatant fluids (medium, 10 μl) were separated on 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and stained with IDS polyclonal antibody.

Table 1. Classification of the mucopolysaccharidoses (MPS)

MPS type	Syndrome name	Enzyme deficiency	Chromosomal location
I	Hurler	α-L-Iduronidase	4p16.3
II	Hunter	Iduronate-2-sulfatase	Xq28
IIIA	Sanfilippo A	Sulfamidase	17q25.3
IIIB	Sanfilippo B	N-Acetyl-α-D-glucosaminidase	17q21
IIIC	Sanfilippo C	Acetyl CoA:α-glucosaminide N-Acetylglucomine 6-sulfatase	14q
IIID	Sanfilippo D	N-Acetylgalactosamine 6-sulfatase	12q14
IVA	Morquio A	N-Acetylgalactosamine 6-sulfatase	16q24
IVA	Morquio B	β-Galactosidase	3p21
VI	Maroteaux-Lamy	N-Acetylgalactosamine 4-sulfatase	5q12
VII	Sly	β-Glucuronidase	7q22
IX	Natowicz	Hyaluronidase	3p21

Table 2. Primers and conditions for PCR amplification of IDS gene

Exon / cDNA	Primer sequence	Annealing temp. (℃)	$MgCl_2$ (mM)	Product (bp)
1	F: ACGAGGAGGTCTCTGTGGCT R: GGAGGAAGGGAGAAGAGATG	59	1.0	240
2	F: TCAGTGTCAGTGCAGGTTAC R: CTGTGCCATCTGACAATAGC	57	1.0	276
3	F: AGATGGCAGACATGTTTTGC R: GAGAACCCAGACTCTGGACA	60	1.0	266
4	F: GGAGTGGGGTGTTGAAAGAC R: TGGTATATAACCAGCTTCACAG	61	1.5	245
5	F: CTTGCACTTAAAAAGCTG R: TGTCACAGCTGTGCTGGA	61	2.5	285
6	F: TTGTGCTTTTGCTAAAAG R: ACGACACTATGTCATCAG	58	2.5	276
7	F: GGCAAGCATTATCTCTGTATGC R: CACACCCATGTTTATGTCAATG	58	1.5	296
8	F: CAAGCTGTGGTATGATGATT R: AGGTGATCTTACTGTCAAGC	58	1.5	276
9	F: GTAACCCATTCTGCTCTGTC R: GCACATCACATTTGCCATCC	58	1.5	541
cDNA	F: ACGAGGAGGTCTCTGTGGCT R: GCACATCACATTTGCCATCC	55	1.5	1782

Table 3. IDS mutations in Taiwanese MPS II patients

Mutation	Nucleotide alteration	Protein alteration	Phenotype	Restriction analysis	Remarks
Mutations found in this study					
231del6	6 bp del	loss of F78 & A79	severe		Novel
A85T	GCC-ACC	Ala-Thr	mild	+*Bsp*1286I	
R88C	CGC-TGC	Arg-Cys	severe	-*Bst*UI	
H138R	CAC-CGC	His-Arg	severe	-*Hph*I	Novel
IVS5+2T>G	gt-gg	false splicing	severe	-*Hph*I	
		12 altered aa, term			
W267C	TGG-TGT	Trp-Cys	mild	-*Bst*NI	Novel
W267X	TGG-TGA	Trp-Stop	severe	-*Bst*NI	Novel
G374sp	GGC-GGT	false splicing	mild	+*Hph*I	
		loss of 20 aa			
1184delG*	1 bp del	frame shift	severe	+*Fnu*4HI	Novel
		44 altered aa, term			
E521X	GAA-TAA	Glu-Stop	severe	-*Sma*I§	
Mutations found previously (Lin et al. 2000)					
N63K	AAT-AAG	Asn-Lys	severe		
IVS2+1G>C	gt-ct	false splicing	severe		
		del of 105 aa			
P228L	CCA-CTA	Pro-Leu	Intermedi-ate		
K347E	AAA-GAA	Lys-Glu	severe		
1055del12*	12 bp del	loss of V353-H356	severe		

R468Q	CGG-CAG	Arg-Gln	severe
R468W	CGG-TGG	Arg-Trp	severe
1421delAG*	2 bp del	frame shift	severe
		17 altered aa, term	
I485R	ATA-AGA	Ile-Arg	severe
G489D	GGC-GAC	Gly-Asp	severe

*Nucleotide number 1 was the first nucleotide to be translated.

§The *Sma*I sites generated by amplification created restriction site PCR using mismatch primers 5'-TTTCTGACATCCATGC**CC**GG-3'.

Table 4. Primers and conditions for PCR amplification DXS1123 and DXS1113

Amplified region	Primer sequence	Annealing temp. (℃)	Product (bp)
DXS1123	F: fam-TGCCTAAATGTTCGCAAGCCCATTC R: ACAAACAGCTGCCTCCTAGAAACCC	58	168-179 bp
DXS1113	F: hex-GGGAGCTTTAGAGATTTTGGTAAAC R: CCCTGTGGAGGACAGCAGCCTGACT	58	154-167 bp

Table 5. Primers for site-directed mutagenesis

Mutation	Primer sequence
N63K	GCTGGTGAGGTCCCCAAA**G**ATTGACCAACTGGC GCCAGTTGGTCAAT**C**TTTGGGGACCTCACCAGC
A85T	CAGCAAGCAGTGTGC**A**CCCCGAGCCGCGTTTC GAAACGCGGCTCGGGG**T**GCACACTGCTTGCTG
R88C	GTGTGCGCCCCGAGC**T**GCGTTTCTTTCCTCAC GTGAGGAAAGAAACGC**A**GCTCGGGGCGCACAC
H138R	CGGTGGGAAAAGTCTTT**C**GCCCTGGGATATCTTC GAAGATATCCCAGGGC**G**AAAGACTTTTCCCACCG
P228L	CCGTTGGGTATCATAAGC**T**ACACATCCCCTTCAG CTGAAGGGGATGTGT**A**GCTTATGATACCCAACGG
W267C	GCCTACAACCCCTG**T**ATGGACATCAGGCAACG CGTTGCCTGATGTCCAT**A**CAGGGGTTGTAGGC
W267X	GCCTACAACCCCTG**A**ATGGACATCAGGCAACG CGTTGCCTGATGTCCAT**T**CAGGGGTTGTAGGC
K347E	GAACATGGAGAATGGGCC**G**AATACAGCAATTTTGATGTTGC GCAACATCAAAATTGCTGTATT**C**GGCCCATTCTCCATGTTC
R468Q	CTATAGCCAGTATCCCC**A**GCCTTCAGACATCCC GGGATGTCTGAAGGC**T**GGGGATACTGGCTATAG
R468W	CTATAGCCAGTATCCC**T**GGCCTTCAGACATCCC GGGATGTCTGAAGGCC**A**GGGATACTGGCTATAG
I485R	GCCGAGTTTAAAAGATAGAAA**G**ATCATGGGCTATTCC GGAATAGCCCATGAT**C**TTTCTATCTTTTAAACTCGGC
G489D	GATATAAAGATCATGG**A**CTATTCCATACGCACCATAGAC GTCTATGGTGCGTATG**T**GAATAG**T**CCATGATCTTTATATC
E521X	GACATCCATGCAGGG**T**AACTGTATTTTGTGGATTCTGAC GTCAGAATCCACAAAA**T**ACAGTT**A**CCCTGCATGGATGTC
1184delG	CAGTTGATGGAGCCAGGCA-GCAATCCATGG CCATGGATTGC-TGCCTGGCTCCATCAACTG
1421delAG	CCTTCAGACATCCCTC--TGGAATTCTGACAAGCC GGCTTGTCAGAATTCCA--GAGGGATGTCTGAAGG

Table 6. Allele frequencies for flanking dinucleotide repeat markers on MPS II and normal chromosomes

	Allele	Chromosomes				*P* value
		MPS II		Normal		(1 *df*)
		no.	%	no.	%	
DXS1113 (nt)	153	0	0.0	1	0.7	
	155	0	0.0	31	20.9	
	157	1	7.1	2	1.4	
	161	0	0.0	8	5.4	
	163	1	7.1	1	0.7	
	165	0	0.0	3	2.0	
	167	9	64.3	80	54.1	0.622
	169	2	14.3	8	5.4	
	171	1	7.1	10	6.8	
	173	0	0.0	4	2.7	
	Total	14		148		
DXS1123 (nt)	167	0	0.0	1	0.7	
	171	3	21.4	20	13.5	
	173	3	21.4	12	8.1	
	175	4	28.6	51	34.5	0.718
	177	4	28.6	48	32.4	0.807
	179	0	0.0	12	8.1	
	181	0	0.0	4	2.7	
	Total	14		148		

