Evaluation of Receptor- Mediated Gene Transfer using an Integrin- Targeting Vector as a Potential Form of Therapy for Lysosomal Storage Diseases

by

Elaine Joanna White

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Unit of Biochemistry, Endocrinology and Metabolism
Institute of Child Health (University College London)
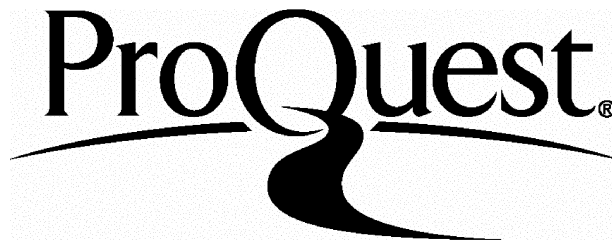30 Guilford Street
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Preface

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Abstract

Lysosomal storage diseases (LSD) result from deficiencies of enzymes or structural proteins involved in the catabolism of macromolecules inside the lysosome. In this study non-viral receptor-mediated gene transfer into fibroblasts from patients with the LSDs, fucosidosis and Fabry disease, which result from deficiencies of α- L- fucosidase and α- galactosidase A respectively, has been investigated as a possible form of therapy for LSDs.

The biochemical and molecular basis of the deficiency of α- L-fucosidase in the fucosidosis patients used in the study was investigated. The residual α- L- fucosidase activity and the amount of material cross-reacting with anti- α- L- fucosidase antibodies were determined. Patient genomic DNA was haplotyped for the Q/R281 polymorphism and analysed by single strand conformation polymorphism analysis (SSCP) followed by sequencing to identify the mutations in the α- L- fucosidase gene.

In this study a non-viral vector, which exploits receptor-mediated endocytosis to target and enter cells, was used for gene delivery. This vector (LID complex) consisted of plasmid DNA complexed with the poly-lysine domain of a peptide containing an integrin- targeting domain and Lipofectin to aid endosomal escape of the complex. Transfection of fucosidosis fibroblasts with the luciferase reporter gene was shown to be less efficient than transfection of normal fibroblasts. Transfection of LSD patient fibroblast cultures with the normal cDNA of the gene that is defective resulted in a large increase in the enzyme activity of the defective enzyme. This activity was mainly secreted into the culture medium suggesting that the introduced gene product is not being correctly targeted to the lysosome. However, secretion of enzyme would be of some advantage for gene therapy if the enzyme were taken up by other cells via the mannose-6-phosphate receptor in the plasma membrane. Transfection of fibroblasts with LID complexes caused a small decrease in the intracellular activity of endogenous lysosomal enzymes and a small increase in their extracellular activity. This finding highlights the need for further basic studies on non-viral gene delivery systems to examine their modes of delivery and their effects on cells.

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List of abbreviations

Amp	ampicillin
BCA	Bicinchoninic acid
BMT	bone marrow transplantation
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
con A	conconavalin A
CMV	cytomegalovirus
CRIM	cross reactive immunological material
DAPI	4',6- diamidino- 2- phenylindole
DTT	dithiothreitol
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ddNTP	dideoxy nucleoside triphosphate (ddATP, ddCTP, ddGTP, ddTTP)
dNTP	deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)
DOPE	Dioleoyl phosphatidylethanolamine
DOPSA	2,3-dioleyloxy-N(sperminecarboxamido)ethyl)-N, N-dimethyl-1-propanaminium trifluoroacetate
DOTMA	N-[1-((2,3,-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
EBV	Epstein Barrr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ELISA	double antibody sandwich enzyme linked immunosorbant assay
ER	endoplasmic reticulum
FCS	Foetal calf serum
GAG	glycosaminoglycan
h	hour
HLS	human liver supernatant
kDa	kilo Dalton
Km	Michaelis- Menten constant
LB	Luria- Bertaini
LTR	long terminal repeat

M	molar
MPR	mannose- 6- phosphate receptor
Mr	molecular weight
MRI	magnetic resonance imaging
mRNA	messenger RNA
MU	methylumbelliferyl
MV4.5	McIlvaine citrate phosphate buffer
LID	Lipofectin- integrin targeting peptide- DNA
OD	optical density
PBS	phosphate- buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
RGD	arginine- glycine- aspartic acid
RLB	reporter lysis buffer
RLU	relative light units
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SSCP	single- strand conformation polymorphism analysis
TBE	tris- borate- EDTA buffer
TBS	tris buffered saline
TE	tris- EDTA buffer
TEMED	N, N, N', N'- tetramethylethylenediamine
TES	tris- EDTA sodium buffer
Tm	annealing temperature
Tris	tris (hydroxymethyl) aminomethane
TSB	transformation storage buffer
UV	ultra violet

Introduction

1.1. Background; morphology and cellular organisation of lysosomes

Lysosomes were first isolated as a membrane-bound cytoplasmic organelle fraction from a rat liver homogenate by differential centrifugation (De Duve et al., 1955). The lysosomal fraction was found to contain five hydrolytic enzymes, which acted on different sets of substrates and had acidic pH optima. This suggested a non-specific lytic function for the organelle and it was named the lysosome ('lytic sub-cellular particle') (De Duve et al., 1955). Lysosomes are now known to contain at least 40 acid hydrolase enzymes, which are responsible for the degradation of almost all classes of macromolecules. They were first visualised by Novikoff using electron microscopy in 1955 (reviewed by Novikoff, 1973; Bainton, 1981). They have a ubiquitous distribution and are found in all nucleated plant and animal cells.

A lysosome can be defined as a membrane- bound cytoplasmic organelle involved in intracellular digestion and processing of a wide range of exogenous and endogenous molecules, by acid hydrolases. The functional and morphological diversity of lysosomes has led to a range of terms being used to indicate the exact status of the lysosome e.g. primary lysosome, secondary lysosome, pre- lysosome, autosome etc. (Novikoff, 1973). The primary role of the lysosomal system is the degradation of both intra- and extracellular macromolecules to their basic constituents as part of a diverse, dynamic intracellular digestive system. Lysosomal enzymes are also involved in peptide processing, activation of hormones and regulation of cholesterol synthesis (Goldstein and Brown, 1977). These functions impose structural constraints upon the lysosome. It has a 9nm thick lipoprotein membrane, which is needed to form a closed compartment for hydrolysis so that hydrolytic enzymes do not encounter other cellular constituents. The lysosomal membrane is impermeable to large molecules therefore, macromolecules destined for degradation enter lysosomes when they are enveloped by a membrane, either as a result of engulfment by the lysosomal membrane or fusion of a digestive vacuole with the lysosome. The macromolecules that are to be degraded are delivered to the

functional lysosomes by endosomes or phagosomes. The delivery of degradable products via endosomes involves the general pathways of endocytosis, micro- and macroautophagy.