Table 7. Haplotypes of MPS II and normal chromosomes

Haplo-type	DXS1113 (nt)	DXS1123 (nt)	Mutation	Chromosomes				*P* (1 *df*)
				MPS II		Normal		
				no.	%	no.	%	
1	153	175		0	0.0	1	1.0	
2-a	155	173	231del6	1	4.2	5	4.9	
2-b	155	177	H138R	1	4.2	6	5.8	
2-m	155	171, 175, 179, 181		0	0.0	13	12.6	
3	157	175	1055del12	1	4.2	0	0.0	
4-a	161	175		0	0.0	2	1.9	
4-b	161	177	G374sp	1	4.2	4	3.9	
5	163	177	K347E	1	4.2	1	1.0	
6	165	171	IVS2+1G>C	1	4.2	0	0.0	
6-m	165	175, 177		0	0.0	2	1.9	
7-a	167	171	R88C, W267C, R468Q	3	12.5	7	6.8	
7-b	**167**	**173**	**R468W (3)**	**3**	**12.5**	**3**	**2.9**	**0.004**
7-c	167	175	P228L, R468Q (2), IVS5+2T>G	4	16.7	21	20.4	
7-d	167	177	A85T, N63K, W267X, G489D, 1184delG	5	20.8	18	17.5	
7-m	167	167, 179, 181		0	0.0	7	6.8	
8	169	171	I485R	1	4.2	0	0.0	
8-m	169	175, 177, 179		0	0.0	3	2.9	
9	171	177	E521X, 1421delAG	2	8.3	3	2.9	
9-m	171	175, 179, 181		0	0.0	5	4.9	
10	173	177		0	0.0	2	1.9	
				n = 24		n = 103		

The R468W haplotype associated with 3 Taiwanese MPS II mutation is numbered in bold.

Table 8. Expression of IDS in transfected COS-7 cells

Transfected vector	Iduronate-2-sulfatase activity (nmole/h/mg protein)	Total (% of wild type)
pcDNA3	12.2	0.0
pcDNA3-IDS	530.2	100.0
pcDNA3-IDS/N63K	12.1	0.0
pcDNA3-IDS/A85T	26.7	2.8
pcDNA3-IDS/R88C	14.8	0.5
pcDNA3-IDS/H138R	13.2	0.2
pcDNA3-IDS/P228L	23.6	2.2
pcDNA3-IDS/W267C	32.4	3.9
pcDNA3-IDS/K347E	16.7	0.9
pcDNA3-IDS/R468Q	13.3	0.2
pcDNA3-IDS/R468W	15.3	0.6
pcDNA3-IDS/I485R	12.5	0.1
pcDNA3-IDS/G489D	14.8	0.5
pcDNA3-IDS/231del6	12.3	0.0
pcDNA3-IDS/W267X	12.0	0.0
pcDNA3-IDS/1055del12	12.1	0.0
pcDNA3-IDS/1184delG	12.4	0.0
pcDNA3-IDS/1421delAG	12.1	0.0
pcDNA3-IDS/E521X	12.0	0.0

The IDS activities in COS-7 cells are the mean of three independent experiments, with a

range less than 5%. The endogenous IDS activity in COS-7 cells was subtracted before the percentage of wild type was calculated.

Table 9. Expression of PEIa-IDS and IDS-PEIa in transfected COS-7 cells

Transfected vector	Lysate (nmol/h/mg protein)	Fold increase	Medium (nmol/h/ml)	Fold increase
pcDNA3	7.5		0.5	
pcDNA3-IDS	381.6	50	24.6	48
pcDNA3-PEIa-IDS	405.0	53	55.2	109
pcDNA3-IDS-PEIa	400.8	52	53.4	106

The IDS activities in COS-7 cell lysates and supernatant fluids are the mean of three independent experiments, with a range less than 5%.

Table 10. Uptake of PEIa-IDS and IDS-PEIa by COS-7 cells

Transfected vector	Lysate		Medium	
	−M-6-P	+M-6-P	−M-6-P	+M-6-P
	(nmol/h/mg protein)		(nmol/h/ml)	
pcDNA3	7.7	7.3	0.4	0.3
pcDNA3-IDS	135.6	6.7	20.4	14.5
pcDNA3-PEIa-IDS	186.6	7.2	46.9	34.2
pcDNA3-IDS-PEIa	166.2	7.0	44.9	33.8

The IDS activities in COS-7 cell lysates and supernatant fluids are the mean of three independent experiments, with a range less than 5%.

Part II: Characterization of LDL Receptor Gene Mutations in Familial Hypercholesterolemia

第二部份：家族性高膽固醇血症LDL受體基因突變的記述

Introduction

Lipoproteins

Many lipids, such as cholesterol, cholesterol esters, phospholipids, and triacylglycerols, and lipid-soluble substances, such as fat-soluble vitamins, are transported through the aqueous circulation by lipoprotein particles. Plasma lipoproteins can be divided into six major classes: very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), high density lipoprotein (HDL), chylomicrons, and chylomicron remnants. The first four classes, VLDL, IDL, LDL, and HDL, are derived from the liver and they can be found in the plasma of both fasted and nonfasted subjects. The last two classes, chylomicrons and chylomicron remnants, are derived from the small intestine and they can only be found after a fatty meal in the plasma. LDL, which is the end product from lipolysis of VLDL and IDL processing by lipoprotein lipase, is the major cholesterol-transporting lipoprotein in humans, carrying about two-thirds of the total plasma cholesterol. The lipid composition of LDL is ~35% cholesteryl ester, ~12% cholesterol, ~8% triglycerides, and 20% phospholipids. The cholesterol delivered by LDL is being endocytosed followed by hydrolysis within the endosome (Brown and Goldstein, 1986).

Cholesterol metabolism

The cholesterol pool of the human body comes from two sources: the absorption of dietary cholesterol and the *de novo* biosynthesis of cholesterol in all tissues (Mayes, 1998). All mammalian cells can synthesize the required cholesterol for themselves, but only some are synthesized locally. Most cholesterol used by peripheral tissues are originated from the liver (Spady and Dietschy, 1983). The *de novo* synthesis of cholesterol from acetyl-CoA in liver and other body tissues provides about three quarters of the cholesterol in the body. The rate-limiting step in the *de novo* synthesis is the conversion of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA reductase) to mevalonate, which is catalyzed by HMG-CoA reductase. The inhibition of this reaction is the primary

strategy for regulating cholesterol biosynthesis. HMG-CoA reductase is located in the endoplasmic reticulum and the enzyme is regulated by several mechanisms: the feedback inhibition from high cholesterol level acting on the activity of preexisting HMG-CoA reductase, degradation of existing HMG-CoA reductase accelerated by high cholesterol level, decreasing the amount of mRNA coding for HMG-CoA reductase by high cholesterol level, and inhibition of the enzyme by phosphorylation of HMG-CoA reductase and activation of the enzyme by dephosphorylation of HMG-CoA reductase (Champe and Harvey, 1994). The membrane level of free cholesterol in peripheral cells is tightly regulated by esterification or sequestration in lipoproteins. Most cells can esterify and store excess cholesterol via the acyl CoA:cholesterol acyltransferase (ACAT) reaction (Suckling and Stange, 1985).

Hyperlipidemia and atherosclerosis

Elevated plasma cholesterol concentrations, especially LDL, are strongly correlated with atherosclerotic coronary heart disease (CHD) (Freedman et al., 1993; Toshima et al., 2000). A high level of plasma LDL usually originates from excessive lipoprotein secretion from the liver or defects in plasma LDL clearance process (Lougheed and Steinbrecher, 1996). The elevated plasma LDL would increase the risk of LDL being trapped in the intima and oxidized by free radicals generated by adjacent cells, including endothelial cells, smooth muscle cells, and isolated macrophages. Since the oxidized LDL is able to impair endothelium-dependent relaxation and act as a chemoattractant substance for monocytes, it is highly atherogenic. When endothelial cells are being stimulated by oxidized LDL, they secrete cytokines and cause accumulations of monocytes and macrophages, leading to the formation of foam cells. The foam cells form the core of the atherosclerotic plaque, which is originated from macrophages and smooth muscle cells through the cholesterol loading process. The scavenger receptors in macrophages can recognize oxidized LDL but not native LDL (Cotran et al. 1994). The internalization of oxidized LDL by scavenger receptors in macrophages is not subject to feedback regulation and the uptake is not saturated, thus, large amounts of modified LDL would accumulate to form foam cells.

Familial defective apolipoprotein B-100 (FDB)

Both VLDL and its product, LDL, contain a copy of a large structural apolipoprotein B-100 (apo B-100) (Elovson et al., 1988). The C-terminal segment of apo B-100 on the surface of these particles can be recognized by hepatic and extrahepatic LDL receptors. This interaction is responsible for ~75% of the clearance of LDL in the plasma, primarily through the liver (Lund et al., 1989). Increased LDL concentrations resulting from inefficient clearance of LDL particles by the receptors may cause by the defect in apo B-100. This defect in the ligand, familial defective apolipoproteinB-100 (FDB), is a dominantly inherited genetic disease. Three forms of FDB have been described genetically: a CGC-to-CAG change in apo B-100 codon 3500 (R3500Q), a CGC-to-TGC change in codon 3531 (R3531C), and a CGG-to-TGG change in codon 3500 (R3500W). These three mutations affect the function of apo B-100 by decreasing the binding affinity of LDL receptors (Innerarity et al., 1987; Gaffney et al., 1995; Pullinger et al., 1995).

Familial hypercholesterolemia (FH)

Another reason for inefficient clearance of LDL particles is that a defect has occurred in the LDL receptor. This class of genetic disorder is an autosomal dominant familial hypercholesterolemia (FH), and it is characterized by increased LDL cholesterol levels in plasma, which will then cause cholesterol deposition in tissues in the forms of tendon xanthomas and atheroma, leading to premature arteriosclerosis and CHD (Goldstein and Brown, 1989). Heterozygous FH is one of the most common inborn errors of metabolism, with a frequency of ~ 1/500 in most populations. Heterozygous carriers may have up to three-fold elevations in plasma cholesterol, ranging from 300 to 600 mg/dl. The homozygous FH occurs in about one in a million individuals and is more severely affected than the heterozygote carrier, having circulating LDL level six to eight times higher than normal (from 600 — >1,200 mg/dl) and usually have heart attacks before the age of 20 (Goldstein et al, 2001).

The LDL receptor gene: relation of exons to protein domains

The human LDL receptor gene consists of 18 exons, spanning 45 kb on chromosome 19p13 (**Figure 1**) (Sudhof et al., 1985). Half of the 5.3 kb mRNA comprises a long 3' untranslated region, which encodes a protein with 860 amino acids. At the N terminal end, 21 hydrophobic amino acids comprise the signal sequence, which is cleaved from the protein during the translocation process into the endoplasmic reticulum (ER). The resultant 839-amino acid protein (120 kDa precursor receptor) contains 1~2 N-linked and 9~18 O-linked carbohydrate chains, which undergo several carbohydrate processing reactions in the Golgi apparatus en route to the cell surface, resulting in a 160 kDa mature receptor (Cummings et al., 1983).

Many of the LDL receptor exons share an evolutionary history with exons from other genes; an observation suggested that the LDL receptor gene was assembled by exon shuffling. **Figure 2** shows how exons of the gene are correlated with the functional domains of the mature protein. The signal sequence is encoded by exon 1 and the ligand-binding domains encoded by exons 2 ~ 6 are made by 7 ~ 40 amino acid repeats (R1 ~ R7), which form two loops joined by disulfide bonds. The domain contains clusters of negatively charged amino acids, which bind the lipoprotein particles to ligands apo B-100 and apo E. Exons 7 ~ 14 encode EGF-precursor homology domain, which is 33% identical to the human epidermal growth factor precursor, containing three EGF-like repeats: tandem pairs A-B and C, which form two pairs of short antiparallel β strands. This domain is involved in positioning the ligand-binding domain on the cell surface, and plays an important role in acid dependant dissociation of the lipoprotein after the endocytosis process. Exon 15 encodes 58 amino acids, which are riched in serine and threonine residues; many of which serve as attachment sites for O-linked carbohydrate chains. The membrane spanning domain, encoded by exons 16 and 17, anchors the protein in the cellular membrane. The cytoplasmic domain, encoded by exons 17 and 18, is critical to the ligand uptake and signaling functions. The tetra-amino-acid NPXY motif within the cytoplasmic domain forms a tight hairpin conformation to mediate the

interaction between the receptor tails and the endocytosis mechanism, and to serve as a docking site for cytoplasmic adaptor and scaffold proteins (Yamamoto et al., 1984).

The LDL receptor pathway

The LDL receptor sequential pathway is summarized in **Figure 3**. The receptor begins its life in the endoplasmic reticulum, and then it travels to the Golgi complex, cell surface, coated pit, endosome, and back to the surface. On the cell surface, LDL receptor binds to LDL particles that contain apo B-100. The LDL particles are internalized via clathrin-coated pit on the plasma membrane. The coated pits "pinch off" from the membrane to form coated vesicles, and then they fuse together to lose their clathrin protein coat and become endosomes. Proton pumps acidify the endosomes, causing lipoprotein release from the receptor. Within a few minutes, the receptor is recycled to the cell surface, where it can be used for another round of endocytosis. The lipoproteins are delivered to lysosomes, where enzymes degrade the apolipoproteins into their constituent amino acids and hydrolyze the cholesteryl esters to free cholesterol. The released cholesterol is used in the synthesis of cell membranes, and some specialized cells, and it is also a precursor for sterol end products.

Regulation of LDL receptor synthesis

The amount of LDL receptor synthesis in cells is strongly regulated by the need or availability of cholesterol. When intracellular cholesterol raised, the expression of LDL receptors is down-regulated; excess cholesterol also down-regulates the expression of HMG-CoA reductase, which is the major rate limiting enzyme in cholesterol biosynthesis and the primary target of modern lipid-lowering drugs, such as statines. Also, the activity of ACAT, a lipid droplet enzyme that re-esterifies cholesterol into cholesteryl esters in the cytoplasm, is increased when intracellular cholesterol increased. By these mechanisms, cells are able to maintain sufficient amount of intracellular cholesterol for various

functions (**Figure 3**).

LDL receptor mutations

At the present, more than 840 mutations, including gross deletions, minor deletions, insertions, point mutations, and splice-site mutations, have been reported to scatter over the LDL receptor gene (human mutation databases http://www.uwcm.ac.uk/ and http://www.umd.necker.fr) (Villeger et al, 2002; Soutar et al., 2003). Among these, *Alu* sequences are always involved in deletions or insertions of the LDL receptor gene (Lehrman et al., 1985, 1987). The mutations characterized at the molecular level can be divided into five different classes (**Figure 4**) (Hobbs et al, 1992). Class 1 mutations (null alleles) affect the receptor synthesis, which means that no receptor can be detected in the affected subjects. Class 2 mutations (transport defective alleles) are the most common LDL receptor gene mutations. Patients in this class have a relative LDL receptor deficiency at the cellular surface, because the intracellular transport between endoplasmic reticulum and Golgi apparatus is either completely blocked (class 2A) or delayed (class 2B). Class 3 mutations (binding defective alleles) prevent receptors from binding LDL through ligands apo B/E. Class 4 mutations (internalization defective alleles) still conserve the binding ability on LDL particles, but fail to internalize the bound LDL. Class 4 can be either complete (4A) or partial (4B).Class 5 mutations (recycling defective alleles) produce receptors that bind and internalize LDL normally, but fail to discharge the LDL in endosomes and recycle them to the cellular surface.

Aims

Previously, 170 unrelated hyperlipidemic Chinese and two clinically diagnosed familial hypercholesterolemia (FH) patients were screened by multiplex polymerase chain reaction (PCR), long PCR, and single strand conformation polymorphism (SSCP) analyses to detect mutations in the LDL receptor gene. Eight point mutations (W-18X, D69N, R94H, E207K, C308Y, I402T, A410T, and A696G), two gross deletions (Del e3-5 and Del e6-8), and one polymorphism (I602V) were found (**Table 1**) (Huang, 2001; Lin et al., 2002). In this study, the effect of seven point mutations and two gross deletions on processing and stability of LDL receptor was characterized by transient expression in COS-7 cells.

Materials and Methods

cDNA constructs

pcDNA3-LDLR contains a 2.8 kb *Bam*HI-*Sma*I LDL receptor coding region (cDNA -19~2804, where +1 represents the first nucleotide to be translated) cloned in *Bam*HI and *Eco*RV sites of pcDNA3 (Huang, 2001). The cDNA constructs containing D69N, C308Y, I402T, A410T, and A696G changes were from Huang et al., 2001. The point changes in pcDNA3-LDLR/R94H and pcDNA3-LDLR/E207K were made by using QuickChange XL system (Stratagene) (sequences of primers are listed in **Table 2**) and confirmed by DNA sequencing. pcDNA3-LDLR/Del e3-5 was derived from pcDNA3-LDLR by replacing a 759-bp *Hae*II-*Nci*I fragment (cDNA 150~908) with a 552-bp fragment excised from the deleted exons 2-6 long PCR clone of patient D72 (substitution of exons 3-5 with 420-bp intron). pcDNA3-LDLR/Del e6-8 was derived from pcDNA3-LDLR by replacing a 559-bp *Eco*RI-*Bsu*36I fragment (cDNA 719~1277) with a 1503-bp fragment from the deleted exons 5-9 long PCR clone of patient D254 (substitution of exons 6-8 with 1313-bp intron). The replaced sequences were confirmed by restriction enzyme digestion and DNA sequencing.

COS-7 cells transfection

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FCS, 100 μg/ml penicillin, 100 μg/ml streptomycin at 37℃ in a 5% CO_2/air atmosphere. Wild-type and mutant cDNA expression constructs (10 μg) were introduced into COS-7 cells by electroporation. To inhibit the endogenous LDL receptor expression, cells were incubated in medium containing 0.2 μg/ml 25-hydroxycholesterol for 24 hr prior to transfection. Forty-eight hr later, total RNA and proteins from transfected cells were prepared.

Reverse transcription-PCR (RT-PCR)

Total RNA were prepared from transfected cells using Trizol reagent (Invirtrogen) according to the manufacturer's instructions. The level of LDL receptor mRNA in transfected cells was quantitated by RT-PCR using the ThermoScript^{TM} RT-PCR system (Gibco). Sense primer 5'-GGTGTGGACATCTACTCGC-3' and the antisense primer 5'-TCTCAGGAAGGGTTCTGGG-3' were used to amplify 983-bp 3' cDNA fragment. The amplified products were examined by 1.0% agarose gel electrophoresis.