The lysosomal membrane also contains transport proteins that carry the final products of macromolecular degradation such as amino acids, fatty acids, carbohydrates and nutrients out of the lysosome for re- use or excretion (Kornfeld and Mellman, 1989). The lysosomal membrane proteins are unusually highly glycosylated and it is postulated that the glycans protect the inner side of the lysosomal membrane from digestion by the lysosomal hydrolases in the lumen (Holtzmann, 1989). In order to maintain the lumen at the low pH required for optimal function of the hydrolases, the lysosomal membrane contains an ATP-dependent proton pump, which uses energy from ATP hydrolysis to pump H^+ into the lumen. The low pH of this compartment also aids the killing of microbes taken up by phagocytes and promotes denaturation and conformational changes in macromolecules thus rendering them more accessible for degradation (Holtzmann, 1989).

Lysosomes contain a battery of hydrolytic enzymes, and a few other enzymes, as well as other appropriate cofactors necessary for the degradation of all naturally occurring macromolecules and complex molecules of intra- and extracellular origin. All hydrolytic enzymes and other lysosomal proteins are glycoproteins synthesised in the endoplasmic reticulum (ER) and transported to lysosomes via the Golgi network for necessary processing and maturation. The vesicles containing these lysosomal proteins are transported from the trans-Golgi network and are fused with the pre-lysosomal compartments such as the endosomes to form the lysosome. Final maturation takes place within the lysosome and the enzymes assume their active conformation. The lysosome may further fuse with other endosomes or phagosomes.

1.2. Lysosomal enzymes

Lysosomal enzymes include a variety of nucleases, proteases, glycosidases, lipases, phospholipases, sulphatases and phosphatases. A list of lysosomal enzymes is given in the Appendix 1.1. Most of the enzymes have been purified to homogeneity, cloned and their amino acid sequence determined or deduced (Gieselmann, 1995). There is a low sequence homology

within each group of enzymes and they do not have any characteristic cDNA or amino acid sequences except for a similar signal peptide sequence. However, there is generally high level of homology for the same enzyme across species, suggesting they have evolved from a common ancestral gene.

1.2.1. Synthesis and processing of soluble lysosomal enzymes

Soluble lysosomal enzymes are synthesised on ribosomes attached to the rough endoplasmic reticulum (ER) as larger inactive precursors (prepropolypeptides). The prepropolypeptide contains a signal sequence, a stretch of 15-30 mainly hydrophobic amino acids localised in the N- terminus of the nascent polypeptide. This signal sequence mediates cotranslational translocation to the ER lumen by interaction with the signal recognition particle (SRP) and SRP- receptor (Erickson et al., 1984). Following entry to the ER lumen the signal peptide is cleaved off by a signal peptidase, prior to the completion of translation of the mRNA (Von Figura and Hasilik, 1986).

As the polypeptide chain enters the ER it is co-translationally N-glycosylated by the transfer of a pre-assembled oligosaccharide ($Glc_3Man_9Glc NAc_2$) from the dolichol pyrophosphate carrier to selected asparagine residues in the canonical N-glycosylation sites (Asn-X-Thr-, where X can be any amino acid except proline) (Pless and Lennarz, 1977; Kronquist and Lennarz, 1978; Kornfeld and Mellman, 1989; Gieselmann, 1995; Braulke, 1996). The polypeptide linked N- glycans are then processed by the removal of three glucose residues and one mannose residue by glucosidase I and II and α-mannosidase I within the ER. The polypeptide also undergoes other modifications such as disulphide bond formation, folding and oligomerisation of polypeptides.

The polypeptide is then transferred to the Golgi apparatus by a vesicular mechanism where it undergoes further modifications, which include processing of N- linked oligosaccharide chains to hybrid or complex type (Kornfeld and Mellman, 1989). α- Mannosidase I trims the chain down to 5 mannoses, then GlcNAc transferase I adds a GlcNAc and α- mannosidase II then removes two further mannoses generating $GlcNAc\ Man_3\ GlcNac_2$. Finally, further GlcNAc, GalNAc, sialic acid and sometimes fucoses are added by the appropriate glycosyl transferases (Beyer et al., 1981; Kornfeld and Kornfeld, 1985).

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1.2.2. Targeting of lysosomal enzymes

A major event in the processing of lysosomal enzymes for their delivery to the lysosome is the acquisition of the mannose- 6- phosphate (M6P) recognition signal by phosphorylation of the C-6 of one or two mannose residues in high mannose oligosaccharide chains of the precursor polypeptides (Hancock and Dawson, 1989; Gieselman, 1995). It occurs in a two- step reaction. First, N- acetylglucosamine- 1- phosphotransferase (phosphotransferase, EC2.7.8.17) recognises a conformational protein signal common to all soluble lysosomal proteins and transfers N- acetylglucosamine- 1- phosphate from UDP-GlcNAc to one or more mannose residues, generating phosphodiester forms. The second enzyme, N- acetylglucosamine- 1- phosphodiester- α- N- acetylglucosaminidase (EC3.1.4.45), which is located in a later mid- Golgi compartment , removes the terminal N- acetylglucosamine residue, thereby generating the mannose- 6- phosphate recognition signal (Kornfeld and Mellman, 1989).

The mannose- 6- phosphate residues function as a high- affinity recognition signal for mannose- 6- phosphate receptors (MPRs) in the Golgi apparatus. These receptors shuttle between the trans Golgi network and the pre- lysosomal compartment. Two MPRs are known, both of which are transmembrane glycoproteins; MPR46 (cation- dependent) and MPR300 (cation independent) (Holtzmann, 1989; Kornfeld and Mellman, 1989). They differ in ligand binding properties, transport function and in their efficiency. Both MPRs have a role in intracellular sorting of lysosomal enzymes (Koster et al., 1993). MPR 300 is also located on the plasma membrane. As well as binding to mannose- 6- phosphate ligands it also binds to insulin like growth factor II (IGFII) at a different binding domain. Exogenous enzymes containing mannose- 6- phosphate ligands can bind to MPR300 on the plasma membrane and be taken up by receptor- mediated endocytosis (Watanabe et al., 1990).

The sorting of enzymes is believed to occur in the trans- Golgi network followed by translocation of clathrin- coated vesicles containing the MPR- ligand complexes, to a pre- lysosomal compartment. The low pH in these endosomes, created by an ATP- driven proton pump, causes the receptor and precursor enzyme to dissociate (Schmid et al., 1989). This allows recycling of the receptor

to the Golgi apparatus. During the maturation of precursor forms of lysosomal hydrolases their oligosaccharides become dephosphorylated.

The physiological importance of mannose- 6- phosphate residues in targeting lysosomal enzymes is observed in the disease mucolipidosis II or I-cell disease. In this disease the correct targeting of lysosomal enzymes is affected due to a deficiency of the phosphotransferase. This defect leads to the secretion of most of the lysosomal enzymes by many cell types, including fibroblasts (Hasilik et al., 1981).

There is evidence for a M6P independent targeting pathway, which is not yet fully understood but may be particularly important in the nervous system (Hancock and Dawson, 1989). Its existence has been shown in studies of mucolipidosis II where the phosphotransferase is absent but some cell types, such as hepatocytes and B- lymphocytes have normal lysosomal enzyme activities. It seems to be a minor pathway and accounts for approximately 5-10% of lysosomal enzyme delivery (Kornfeld and Mellman, 1989). Evidence is increasing that at least in some mammalian cells some soluble lysosomal enzymes are targeted to lysosomes by a mechanism dependent on a protein signal rather than on mannose- 6- phosphate receptors. This signal could be constituted by a short amino acid sequence or by a conformational signal.

1.2.3. Regulation of lysosomal enzyme activity

The synthesis and turnover of lysosomal enzymes is generally considered to be constitutive although possibly regulated by mRNA induction (Hancock and Dawson, 1989). Since the expression of particular enzymes varies in different cells and conditions it is unlikely that the expression is regulated by transcription and translation control elements. Nevertheless, some genes have upstream control elements found in certain housekeeping genes (Neufield, 1991). An acidic pH environment and cofactors are of primary importance for these enzymes (Hancock and Dawson, 1989). A number of lysosomal enzymes require divalent cations in order to express full activity e.g. α- mannosidase. In some pathways there is physical association and organisation of proteins. For instance, β- galactosidase forms a complex with α-neuraminidase and the protective protein, cathepsin A, and also requires the activator protein saposin C when acting on a lipid substrate (Pshezhetsky and

Porter, 1996). This suggests that the specificity of β- galactosidase can be modified by association with other proteins. Some lysosomal hydrolases associate with non- enzymic proteins and formation of non- covalent multimeric complexes may organise and co-ordinate the action of the enzymes within a catabolic pathway and protect against proteolytic degradation.

1.3. Lysosomal storage diseases

The concept of a lysosomal storage disorder as the result of a deficiency of a lysosomal hydrolase was first introduced by Hers in 1965 on the basis of a deficiency of the enzyme α-glucosidase (acid maltase) being responsible for the fatal glycogen storage disease type II (Hers, 1965). As the degradation of a macromolecule requires stepwise removal of a moiety by a specific hydrolase, the reaction product of one enzyme becomes the substrate for the next enzyme in the pathway. The failure to remove a terminal residue in this stepwise reaction, due to the deficiency of a specific hydrolase, renders the product un-degradable by the other enzymes. This results in the progressive accumulation in the lysosome of catabolic intermediates, which are unable to pass through the lysosomal membrane. This intralysosomal storage leads to the malfunction of the cells and organs and to the progressive clinical symptoms of the lysosomal storage diseases. The pathogenesis of lysosomal storage diseases is poorly understood.

The main cause of lysosomal storage diseases is a defect in a gene encoding a lysosomal enzyme. Other causes are defects in lysosomal membrane transport, non- enzymic protein cofactors and processing of lysosomal enzymes. A defect in lysosomal membrane transport system can prevent entry of substrates or the release of breakdown products from the lysosomes e.g. cystinosis where there is a failure in the transport mechanism which removes cystine from the lysosomes. A deficiency of an activator or a protective protein may also produce a deficiency of the enzymic activity e.g. galactosialidosis which is caused by a deficiency of a 32kDa protein which normally stabilises β-D- galactosidase and α- neuraminidase and protects them from intralysosomal proteolytic degradation (D'Azzo et al., 1982). A defect in the targeting processes responsible for the delivery of the enzyme to lysosomes can produce a loss of intra-lysosomal activity e.g. mucolipidosis II where a

deficiency of N- acetyl glucosaminyl phosphotransferase leads to incorrect targeting of the lysosomal enzymes. The lysosomal storage disease multiple sulphatase deficiency is caused by a defect in a novel post- translational modification. This modification normally oxidises the thiol group of a cysteine that is conserved across all eukaryotic sulphatases generating a 2- amino- 3- oxopropanoic acid residue which is essential to function (Von Figura et al., 1998).

1.3.1. Molecular genetics and phenotypic variation in lysosomal storage diseases

All lysosomal storage diseases have an autosomal recessive pattern of inheritance except for the X- linked recessive Hunter and Fabry diseases (Galjaard and Reuser, 1984). Lysosomal storage diseases commonly display phenotypic heterogeneity (Gieselman, 1995) and are often divided into different forms depending on their clinical severity and the age of onset of clinical symptoms (severe infantile, intermediate juvenile and adult). However, in most disorders these distinctions are probably artificial because there is a continuum of clinical symptoms between the extremes. It has been shown for many disorders that defects in the same gene can cause very different phenotypes (reviewed by Gieselman, 1995). For example the neurodegenerative features of GM1-gangliosidosis and the skeletal abnormalities in Morquio B disease are both due to deficiencies of β-galactosidase and the clinical symptoms depend on the nature of the mutation (Oshima et al., 1994).

The phenotypic variation in lysosomal storage diseases is therefore largely due to genotypic heterogeneity and within each disease a multiplicity of disease alleles has been noted. Some studies of correlation between genotype and phenotype reveal that the age of onset and severity of disease are related to the residual enzyme activity in the patient. However, in most cases the relationship is not so clear, for example in patients with mucopolysaccharidosis type I (MPS I) residual enzymic activity of less than 1% of the control activity of α-iduronidase may lead to the severe Hurler phenotype or to the less severe Scheie phenotype (Galjaard and Reuser, 1984). However, these studies measure enzyme activity using artificial substrates and may not reflect the true residual activity which probably correlates with phenotype.