Western blot analysis

The expressed LDL receptor proteins were examined by Western blot analysis. Transfected cell were washed with PBS twice and collected. The cell lysates were prepared by freeze thawing, in the presence of a protease inhibitor cocktail (Complete Mini, Roche, Mannheim, Germany). Protein concentrations in the lysates were measured with a Protein Assay Dye reagent (Bio-Rad), using bovine serum albumin as a standard. After boiling for 10 min, proteins (10 μg) were loaded onto a 10% SDS-polyacrylamide gel, separated electrophoretically, and transferred to a nitrocellulose membrane (SWSS, Inc., Chicago, IL). After blocking with 5% skim milk for 1 hr at room temperature, membranes were incubated with rabbit anti-LDL receptor polyclonal antibody (1:400 dilution, Progen) at room temperature for 1 hr. After washing, blots were then incubated with a alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (1:3000, Boehringer Mannheim) for 1 hr at room temperature. Blots were then developed using nitroblue tetrazolinm (NBT)/ 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as a substrate.

Flow cytometric analysis

For flow cytometric analysis, cells were harvested by trypsin treatment 48 hr after transfection and cells in suspension were incubated with 0.4% heparin for 30 min to remove any surface-bound LDL. To quantitate the amount of receptor on the cell surface, the cells were incubated for 30 min at 4°C in primary antibody diluted 1: 60 in PBS, washed in PBS, and incubated further for 30 min at 4°C in FITC-conjugated goat

anti-rabbit antibody (Zymed) also diluted 1: 60 in PBS. To measure the LDL receptor function, the cells were incubated for 30 min at 4°C in DiI-LDL (Molecular Probes) diluted in DMEM to a concentration of 4 μg/ml, washed with PBS, and incubated further for 30 min at 37°C. All cells were subsequently washed and resuspended in PBS for FACS analysis. Cells were analyzed in a FACStar flow cytometer (Becton-Dickinson) equipped with an argon laser operating at 488 nm (antibody) or 514 nm (DiI). A forward scatter gate was established to exclude dead cells and cell debris from the analysis. 10^4 cells were analyzed in each sample.

Immunofluorescence and microscopy

The intracellular receptor protein was visualized by staining cells fixed in 4% paraformaldehyde and permeabilized in 70% ethanol. Cells were incubated for 60 min at room temperature with primary antibody diluted 1:50 in 1% BSA in TBS, washed in PBS, and incubated for 60 min at room temperature in FITC-conjugated secondary antibody diluted 1:50 in 1% BSA in TBS, washed in PBS. Lysosomes were detected using lysotracker (Molecular Probes) at 0.1 μmol/L for 60 min. The stained cells were analysed using a Leica TCS confocal laser scanning microscope.

Results

Expression of LDL receptor cDNA mutants

The effects of seven point changes (D69N, R94H, E207K, C308Y, I402T, A410T, and A696G), two gross deletions (Del e3-5 and Del e6-8) on LDL receptor function were investigated by transient expression in COS-7 cells. In recombinant pcDNA3 plasmids, the entire coding region of LDL receptor is under the control of CMV promoter. Upon transfection into COS-7 cells, the recombinant plasmid reproduces via SV40 origin and transiently expresses high level of LDL receptor protein. Approximately equal levels of LDL receptor mRNA derived from each variant was expressed as quantitated by RT-PCR (**Figure 5**). The LDL receptor protein that derived from each variant was examined by immunostaining of Western blot with LDL receptor polyclonal antibody (**Figure 6**). No nonspecific polypeptide was detected in pcDNA3 vector-transfected cells (lane 1). 160 kDa mature and 120 kDa precursor proteins as well as an intermediate form were detected in wild type receptor cDNA-transfected cells (lane 2). A novel intermediate protein that is probably a degradation product and an apparent reduction in mature receptor protein were seen in D69N transfected cells (lane 3). Although the same molecular weight receptor proteins were seen with R94H, E207K, C308Y, I402T and A410T mutations, the amounts of mature protein detected were reduced (lanes 4-8). For A696G, both the processing and the level of the mature protein were close to those of wild type (lane 9). Conversely, no receptor protein for Del e3-5 and a defect receptor protein for Del e6-8 were detected (lanes 10-11).

LDL receptor function

The amounts and activity of the LDL receptor variants were examined by flow cytometer analysis using Antibody at 4°C and DiI-LDL at 37°C. Analyses of antibody-labeled cells and activity measurements revealed that A696G encodes receptors in amounts and activity comparable with the wild type receptor (98% and 93%, respectively). Del e3-5 encodes receptors that cannot be detected and have no detectable

residual activity. All other mutations impair the function, encoding receptors in reduced amounts (13%~64%) and displaying approximately 12%~64% residual activity (**Table 3**).

Fluorescence microscopy of cells expressing the wildtype or the mutant LDL receptor was performed. As shown in **Figure 7A**, the wild-type and A696G receptor proteins were located in a ring-shaped structure representing the cell surface, whereas most of the mutant receptors accumulated intracellularly. Colocalization studies using a lysosome staining dye (**Figure 7B**) further established that A410T and Del e6-8 mutant proteins are retained in the endosomal/lysosomal regions, whereas D69N, R94H, E207K, C308Y, and I402T produce proteins that seem to be localized in the endoplasmic reticulum (ER) (**Figure 7C**).

Discussion

Mutation characterization

In the present study, mutations identified in Taiwanese hyperlipidemia patients were characterized to verify if the mutations are indeed the pathological lesion causing disease. Four complementary experiments were used to characterize the mutations: LDL receptor amount and activity measurement (**Table 3**), RT-PCR study to examine the LDLR mRNA stability, immunoblot analysis to visualize the LDL receptor protein maturation (**Figure 6**), and confocal microscopy examination to reveal the LDLR targeting (**Figure 7**). Approximately equal levels of LDL receptor mRNA derived from each variant was expressed as quantitated by RT-PCR (**Figure 5**). The possible effects of each mutation on the synthesis, post-translational processing, ligand binding activity, internalization, or recycling of the LDL receptor are discussed as the following.

(1) D69N mutation. The D69N mutation affects the highly conserved aspartate residue 69 in the second cysteine-rich repeats in the binding domain (Yamamoto et al., 1984; Sudhof et al., 1985). The novel intermediate and reduced mature receptor protein (**Figure 6**) indicate abnormal processing and delayed transport of newly synthesized receptors to the cell surface (class 2B), similar to D69G and D69Y mutations reported earlier (Hobbs et al., 1992; Rubinsztein et al., 1993). The LDL receptor was retained in ER with 55% activity (**Table 3**). The mutation was found in two Chinese patients in Hong Kong and Malaysia and a British patient previously (Day et al., 1997; Mak et al., 1998; Khoo et al., 2000). Two patients (A133 and L41) originating from Fukien province of China were noted to have this mutation.

(2) R94H mutation. The R94H mutation is located in the third cysteine-rich repeats in the binding domain. Although evolutionarily conserved among human, rat, rabbit, and cow (Russell et al., 1984; Yamamoto et al., 1984, 1986; Lee et al., 1989), arginine$_{94}$ is not conserved among the seven cysteine-rich repeats (Sudhof et al., 1985). The mutation

results in slower processing and delayed transport (class 2B). The mutation was also found in two Chinese patients and a Japanese patient (Khoo et al., 2000; Varret et al., 1998).

(3) E207K mutation. The E207K mutation is located in the fifth cysteine-rich repeats in the binding domain. The mutation affects the highly conserved negatively charged Ser-Asp-Glu triplet at the COOH-terminus of all ligand-binding repeats (Sudhof et al., 1985) and results in severely slow processing and delayed transport (class 2B). This mutation occurs at a CpG hot spot in which the G is mutable to A, C, or T (Hobb et al., 1992). Such mutation has been detected in different ethnic groups (Leitersdorf et al., 1990; Hobb et al., 1992; Kotze et al., 1997; Mak et al., 1998; Pimstone et al., 1998) and might have occurred independently during evolution.

(4) C308Y mutation. For C308Y, the cysteine residue 308 in the cysteine-rich repeat A of the EGF precursor homology domain is highly conserved and is important for the correct folding of the LDL receptor protein for its function (Sudhof et al., 1985). The disruption of the disulfide linkage formation may lead to the reduced mature protein (class 2B) (**Figure 6**), similar to other transport defect mutations nearby, C297Y (Hobb et al., 1992) and C317S (Maruyama et al., 1995). The LDL receptor activity was reduced to 31% (**Table 3**). Previously, the C308Y mutation was found in two Chinese patients in Hong Kong (Mak et al., 1998) and two Chinese patients in Malaysia (Khoo et al., 2000).