The clinical variability between patients with identical genotypes, in lysosomal storage diseases like fucosidosis, suggests other genetic or epigenetic factors are involved and makes the prognosis difficult to judge. Many patients with a lysosomal storage disease have unique, rare mutations. When the same mutation(s) is found in more than one patient it tends to be within a restricted ethnic group, due to a founder effect or where there is consanguinity. Panethnic mutations may also be due to "hot spots" within genes.

Low levels of activity in heterozygotes for lysosomal storage diseases is sufficient to prevent storage and in fact very little residual activity may protect against symptoms. Conzelman and Sandhoff (1983) proposed a model for 'critical threshold of activity' above which the enzyme can keep up with substrate influx and below which accumulation of undegraded substrate occurs. This critical threshold may differ among cell types or in cells at different stages of development. This explains why different mutations may produce proteins with small differences in residual activity which could affect the rate of storage and therefore affect ages of onset, disease progression and symptoms in individuals (reviewed by Neufeld, 1991; Kolter and Sandhoff, 1998).

1.3.2. Classification of lysosomal storage diseases according to storage material and enzyme defect

The lysosomal storage disorders have been classified according to the catabolic pathway affected and the nature of the predominant storage material (Gieselman, 1995). The main groups are the mucopolysaccharidoses, glycoproteinoses, sphingolipidoses and glycogen storage diseases. There is some overlap of storage material between groups since some lysosomal enzymes are specific for hydrolysis of a specific linkage, which may be found in different macromolecules e.g. the α-fucosidic linkage is found in both glycolipids and glycoproteins (Hers, 1973). Likewise, within different groups, similar storage and excretion products and ultrastructural appearance of storage material have been reported (Goebel and Lake, 1998).

1.4. Fucosidosis

Fucosidosis (McKusick number 23 000) is an autosomal recessive disorder resulting from mutations in the human FUCA 1 gene, which encodes

the lysosomal hydrolase α- L- fucosidase (EC3.2.1.51). Historically, the disorder was first recognised clinically by Durand and colleagues in two Italian siblings (Durand et al., 1966). Subsequent studies by this group and others demonstrated the presence of fucose- containing material in the liver and brain tissues of the patients and the underlying enzyme defect was identified as a deficiency of lysosomal α- L- fucosidase (Durand et al., 1966; Van Hoof and Hers, 1968).

1.4.1. Clinical features

Historically, fucosidosis was classified into two types; type I and type II (Willems et al., 1988). Type I comprises a minority of patients with severe symptoms presenting at age 5-12 months. The patient dies from pulmonary infections and pneumonia in the first decade of life. Type II is a milder disorder with the patients presenting with symptoms one to two years after birth and generally surviving into adulthood. The division into two types is arbitrary since a range of clinical phenotypes has been observed between these two extremes (Willems et al., 1988). The main neurological involvement in patients includes psychomotor delay, seizures, loss of hearing and visual acuity, emotional disturbances and progressive neurological deterioration with loss of mental and motor abilities leading to dementia and a vegetative state. There are also skeletal problems such as coarse facial features, growth retardation, dysostosis multiplex, joint contractures and kyphoscoliosis, a deformity of the spine with flexion and lateral curvature. The spleen is usually enlarged. Angiokeratoma corporis diffusum, which is a purple, pin-head size rash first seen on the scrotum and the lower extremities and later on the abdomen, upper extremities and back may also be present. Immunologically patients suffer from recurrent infections and usually die from respiratory disturbances and infections (Cantz and Ulrich Bott, 1990; Willems et al., 1991).

Histopathology of fucosidosis

Enlargement of brain, heart, liver, spleen and pancreas have been observed on autopsy examination (Thomas and Beaudet, 1998). There is extensive vacuolation in several tissues (Kouseff et al., 1976; Libert et al., 1976; Kornfeld et al., 1977) and some cells have large irregular intracellular spaces

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caused by fusion of ruptured vacuoles. Liver biopsy studies showed foamy cytoplasm in some hepatocytes and Kupffer cells (Thomas and Beaudet, 1998).

1.4.2. Biochemical features

Deficiency of α- L- fucosidase

Normal human α- L- fucosidase has been purified from autopsy liver sample using affinity chromatography by Alhadeff et al (1975a). It is a tetramer, with each subunit having a molecular weight of about 58kDa. The enzyme has a broad pH range with two optima, pH 4-5 and pH 6.5 (Alhadeff and Andrews Smith, 1978; Willems et al., 1991). Km values have been reported to range from 0.17mM- 0.53mM for the p- nitrophenyl substrate depending on the isoenzyme (Alhadeff et al., 1978c). The enzyme has been shown to have 4 active sites per tetrameric α- L- fucosidase molecule (White et al., 1987; Thomas and Beaudet, 1998) and a general acid- base mechanism of catalysis has been suggested with proton transfer as the rate limiting step (White et al., 1987).

Expression of α- L- fucosidase commences as early as 5-7 weeks of gestation in the liver and activity has also been found in amniotic fluid (Wiederschain et al., 1971). α- L- fucosidase activity in serum, peaks at 10-15 days after birth, remains high into the second month and gradually decreases in the first year to a level, which remains relatively constant for the rest of life (Vaysse et al., 1990).

α- L- fucosidase exists as multiple molecular forms which are encoded by a single locus (Alhadeff et al., 1974a). Isoelectric focusing (IEF) profiles of the α- L- fucosidase in most cell types are similar with 6-9 isoforms whose isoelectric points range between pH5-7 (Alhadeff et al., 1974a; Turner et al., 1974, 1975b; Thorpe and Robinson, 1975). The multiple forms of α- L- fucosidase result mainly from the presence of different numbers of sialic acid residues on the glycans (Di Matteo et al., 1976; Alhadeff and Freeze, 1977; Alhadeff et al., 1978c), as only 2-3 predominant isoenzymes remain after neuraminidase treatment (Turner et al., 1974). The IEF patterns of α- L- fucosidase were ascribed to three phenotypic groups and their inheritance was consistent with the segregation of two codominant alleles at a single autosomal locus, named Fu1 and Fu2 where Fu2 is the minor allele. Fu1 encodes for the

cathodal enzyme form, Fu2 the anodal form and Fu1/ Fu2 heterozygotes a mixture of the two. The polymorphism was mapped to Chr1p34.1 within the α- L- fucosidase structural gene, FUCA1. It was suggested that the production of the three isoform phenotypes may be due to a polymorphism at the DNA level in the α- L- fucosidase gene producing small alterations in the polypeptide backbone. This was further substantiated by studies on the polymorphism (Cragg et al., 1994; Yang and DiCioccio, 1994). The polymorphism is Q/R281, an A to G transition which causes substitution of an arginine for a glutamine. This substitution causes an increase in the positive charge in the enzyme polypeptide which affects its electrophoretic mobility but not its catalytic activity. This is superimposed on the structural microheterogeneity due to different post-translational modifications, such as N- glycosylation, of the enzyme polypeptide (Johnson et al., 1992).