(5) I402T and A410T mutations. Mutations I402T and A410T were between cysteine-rich repeats B and C of the EGF precursor homology domain. The domain serves to position the ligand-binding domain to bind LDL on the cell surface. It is also required for the acid-dependent dissociation of lipoproteins from the receptor in the endosome during receptor recycling. The amount of receptor protein produced by both mutations was reduced (**Figure 6**). I402T has reported as pathogenic in mediating uptake and degradation of LDL (Ekstrom et al., 2000). The LDL receptor was retained in ER with 54% activity (class 2B) (**Table 3**). A410T may be associated with a recycling-deficient phenotype (Hobbs et al., 1990). The mutant receptor retained 20%

activity (**Table 3**) and gave rise to an endosomal/lysosomal staining pattern (class 5) (**Figure 7**). Both I402T and A410T mutations have been reported previously in different ethnic groups (Hobbs et al., 1990; Ekstrom et al., 1995; Khoo et al., 2000). Due to the geographical distance and historic ethnic differences, it is probable that the two mutations have arisen independently in Chinese.

(6) A696G mutation. The A696G in the O-linked sugars domain was novel. The point change was not detected in our normolipidemic controls. A696G is a conservative amino acid substitution and alanine at 696 is not conserved across species among human, rat, rabbit, and cow (Yamamoto et al., 1984, 1986; Russell et al., 1984; Lee et al., 1989). Although patient D162 had elevated cholesterol value (6.77 mmol/l) in addition to hypertension, A696G is a rare sequence variation which does not affect LDL receptor function (**Figure 6** and **Table 3**).

(7) Del e3-5 and Del e6-8 deletions. Two novel deletions were characterized. Del e3-5 is caused by misalignment of *Alu* sequences in intron 2 and intron 5 and predicted to cause deletion of 209 aa (44-252), including cysteine-rich repeats II to VI in the binding domain (Sudhof et al., 1985). The resultant defect receptor protein cannot to be detected and is unable to bind with the LDL (class 2A) (**Figure 6** and **Table 3**). A similar misalignment of *Alu* sequences in introns 5 and 8 is responsible for Del e6-8. The mutation is predicted to cause deletion of 123 aa (252-374) including the seventh disulfide-rich repeat in the binding domain and cysteine-rich repeats A and B in the EGF precursor homology domain (Sudhof et al., 1985). The resultant defect receptor retained 12% of its activity and was localized in the endosomal/lysosomal regions (class 5) (**Table 3 and Figure 7**).

Conclusions

In summary, the effect of 9 mutations on LDL receptor activity was examined by transient expression and western analysis. In FH, a complex genetic disease, to clearly define individual gene defects in patients is of prognostic value, making early detection,

optimal treatment approaches, and a reduction in the risk factors associated with CAD highly possible.

References

Brown, M. S. and Goldstein, J. L. (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science* 232(4746):34-47.

Cai, H. J., Fan, L. M., Huang, M. G., Chen, X. Y., Liu, G. Q. and Chen, Q. (1985) Homozygous familial hypercholesterolemic patients in China. *Atherosclerosis* 57(2-3):303-12.

Castelli, W. P., Doyle, J. T., Gordon, T., Hames, C. G., Hjortland, M. C., Hulley, S. B., Kagan, A. and Zukel, W. J. (1977) HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. *Circulation* 55(5):767-72.

Champe, P.C., and Harvey, R.A. (1994) Cholesterol and steroid metabolism. *In* Lippincott's illustrated reviews: biochemistry. 2nd ed. *Edited by* P.C. Champe and R.A. Harvey. J.B. Lippincott Company, Philadelphia, Pa. pp. 205–228.

Chen, W. J., Goldstein, J. L. and Brown, M. S. (1990) NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J Biol Chem* 265(6):3116-23.

Cotran, R.S., Kumar, V., and Robbins. S.L. (1994) *In* Robbins pathologic basis of disease. 5th ed. *Edited by* R.S. Cotran, V. Kumar, and S.L. Robbins. W.B. Saunders, Philadephia, Pa. pp. 480-2.

Cummings, R. D., Kornfeld, S., Schneider, W. J., Hobgood, K. K., Tolleshaug, H., Brown, M. S. and Goldstein, J. L. (1983) Biosynthesis of N- and O-linked oligosaccharides of the low density lipoprotein receptor. *J Biol Chem* 258(24):15261-73.

Day, I. N., Whittall, R. A., O'Dell, S. D., Haddad, L., Bolla, M. K., Gudnason, V. and Humphries, S. E. (1997) Spectrum of LDL receptor gene mutations in heterozygous familial hypercholesterolemia. *Hum Mutat* 10(2):116-27.

Ekstrom, U., Abrahamson, M., Sveger, T., Lombardi, P. and Nilsson-Ehle, P. (1995) An efficient screening procedure detecting six novel mutations in the LDL receptor gene in Swedish children with hypercholesterolemia. *Hum Genet* 96(2):147-50.

Ekstrom, U., Abrahamson, M., Sveger, T., Sun, X. M., Soutar, A. K. and Nilsson-Ehle, P. (2000) Expression of an LDL receptor allele with two different mutations (E256K and I402T). *Mol Pathol* 53(1):31-6.

Elovson, J., Chatterton, J. E., Bell, G. T., Schumaker, V. N., Reuben, M. A., Puppione, D. L., Reeve, J. R., Jr. and Young, N. L. (1988) Plasma very low density lipoproteins contain a single molecule of apolipoprotein B. *J Lipid Res* 29(11):1461-73.

Fass, D., Blacklow, S., Kim, P. S. and Berger, J. M. (1997) Molecular basis of familial

hypercholesterolaemia from structure of LDL receptor module. *Nature* 388(6643):691-3.

Gaffney, D., Reid, J. M., Cameron, I. M., Vass, K., Caslake, M. J., Shepherd, J. and Packard, C. J. (1995) Independent mutations at codon 3500 of the apolipoprotein B gene are associated with hyperlipidemia. *Arterioscler Thromb Vasc Biol* 15(8):1025-9.

Genest, J. Jr. (2002) Genetics and prevention: a new look at high-density lipoprotein cholesterol. *Cardiol Rev* 10(1):61-71.

Goldstein, J., Hobbs, H. and Brown, M. (1995) Familial hypercholesterolemia. *In* The Metabolic Basis of Inherited Diseases. 6th edition. Scriver, E. R., Beaudet, A. L., Sly, W. S. and Valle, D. editors. McGraw-Hill Book Co., New York. 1981–2030.

Goldstein, J., Hobbs, H. and Brown, M. (2001) Familial hypercholesterolemia. *In* The Metabolic and Molecular Bases of Inherited Disease. Valle, D. ed. Vol 2. 8th ed. New York, NY:McGraw-Hill. 2863–913.

Goldstein, J. L. and Brown, M. S. (2001) Molecular medicine. The cholesterol quartet. *Science* 292(5520):1310-2.

Huang, Ai-Chun. (2001) Molecular studies of Familial Hypercholesterolemia. National Taiwan Normal University Master thesis.

Hobbs, H. H., Russell, D. W., Brown, M. S. and Goldstein, J. L. (1990) The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. *Annu Rev Genet* 24:133-70.

Hobbs, H. H., Brown, M. S. and Goldstein, J. L. (1992) Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum Mutat* 1(6):445-66.

Howell, B. W., Lanier, L. M., Frank, R., Gertler, F. B. and Cooper, J. A. (1999) The disabled 1 phosphotyrosine-binding domain binds to the internalization signals of transmembrane glycoproteins and to phospholipids. *Mol Cell Biol* 19(7):5179-88.

Innerarity, T. L., Weisgraber, K. H., Arnold, K. S., Mahley, R. W., Krauss, R. M., Vega, G. L. and Grundy, S. M. (1987) Familial defective apolipoprotein B-100: low density lipoproteins with abnormal receptor binding. *Proc Natl Acad Sci USA* 84(19):6919-23.

Khoo, K. L., van Acker, P., Defesche, J. C., Tan, H., van de Kerkhof, L., Heijnen-van Eijk, S. J., Kastelein, J. J. and Deslypere, J. P. (2000) Low-density lipoprotein receptor gene mutations in a Southeast Asian population with familial hypercholesterolemia. *Clin Genet* 58(2):98-105.

Kotze, M. J., Loubser, O., Thiart, R., de Villiers, J. N., Langenhoven, E., Theart, L., Steyn, K., Marais, A. D. and Raal, F. J. (1997) CpG hotspot mutations at the LDL receptor locus are a frequent cause of familial hypercholesterolaemia among South African Indians. *Clin*

Genet 51(6):394-8.

Lee, L. Y., Mohler, W. A., Schafer, B. L., Freudenberger, J. S., Byrne-Connolly, N., Eager, K. B., Mosley, S. T., Leighton, J. K., Thrift, R. N., Davis, R. A. and et al. (1989) Nucleotide sequence of the rat low density lipoprotein receptor cDNA. *Nucleic Acids Res* 17(3):1259-60.

Lehrman, M. A., Schneider, W. J., Sudhof, T. C., Brown, M. S., Goldstein, J. L. and Russell, D. W. (1985) Mutation in LDL receptor: *Alu-Alu* recombination deletes exons encoding transmembrane and cytoplasmic domains. *Science* 227(4683):140-6.

Lehrman, M. A., Goldstein, J. L., Russell, D. W. and Brown, M. S. (1987) Duplication of seven exons in LDL receptor gene caused by *Alu-Alu* recombination in a subject with familial hypercholesterolemia. *Cell* 48(5):827-35.

Leitersdorf, E., Tobin, E. J., Davignon, J. and Hobbs, H. H. (1990) Common low-density lipoprotein receptor mutations in the French Canadian population. *J Clin Invest* 85(4):1014-23.

Levy, R. I. (1981) Declining mortality in coronary heart disease. *Arteriosclerosis* 1(5):312-25.

Lin, W. L., Tai, D. Y., Lo, E., Lee-Chen, G. J. (2002) The mutation underlying familial hypercholesterolemia in a Taiwanese family. *BioFormosa* 37(1):1-6.

Lougheed, M. and Steinbrecher, U. P. (1996) Mechanism of uptake of copper-oxidized low density lipoprotein in macrophages is dependent on its extent of oxidation. *J Biol Chem* 271(20):11798-805.

Lund, H., Takahashi, K., Hamilton, R. L. and Havel, R. J. (1989) Lipoprotein binding and endosomal itinerary of the low density lipoprotein receptor-related protein in rat liver. *Proc Natl Acad Sci U S A* 86(23):9318-22.

Mak, Y. T., Pang, C. P., Tomlinson, B., Zhang, J., Chan, Y. S., Mak, T. W. and Masarei, J. R. (1998) Mutations in the low-density lipoprotein receptor gene in Chinese familial hypercholesterolemia patients. *Arterioscler Thromb Vasc Biol* 18(10):1600-5.

Manninen, V., Tenkanen, L., Koskinen, P., Huttunen, J. K., Manttari, M., Heinonen, O. P. and Frick, M. H. (1992) Joint effects of serum triglyceride and LDL cholesterol and HDL cholesterol concentrations on coronary heart disease risk in the Helsinki Heart Study. Implications for treatment. *Circulation* 85(1):37-45.

Maruyama, T., Miyake, Y., Tajima, S., Harada-Shiba, M., Yamamura, T., Tsushima, M., Kishino, B., Horiguchi, Y., Funahashi, T., Matsuzawa, Y. and et al. (1995) Common mutations in the low-density-lipoprotein-receptor gene causing familial hypercholesterolemia in the Japanese population. *Arterioscler Thromb Vasc Biol*

15(10):1713-8.

Mayes, P.A. (1998) Cholesterol synthesis, transport, and excretion. *In* Harper's biochemistry. 23rd ed. *Edited by* R.K. Murray, D.K. Granner, P.A. Mayes, and V.W. Rodwell. Appleton & Lange, Norwalk, Conn. pp. 266-78.

Pimstone, S. N., Sun, X. M., du Souich, C., Frohlich, J. J., Hayden, M. R. and Soutar, A. K. (1998) Phenotypic variation in heterozygous familial hypercholesterolemia: a comparison of Chinese patients with the same or similar mutations in the LDL receptor gene in China or Canada. *Arterioscler Thromb Vasc Biol* 18(2):309-15.

Pittman, R. C., Carew, T. E., Attie, A. D., Witztum, J. L., Watanabe, Y. and Steinberg, D. (1982) Receptor-dependent and receptor-independent degradation of low density lipoprotein in normal rabbits and in receptor-deficient mutant rabbits. *J Biol Chem* 257(14):7994-8000.

Pullinger, C. R., Hennessy, L. K., Chatterton, J. E., Liu, W., Love, J. A., Mendel, C. M., Frost, P. H., Malloy, M. J., Schumaker, V. N. and Kane, J. P. (1995) Familial ligand-defective apolipoprotein B. Identification of a new mutation that decreases LDL receptor binding affinity. *J Clin Invest* 95(3):1225-34.

Rubinsztein, D. C., Jialal, I., Leitersdorf, E., Coetzee, G. A. and van der Westhuyzen, D. R. (1993) Identification of two new LDL-receptor mutations causing homozygous familial hypercholesterolemia in a South African of Indian origin. *Biochim Biophys Acta* 1182(1):75-82.

Russell, D. W., Schneider, W. J., Yamamoto, T., Luskey, K. L., Brown, M. S. and Goldstein, J. L. (1984) Domain map of the LDL receptor: sequence homology with the epidermal growth factor precursor. *Cell* 37(2):577-85.

Russell, D. W., Lehrman, M. A., Sudhof, T. C., Yamamoto, T., Davis, C. G., Hobbs, H. H., Brown, M. S. and Goldstein, J. L. (1986) The LDL receptor in familial hypercholesterolemia: use of human mutations to dissect a membrane protein. *Cold Spring Harb Symp Quant Biol* 51 Pt 2(811-9.

Soutar, A. K. (1998) Update on low density lipoprotein receptor mutations. *Curr Opin Lipidol* 9(2):141-7.

Soutar, A. K., Naoumova, R. P. and Traub, L. M. (2003) Genetics, clinical phenotype, and molecular cell biology of autosomal recessive hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 23(11):1963-70.

Spady, D. K. and Dietschy, J. M. (1983) Sterol synthesis in vivo in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster, and rat. *J Lipid Res* 24(3):303-15.

Suckling, K. E. and Stange, E. F. (1985) Role of acyl-CoA: cholesterol acyltransferase in

cellular cholesterol metabolism. *J Lipid Res* 26(6):647-71.

Sudhof, T. C., Goldstein, J. L., Brown, M. S. and Russell, D. W. (1985) The LDL receptor gene: a mosaic of exons shared with different proteins. *Science* 228(4701):815-22.

Sun, X. M., Patel, D. D., Webb, J. C., Knight, B. L., Fan, L. M., Cai, H. J. and Soutar, A. K. (1994) Familial hypercholesterolemia in China. Identification of mutations in the LDL-receptor gene that result in a receptor-negative phenotype. *Arterioscler Thromb* 14(1):85-94.

Toshima, S., Hasegawa, A., Kurabayashi, M., Itabe, H., Takano, T., Sugano, J., Shimamura, K., Kimura, J., Michishita, I., Suzuki, T. and Nagai, R. (2000) Circulating oxidized low density lipoprotein levels. A biochemical risk marker for coronary heart disease. *Arterioscler Thromb Vasc Biol* 20(10):2243-7.

Varret, M., Rabes, J. P., Thiart, R., Kotze, M. J., Baron, H., Cenarro, A., Descamps, O., Ebhardt, M., Hondelijn, J. C., Kostner, G. M., Miyake, Y., Pocovi, M., Schmidt, H., Schuster, H., Stuhrmann, M., Yamamura, T., Junien, C., Beroud, C. and Boileau, C. (1998) LDLR Database (second edition): new additions to the database and the software, and results of the first molecular analysis. *Nucl Acid Res* 26(1):248-252.

Villeger, L., Abifadel, M., Allard, D., Rabes, J. P., Thiart, R., Kotze, M. J., Beroud, C., Junien, C., Boileau, C. and Varret, M. (2002) The UMD-LDLR database: additions to the software and 490 new entries to the database. *Hum Mutat* 20(2):81-7.

Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L. and Russell, D. W. (1984) The human LDL receptor: a cysteine-rich protein with multiple *Alu* sequences in its mRNA. *Cell* 39(1):27-38.

Yamamoto, T., Bishop, R. W., Brown, M. S., Goldstein, J. L. and Russell, D. W. (1986) Deletion in cysteine-rich region of LDL receptor impedes transport to cell surface in WHHL rabbit. *Science* 232(4755):1230-7.

Yeagle, P. L. (1988) Cholesterol and the cell membrane. In *Biology of Cholesterol* (Yeagle, P., ed) pp. 121-146, CRC Press, Boca Raton, Florida 3. Fielding, C. J.

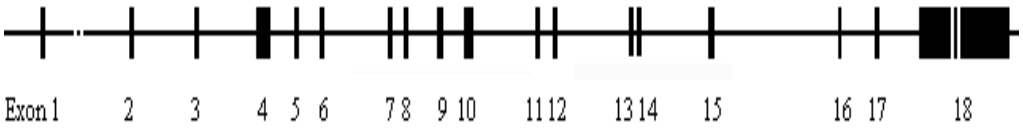


Figure 1. Structure of the LDL receptor gene. The 18 exons are illustrated by small black boxes.