α- L- fucosidase is involved in the breakdown of glycoproteins and glycolipids (Figure 1.1) and a deficiency would lead to incomplete digestion of these macromolecules. A deficiency of α- L- fucosidase has been detected in all tissues including liver, brain, lung, fibroblasts and leukocytes of fucosidosis patients (Cantz and Ulrich Bott, 1990; Durand et al., 1982). The residual enzymic activity in patients is less than 5% of normal control levels in fibroblasts, leukocytes and EBV- transformed lymphoblastoid cell lines (Thorpe and Robinson, 1978; Alhadeff and Andrews- Smith, 1980b; Johnson and Dawson, 1985; Willems et al., 1991).

Low plasma activity polymorphism

A small number of the population have plasma α- L- fucosidase activities between 10-30% of the control mean and yet are phenotypically normal (Ramage and Cunningham, 1975; Ng et al., 1976). The low activity polymorphism is inherited in an autosomal recessive manner and has been linked to the FUCA 2 locus on chromosome 6 (Eiberg et al., 1984). FUCA2 is genetically independent of FUCA1 and it is not clear if FUCA2 is a structural or a regulatory gene.

NeuAc$^{\alpha 2}$—^{6}Gal$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{2}Man$^{\alpha 1}$ \
                                            Fuc$^{\alpha 1}$ \
                 $_3$^{6}Man$^{\beta 1}$—^{4}Glc^{6}NAc$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—Asn \
NeuAc$^{\alpha 2}$—^{6}Gal$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{2}Man$^{\alpha 1}$

↓ Proteases

NeuAc$^{\alpha 2}$—^{6}Gal$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{2}Man$^{\alpha 1}$ \
                                            Fuc$^{\alpha 1}$ \
                 $_3$^{6}Man$^{\beta 1}$—^{4}Glc^{6}NAc$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—Asn \
NeuAc$^{\alpha 2}$—^{6}Gal$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{2}Man$^{\alpha 1}$

↓ α- L- fucosidase

NeuAc$^{\alpha 2}$—^{6}Gal$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{2}Man$^{\alpha 1}$ \
                 $_3$^{6}Man$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—Asn \
NeuAc$^{\alpha 2}$—^{6}Gal$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{2}Man$^{\alpha 1}$

↓ Aspartyl- N- acetylglucosamine

NeuAc$^{\alpha 2}$—^{6}Gal$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{2}Man$^{\alpha 1}$ \
                 $_3$^{6}Man$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{4}GlcNAc \
NeuAc$^{\alpha 2}$—^{6}Gal$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{2}Man$^{\alpha 1}$

↓ Endo- N- acetylglucosaminidase

NeuAc$^{\alpha 2}$—^{6}Gal$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{2}Man$^{\alpha 1}$ \
                 $_3$^{6}Man$^{\beta 1}$—^{4}GlcNAc \
NeuAc$^{\alpha 2}$—^{6}Gal$^{\beta 1}$—^{4}GlcNAc$^{\beta 1}$—^{2}Man$^{\alpha 1}$

    ←— α- Neuraminidase \
    ←— β- Galactosidase \
    ←— β- Hexosaminidase \
    ←— α- Mannosidases

Man$^{\beta 1}$—^{4}GlcNAc

↓ β- Mannosidase

Man + GlcNAc

Figure 1.1 Lysosomal degradation of asparagine- linked oligosaccharide chains of glycoproteins. Gal, Man, Fuc, GalNAc, NeuAc and Asn are galactose, mannose, fucose, N-acetylgalactosamine, N- acetylneuraminic acid and asparagine respectively

Storage products

The methyl- pentose, L- fucose, is a component of many important biological molecules, in which it is usually linked α (1>2) to galactose, α (1>3) to N- acetylglucosamine or α (1>6) to N- acetylglucosamine. The accumulation of fuco- glycoconjugates (fuco- glycopeptides, fuco- oligosaccharides, fuco- sphingolipids and keratan sulfate) has been reported in lysosomes of most tissues such as brain, fibroblasts and spleen and in body fluids like urine and serum (Tsay et al., 1976; Michalski et al., 1991). Histological examination demonstrates storage in most tissues (Willems et al., 1991). The actual storage material found in fucosidosis cells depends on the distribution and turnover of glycoconjugates in that particular tissue e.g. there is major storage of glycolipid in liver but little accumulation in brain. The major storage material in pancreas and liver is a fucose- containing ceramide pentahexoside. In the brain the main storage material was reported to be a fucose- containing decasaccharide but this is unconfirmed (Tsay et al., 1976; Tsay and Dawson, 1976). There is an increase of fucose- containing specific blood group substances with abnormal antigenicity in fucosidosis since α- L- fucosidase is important in the catabolism of fucose- containing Lewis blood group substances (Durand et al., 1982).

80-90% of the storage material consists of fucosylated glycoasparagines (Willems et al., 1991). Fucosidosis patients also excrete fucose- containing glycopeptides and oligosaccharides in their urine. Most of these abnormal metabolites have fucose α (1>6) linked to N- acetylglucosamine (Michalski et al., 1991). About 30 different glycopeptides have been isolated in urine from patients (Strecker et al., 1977, 1978; Yamashita et al., 1979). At least 11 different oligosaccharides have also been identified which are probably cleavage products of the glycopeptides (Tsay and Dawson, 1976; Tsay et al., 1976; Strecker et al., 1977; Lundblad et al., 1978; Nishigaki et al., 1978; Ng Ying Kin and Wolfe, 1979). These storage products are consistent with the substrate specificity of α- L- fucosidase. A limited amount of mucopolysaccharide and glycolipid also accumulate in tissues and are seen in the urine (Tsay et al., 1976; reviewed by Willems et al., 1991). The main glycolipid seen in the urine of patients is the H- antigen glycolipid (Tsay and Dawson, 1976).