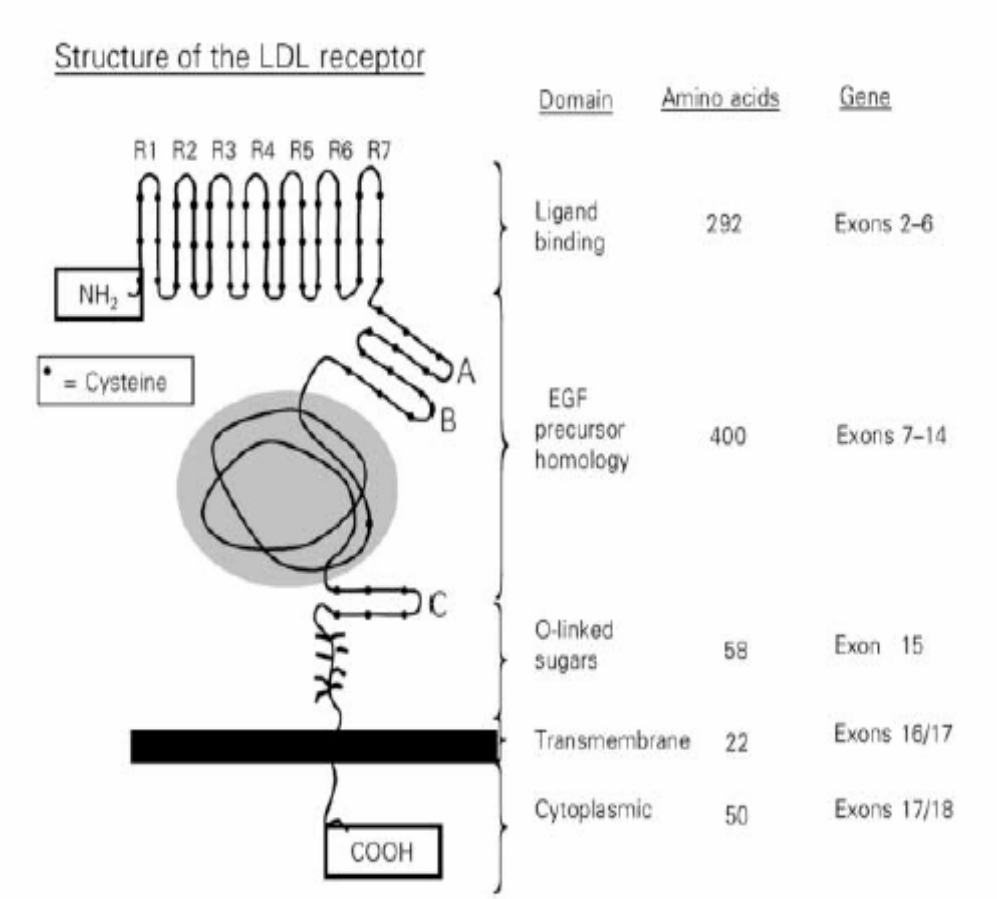


Figure 2. Domain structure of the human LDL receptor protein (left) as well as its relation to the exon organization of the LDL receptor gene (right). Exons 2~6 encode the ligand-binding domain, which is made up of seven ~40-amino acid repeats (R1 ~ R7), each containing six cysteine residues (black circles) that form three intrarepeat disulfide bonds. Exons 7~14 encode the EGF precursor homology domain, which includes cysteine-rich growth factor repeats A, B and C. Exon 15 encodes O-linked sugar domain that is enriched in serine and threonine for O-linked carbohydrate chains. Exon 16 and 5'-end of exon 17 encode hydrophobic amino acids comprising the membrane-spanning domain. The remainder of exon 17 and exon 18 encode amino acids that make up the cytoplasmic domain. (Reprint from Hobbs et al., 1990)

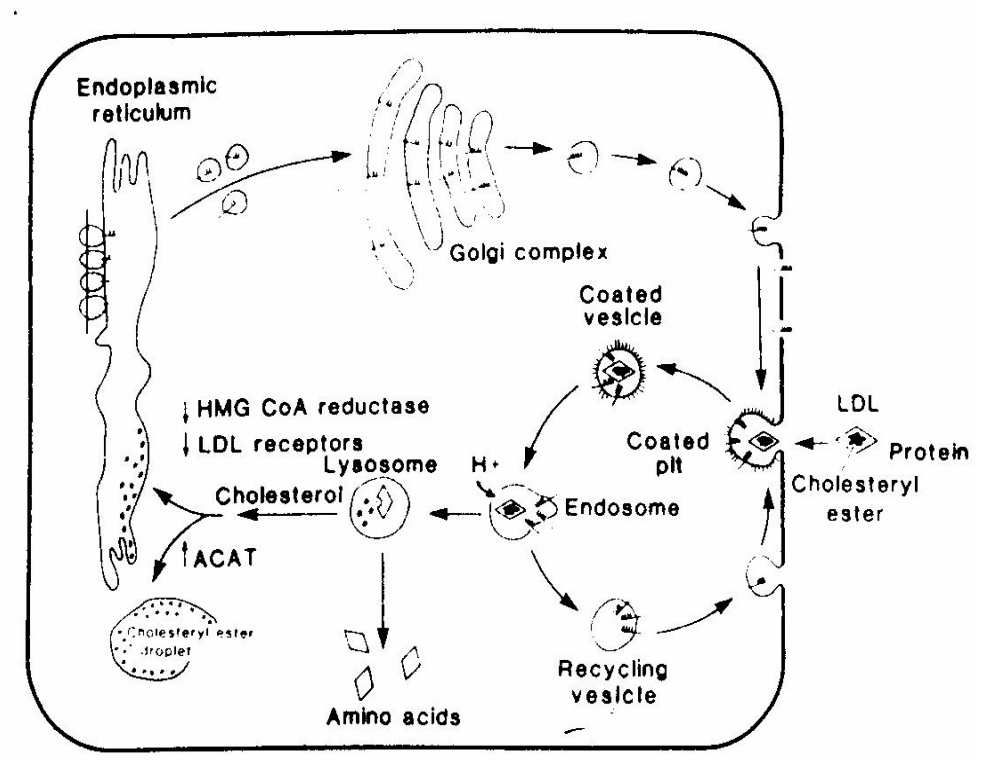


Figure 3. Route of the LDL receptor in mammalian cells. The receptor begins life in the endoplasmic reticulum from which it travels to the Golgi complex, cell surface, coated pit, endosome, and back to the surface. HMG-CoA reductase denotes 3-hydroxy-3-methylglutaryl-CoA reductase; ACAT denotes acyl-CoA: cholesterol acyltransferase (ACAT). (Reprint from Brown and Goldstein, 1985)

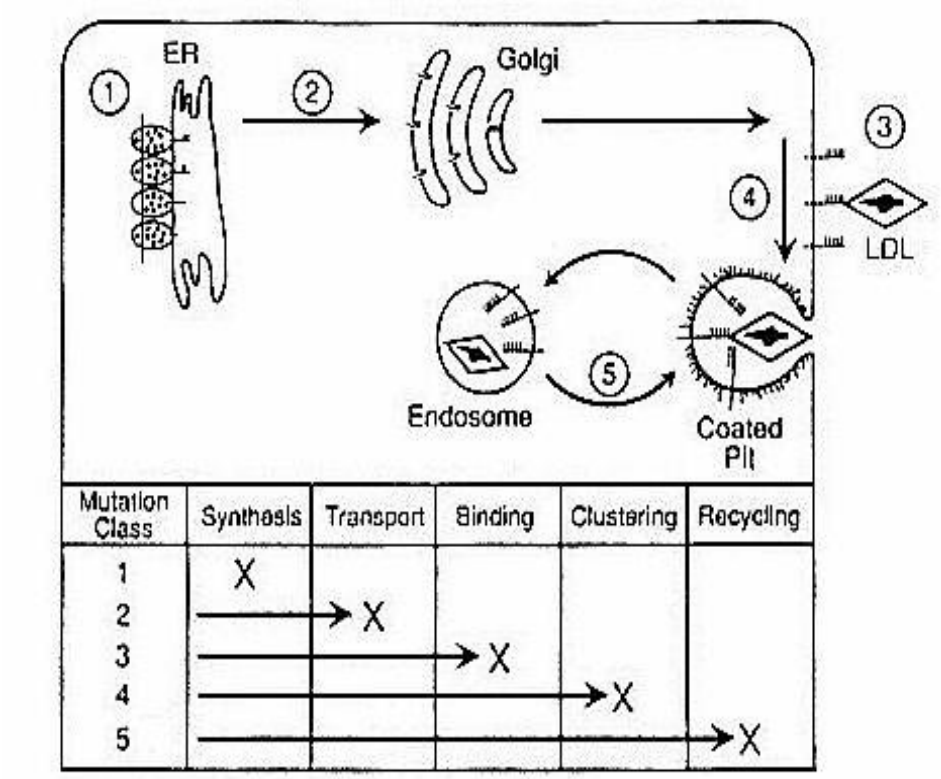


Figure 4. Five classes of mutations at the LDL receptor locus. These mutations disrupt the receptor's synthesis in the endoplasmic reticulum (ER), transport to the Golgi, binding of apolipoprotein ligands, clustering in coated pits, and recycling in endosomes. (Reprint from Hobbs et al., 1990)

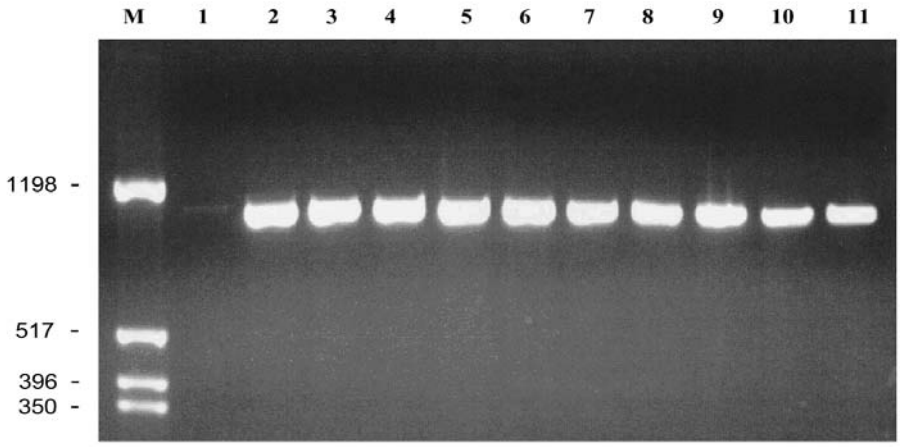


Figure 5. RT-PCR analysis of LDL receptor cDNA variants. The amplified products are examined by 1% agarose gel electrophoresis. Lane 1, pcDNA3; lane 2, wild-type cDNA; lane 3, D69N; lane 4, R94H; lane 5, E207K; lane 6, C308Y; lane 7, I402T; lane 8, A410T; lane 9, A696G. Lane M contains molecular weight markers (*Hin*fI digest of pGEM-4 DNA).

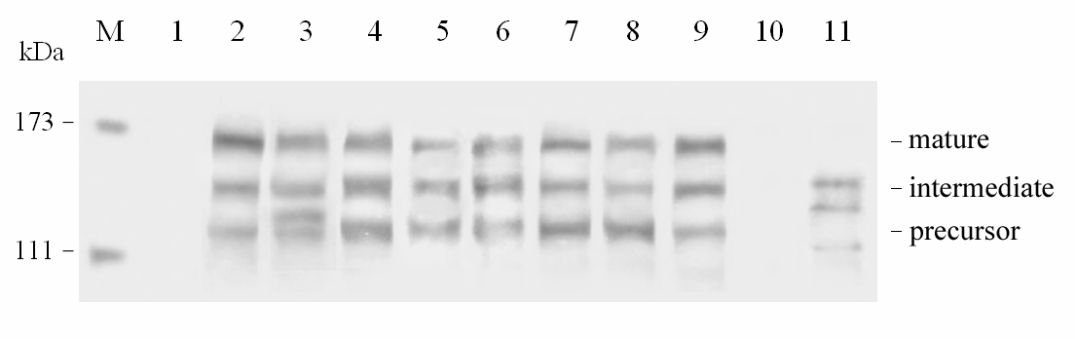


Figure 6. Western analysis of LDL receptor cDNA variants. Total protein (10 μg) was separated on 6% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and stained with LDL receptor polyclonal antibody. Molecular mass of LDL receptor protein is indicated as determined from a plot of migration distances of standard proteins against the logarithms of molecular masses. Lane 1, pcDNA3; lane 2, wild-type; lane 3, D69N; lane 4, R94H; lane 5, E207K; lane 6, C308Y; lane 7, I402T; lane 8, A410T; lane 9, A696G; lane 10, Del e3-5; lane 11, Del e6-8. Lane M contains molecular weight markers.

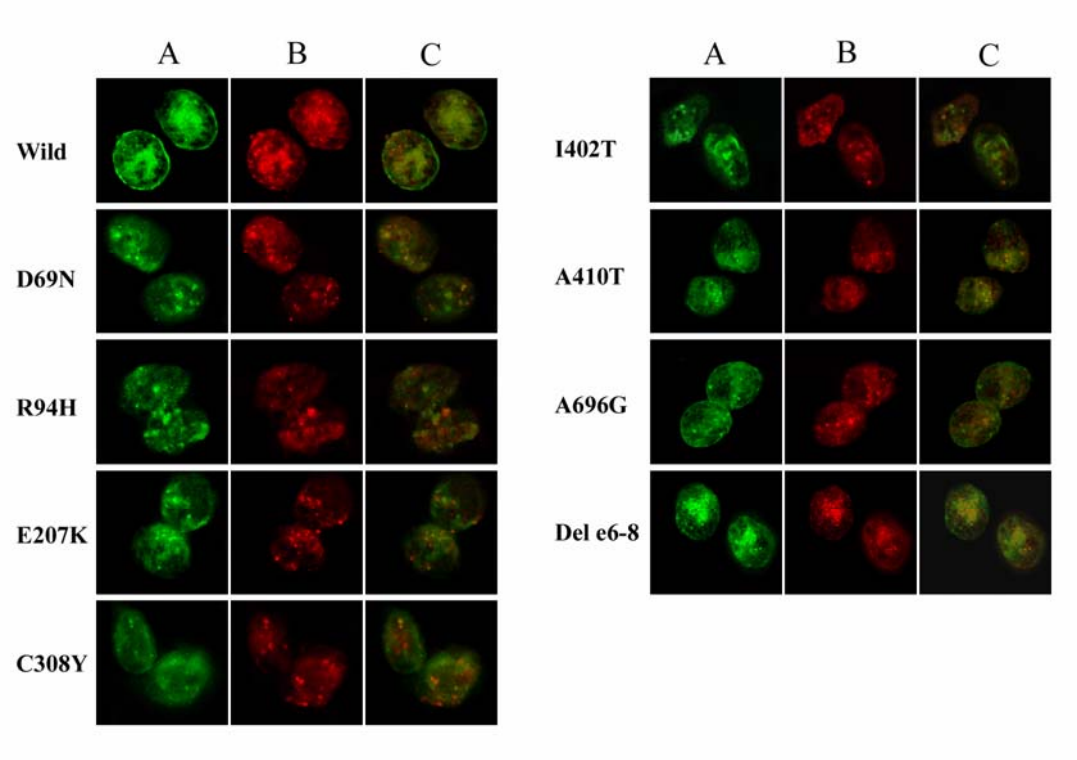


Figure 7. Colocalization analysis of COS-7 cells transfected with pcDNA3-LDLR plasmids. Transfected cells were analyzed by fluorescence microscopy simultaneously for LDL receptor immunofluorescence (green, A) and lysosome fluorescence (red, B). The sum of these two images is also shown C. Original magnification 600.

Table 1. LDL receptor mutations in hyperlipidemic Chinese

Mutation[a]	Nucleotide alteration	Protein alteration	Patient
W-18X	TGG→TGA (exon 1)	Trp_{-18}→Stop	D102
D69N	CGC→CAC (exon 3)	Asp_{69}→Asn	A133, L41
R94H	CGC→CAC (exon 4)	Arg_{94}→His	A144
E207K	GAG→AAG (exon 4)	Glu_{207}→Lys	95-123
C308Y	TGC→TAC (exon 7)	Cys_{308}→Tyr	95-035, D63[b]
I402T	ATC→ACC (exon 9)	Ile_{402}→Thr	D101
A410T	GCT→ACT (exon 9)	Ala_{410}→Thr	D142
I602V[c]	ATC→GTC (exon 13)	Ile_{602}→Val	A107, D96, D194
A696G	GCT→GGT (exon 15)	Ala_{696}→Gly	D162
Del e3-5	Exons 3-5 deleted	Deletion of 209 aa (44-252)	D72
Del e6-8	Exons 6-8 deleted	Deletion of 123 aa (252-374)	D254[b]

[a]Number of amino acids according to Sudhof et al., 1985.

[b]Homozygosity for the mutation.

[c]Normal variant allele, which is presumed to be a polymorphism.

Table 2. Primers for site-directed mutagenesis

Mutation	Primer sequence
R94H	CCCAGGACGAGTTTC**A**CTGCCACGATGGGAAG
	CTTCCCATCGTGGCAG**T**GAAACTCGTCCTGGG
E207K	GCAAGGACAAATCTGAC**A**AGGAAAACTGCGCTGTG
	CACAGCGCAGTTTTCCT**T**GTCAGATTTGTCCTT

Table 3. Flow cytometric measurement of polyclonal antibody binding and DiI-LDL binding and internalization

Transfected vector	Antibody 4°C	DiI-LDL 37°C
pcDNA3	1404 ±29 (0%)	5002 ± 116 (0%)
pcDNA3-LDLR	78377 ± 1095 (100%)	141737 ± 3043 (100%)
pcDNA3-LDLR/D69N	43112 ± 2734 (54%)	80246 ± 1571 (55%)
pcDNA3-LDLR/R94H	50932 ± 1443 (64%)	92067 ± 2114 (64%)
pcDNA3-LDLR/E207K	20107 ± 455 (24%)	34017 ± 730 (21%)
pcDNA3-LDLR/C308Y	25600 ± 323 (31%)	46753 ± 643 (31%)
pcDNA3-LDLR/I402T	47018 ± 255 (59%)	79373 ± 1704 (54%)
pcDNA3-LDLR/A410T	18301 ± 1655 (22%)	32119 ± 656 (20%)
pcDNA3-LDLR/A696G	76796 ± 793 (98%)	132670 ± 2393 (93%)
pcDNA3-LDLR/Del e3-5	1422 ± 35 (0%)	5148 ± 102 (0%)
pcDNA3-LDLR/Del e6-8	11757 ± 164 (13%)	21719 ± 194 (12%)

The cumulated fluorescence signals are the mean of three independent measurements and standard deviation. Values in parentheses represent the percentage of wild type.