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1.4.3. Molecular genetics of fucosidosis

Fucosidosis is an autosomal recessive disorder. There is a high consanguinity rate within affected pedigrees (40% of 45 families) but the incidence of the disease overall is extremely low as would be expected for this mode of inheritance (Willems et al., 1991). A review of 77 reported cases in 1991 showed that there was higher incidence in Italy and the Mexican- Indian population of Colorado and New Mexico, which is perhaps due to the high inbreeding or the intensive screening programmes in these areas (Willems et al., 1991).

The gene encoding α- L- fucosidase (FUCA1) has been mapped to chromosome 1 at position 1p34.1- 36.1 and is composed of 8 exons dispersed over 23 kb (Carritt et al., 1982; Fowler et al., 1986). The full length cDNA, which was cloned and sequenced by Occhiodoro et al (1989), is composed of 2053bp with an open reading frame of 461 amino acids, of which 22 encode a signal peptide. There are 4 potential N- glycosylation sites per monomeric α- L- fucosidase molecule. The known mutations in this gene are shown in Figure 1.2 (Willems et al., 1988; Kretz et al., 1989; Yang et al., 1993; Seo et al., 1993a, 1993b; Williamson et al., 1993; Yang et al., 1993; Seo et al., 1994a, 1994b; Yang et al., 1994; Seo et al., 1995, 1996; Cragg et al., 1997; Fleming et al., 1998; Willems et al., 1999).

1	P5R	Q77X
	G60D	G96fs
	S63L	
2	P141fs	
	K151fs	
3	Y211X	
	S216fs	
4		
5	E253fs	
	S265fs	
	IVS5+1	
6	W382X	
	Y330fs	1030-1095 duplication
	E375X	
7	G401X	
		Deletion exons 7 and 8
8	Q422X	

Figure 1.2 Known mutations in exons 1-8 of the FUCA1 gene.

A putative processed pseudogene, FUCAIP, also exists. It has been mapped to 2q31- q32 and does not have an open reading frame but has 80% homology to FUCA1 in sequence (Fowler et al., 1986; Carritt and Welch, 1987; Darby et al., 1988; Coucke et al., 1991; Kretz et al., 1992).

1.4.4. Animal models

Due to a founder effect, fucosidosis was a relatively common inherited disorder in Springer Spaniels in Australia (approximately 4% of the population) and in the United Kingdom prior to an eradication programme (Hartley et al., 1982; Kelly et al., 1983; Littlewood et al., 1983; Healy et al., 1984). Canine fucosidosis is characterized by progressive ataxia, tremor, dysphagia, wasting and development of blindness and deafness. The affected animals die in a vegetative state aged 3-5 years (Durand et al., 1982; Taylor et al., 1987b; Thomas and Beaudet, 1998).

Canine fucosidosis is an autosomal recessive disorder (Healy et al.,1984). There is less than 5% residual α- L- fucosidase activity in white blood cells and plasma, no immunological cross reacting material in most cells and tissues, accumulation of fucose- containing glycoasparagines in the lysosomes and excretion of these in the urine (Kelly et al., 1983; Abraham et al., 1984; Taylor et al., 1987b; Barker et al., 1988; Thomas and Beaudet, 1998). The ultrastructural appearance of tissues is similar to that in humans with fucosidosis (Hartley et al., 1982; Kelly et al., 1983). The molecular defect underlying canine fucosidosis has been identified (Skelly et al., 1996; Occhiodoro and Anson, 1996). The canine α- L- fucosidase gene spans 12kb and consists of 8 exons. The founder mutation is a 14bp deletion at the start of intron 1 causing a frameshift and the transcription of 25 novel codons in exon 2 before a premature stop codon is reached (Skelly et al., 1996).

Canine fucosidosis appears to be a valid model for the human form although there are differences between the symptoms and storage products (Durand et al., 1982; Kelly et al., 1983; Abraham et al., 1984; Alroy et al., 1985; Taylor et al., 1987b). The model has been used to evaluate bone marrow transplantation and enzyme replacement therapy as discussed in sections 1.6.4.4 and 1.6.2 respectively.

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1.5. Fabry disease

Fabry disease, also known as Anderson- Fabry disease (McKusick number 30150), is caused by a deficiency of the lysosomal α- galactosidase A (E.C. 3.2.1.22), which catalyses the removal of terminal α- galactosyl residues from the non- reducing ends of neutral glycosphingolipids (Brady et al., 1967). This results in the progressive accumulation in the lysosomes of glycosphingolipids with terminal α- linked galactosyl residues. The storage occurs in many body tissues and fluids but especially in the vascular endothelium. Fabry disease is X- linked and the α- galactosidase A gene is located at Xq22 (Desnick et al., 1989). Fabry disease is estimated to affect 1 in 40 000 panethnically.

1.5.1. Clinical features

Clinical features of male patients

Anderson- Fabry disease was first described in male patients in 1898 by Anderson, and independently in the same year by Fabry. The disorder presents with markedly different degrees of severity in different patients (Desnick et al., 1989; Morgan et al., 1990; Radcliffe and Evans, 1990). The clinical features include angiokeratoma and peripheral nerve system manifestation with acute episodic crises or constant discomfort. Ocular features of the disease can include corneal dystrophy, conjuctival and retinal vascular lesions and hypohidrosis. Cardiovascular symptoms are an abnormal electrocardiogram, angina, myocardial ischaemia and heart failure. Cerebrovascular involvement can cause neurological symptoms, transient ischaemia, hemiplegia, haemorrhagic lesions and thrombosis. Electrolyte imbalance, due to renal insufficiency or cerebral damage, probably causes the personality and behavioural changes, confusion and disorientation. Patients can also have proteinuria and progressively impaired renal function. The presenting symptom is usually pain in the extremities and generally occurs in the first ten years of life. Diagnosis is usually made in the teenage years when symptoms such as angiokeratoma present. If patients are not treated death usually occurs through

kidney failure in the fifth decade (Columbi et al.,1967). Atypical variants have milder phenotypes characterised by late onset cardiomyopathy.

Clinical features in female heterozygotes

Female heterozygotes are either asymptomatic or manifest attenuated classic symptoms. This is probably due to variation of the expression of the normal and defective copies of the α- galactosidase A gene, in different cell types, resulting from random X- inactivation. About 70% have corneal dystrophy, 30% angiokeratoma and less than 10% experience pain in the extremities (Desnick et al., 1989).

The α- galactosidase A activity in heterozygotes ranges from undetectable to normal in plasma and white blood cells (Avila et al., 1973; Desnick et al., 1973, 1989). Therefore, enzymic detection of carriers is unreliable.

1.5.2. Biochemical features

Deficiency of α- galactosidase A

α- Galactosidase A catalyses the hydrolysis of terminal α- linked galactosidic linkages in the lysosomal catabolism of glycosphingolipids (Figure 1.3). The enzyme is a homodimer with a molecular weight of about 100kDa, consisting of two 50kDa subunits. Multiple forms are found with slightly different physical properties due to heterogeneity of glycosylation. The optimal activity towards the natural substrate, ceramide trihexoside, is at pH3.8-4.0 with a Km of 0.1-0.2mM. *In vivo* α- galactosidase A requires a sphingosine activator protein, saposin B, as a cofactor (Sandhoff and Klein, 1994). α- Galactosidase A is synthesised as a 50kDa precursor protein and is transported to the lysosome in a mannose- 6- phosphate receptor-dependent manner where it is processed by proteolytic cleavage to a mature form of 46kDa. The residual α-galactosidase A activity in patients is negligible except in atypical variants.

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$$\overset{\displaystyle |\text{Fuc}^{\alpha 1}}{Gal^{\alpha 1}—{}^{3}Gal^{2\ \beta 1}—{}^{4}GlcNAc^{\beta 1}—{}^{3}Gal^{\beta 1}—{}^{4}Glc^{\beta 1}—{}^{1}Cer}$$

α- Galactosidase A

$$\overset{\displaystyle |\text{Fuc}^{\alpha 1}}{Gal^{2\ \beta 1}—{}^{4}GlcNAc^{\beta 1}—{}^{3}Gal^{\beta 1}—{}^{4}Glc^{\beta 1}—{}^{1}Cer}$$

α– L- fucosidase

$$Gal^{\beta 1}—{}^{4}GlcNAc^{\beta 1}—{}^{3}Gal^{\beta 1}—{}^{4}Glc^{\beta 1}—{}^{1}Cer$$

β- Galactosidase

$GalNAc^{\beta 1}—{}^{3}Gal^{\alpha 1}—{}^{4}Gal^{\beta 1}—{}^{4}Glc^{\beta 1}—{}^{1}Cer$ $GlcNAc^{\beta 1}—{}^{3}Gal^{\beta 1}—{}^{4}Glc^{\beta 1}—{}^{1}Cer$

β- N- Acetyl-hexosaminidase   β- N- Acetyl hexosaminidase

$Gal^{\alpha 1}—{}^{4}Gal^{\beta 1}—{}^{4}Glc^{\beta 1}—{}^{1}Cer$

α- Galactosidase A $Gal^{\beta 1}—{}^{4}Glc^{\beta 1}—{}^{1}Cer$

β- Galactosidase

$Glc^{\beta 1}—{}^{1}Cer$

β- Glucosidase

Ceramide

β- Galactosidase

$Gal^{\beta 1}—{}^{1}Cer$

α- Galactosidase A

$Gal^{\alpha 1}—{}^{4}Gal^{\beta 1}—{}^{1}Cer$

Figure 1.3 Lysosomal degradation of neutral glycosphingolipids

Gal, Glc, Fuc, GalNAc, GlcNAc and Cer are galactose, glucose, fucose, N-acetylgalactosamine, N- acetylglucosamine and ceramide respectively

Storage products

The two main storage products are globotriaosylceramide and galabiosylceramide. Globotriaosylceramide is the main substrate and it accumulates in most tissues and cells, particularly vascular endothelia, muscle, kidney, lymph nodes, heart, prostate and autonomic ganglia. Galabiosylceramide is less widely distributed and accumulates in the kidney, pancreas, heart, lung and autonomic ganglia (Desnick et al.,1989). Additional glycosphingolipids are found in some individuals. Patients with blood groups AB and B produce the B or B1 antigens, which contain terminal α- linked galactose (Wherret and Hakomori, 1973).

Histological examination demonstrates accumulation of glycosphingolipids in Fabry patients in nearly all the tissues examined (Desnick et al., 1989). The glycosphingolipids deposits are crystalline and birefringent, with a 'Maltese cross' configuration when observed using polarising microscopy. Ultrastructural studies demonstrate the presence of concentric, lamellar lipid inclusions and other atypical deposits in lysosomes of Fabry patients (Elleder et al., 1990). Glycosphingolipids are also excreted in the urine (Oshima et al., 1990).

1.5.3. Molecular genetics

The α- galactosidase A gene is localised to ChrXq22.1 (Vetrie et al., 1993). The full length cDNA (Bishop et al., 1988) and the gene for α- galactosidase A (Kornreich et al., 1989) have been isolated and sequenced (Genbank X14448 and D00039). The gene is 12kb long and contains 7 exons of 92- 291bp in length. Mutation analysis has been carried out for many patients (Bernstein et al., 1989; Koide et al., 1990; Sakuraba et al., 1990; von Scheidt et al., 1991; Yokoi et al., 1991; Davies et al., 1993; Davies et al., 1994; Davies et al., 1996; Eng et al., 1997). Normal human α- galactosidase A consists of a 429 amino acid precursor protein which contains a 31 amino acid signal peptide and a 398 amino acid mature peptide (Bishop et al., 1991). The mature peptide contains 4 consensus sites for protein N- glycosylation, although only three are utilised (Bishop et al., 1991).

1.5.4. Animal models

At least two knockout mouse models have been created and used to test therapeutic strategies including enzyme replacement therapy, gene therapy and substrate deprivation (Ohshima et al., 1997; Suzuki et al., 1998).

1.6. Therapeutic strategies for lysosomal storage diseases

The primary pathogenic factor in lysosomal storage diseases is the accumulation within lysosomes of either partially digested molecules that are too large to pass through the lysosomal membrane or digestion end products that cannot leave the lysosome because of defects in their transport processes. This leads to lysosomal hypertrophy and the subsequent development of the clinical symptoms depending on the storage material and the cells affected. Theoretically the underlying defects could be corrected at the cellular level by supplying the normal gene product, decreasing the rate of material to be catabolised to a level that can be dealt with by any residual activity or by finding an alternative route for the transport of stored material out of the lysosomes. Over the last three decades, many therapeutic approaches have been investigated *in vitro* and *in vivo* for lysosomal storage diseases. Whilst the outcome of some of these studies has shown some therapeutic potential, many have been unsuccessful. Nonetheless, the scientific information gained from these studies has been invaluable in advancing our knowledge of the treatment of human diseases. In the following section, some of the approaches used as well as their shortcomings and advantages are described.

1.6.1. Retardation of formation of undegradable products or substrate deprivation

N- Butyldeoxynojirimycin (NB-DNJ) inhibits the glucosyltransferase responsible for the first step in the synthesis of glycolipids, in which glucose is attached to the ceramide lipid (Platt et al., 1994a). Therefore, this is potentially an agent for slowing down the rate of synthesis of lipids that accumulate in most sphingolipidoses. NB- DNJ was found to prevent lysosomal glycolipid accumulation in an in vivo model of Gaucher disease (Platt et al., 1994a). The

drug also prevented storage of G_{M2} ganglioside in a non- symptomatic Tay-
Sachs mouse model indicating that NB- DNJ can cross the blood- brain barrier
(Platt et al., 1997). However, NB- DNJ also inhibits the synthesis of lipid linked-
oligosaccharide essential for protein N- glycosylation. Another drug, N-
butyldeoxy- galactonojirimycin (DGJ) was synthesised which inhibits glycolipid
biosynthesis specifically. It was found to be as effective as NB- DNJ in
preventing glycolipid storage in an in vivo Gaucher's disease model (Platt et al.,
1994b; Fan et al., 1999).

1.6.2. Direct enzyme replacement

This approach aims to replenish active enzyme to compensate for the
deficiency or inefficiency of endogenous enzyme. The exogenous enzyme is
delivered to lysosomes by receptor- mediated endocytosis. The major problems
with this approach include the short half life of exogenous enzymes, the
possibility of an immune response, cost of enzyme production and inefficient
delivery to certain cells or specific intracellular sites of action, particularly the
brain, joints and bones. Attempts to overcome some of these problems have
included chemical modification of enzymes to increase uptake by specific cells,
linkage to a protective molecule, covalent linkage to a targeting molecule and
enclosing the enzyme in a protective membrane (reviewed by Rademaker and
Raber, 1989; Desnick, 1991; Langer, 1996).

The first lysosomal storage disease to be treated by enzyme
replacement therapy was type I Gaucher disease, which is non- neuronopathic
and results from a deficiency of β- D- glucocerebrosidase. Purified placental β-
D- glucocerebrosidase was modified enzymatically to expose an increased
number of terminal mannose residues on the glycan side chains, to enhance its
uptake by mannose receptors on macrophages, the principal site of storage.
This modification resulted in 40-70 times more efficient uptake of the enzyme by
macrophages (Furbish et al., 1981). Consequently, symptoms were improved in
patients with a reduction of hepatosplenomegaly and some improvement in
skeletal abnormalities (Barton et al., 1990). About 1500 patients with Gaucher
disease type I are currently being treated worldwide with a similarly modified
recombinant form of the enzyme. Treatment appears to be effective with varying

degrees of remission of symptoms. However, the treatment is very expensive (Barranger et al., 1993).

A recent clinical trial, in which 10 patients with mucopolysaccharidosis type I (MPS I) were treated over 26 weeks with recombinant α- L- iduronidase, demonstrated that the treatment improved the patients' physical abilities, decreased fatigue, joint pain, and liver size and improved quality of life (Kakis,1999).

1.6.3. Organ transplant

Organ transplantation replaces a defective organ and also acts as a source of the deficient enzyme. Organ transplantation has been attempted for Fabry and Gaucher diseases using kidney and liver respectively (Desnick et al., 1973, Sutherland et al., 1980). Early observations suggested that renal transplantation is valuable for Fabry disease but neurological and cardiac complications still progress. Generally the success of such transplants has been limited because of patient death resulting from complications of transplantation (Watts and Gibbs, 1986).

1.6.4. Cell transplant

Transplants of normal cells act as a source of the secreted enzyme which can the be taken up by other cells via mannose- 6- phosphate receptors. Cells which have been transplanted include fibroblasts, amniotic epithelial cells, neural progenitors and bone marrow.

1.6.4.1. Fibroblasts

Olsen et al (1983) suggested that the normal fibroblast transplants may be useful in treatment for lysosomal storage diseases. They are already differentiated and simply act as a source of normal enzyme. Gibbs et al (1983) undertook a clinical trial of multiple fibroblast transplantation for 6 patients with Hurler disease, 2 with Hunter disease and 1 with Sanfilippo B disease. The treatment did not alter the clinical course of the disease in any of the cases.

1.6.4.2. Neural progenitors

The possibility of transplanting cells into the brain has also been explored. Enzyme- competent transformed neural progenitors were

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transplanted into the cerebral ventricles of new- born MPS VII mice and over 90% of treated mice showed a distinct improvement with an average acquired β- glucuronidase activity of 2% in the brain (Snyder et al., 1995).

1.6.4.3. Amniotic epithelial cells

Amniotic epithelial cells have also been used for transplantation in clinical trials. These cells would not be recognised as foreign by the immune system of the patient. Three children with Hunter disease and two with Hurler disease were transplanted with 25- 100 million amniotic cells. The patients tolerated the transplants well but showed no clinical improvement.

1.6.4.4. Bone marrow transplantation

Hobbs and his colleagues (Hobbs, 1981; Hobbs et al., 1981) introduced bone marrow transplant as a method of treating lysosomal storage diseases when they treated a boy with Hurler disease. Bone marrow stem cells are self-perpetuating and give rise to several cell lineages. The advantages of bone marrow transplantation over enzyme replacement therapy are that bone marrow- derived macrophages can cross the blood- brain barrier where they remain as microglial cells and can provide a self- renewing cellular source of enzyme replacement.

Studies on animal models

Bone marrow transplantation in some animal models with lysosomal storage diseases has shown complete or partial biochemical and histological correction depending on the organ and disease. Bone marrow transplantation in α- mannosidosis cats (Walkley et al., 1994), fucosidosis dogs (Taylor et al., 1986a, 1986b, 1987a, 1987b, 1988, 1989a, 1989b, 1989c, 1992), and mucopolysaccharidosis VII mice (Hoogerbrugge et al., 1987; Birkenmeir et al., 1991) has produced satisfactory results showing increased levels of the specific enzyme in different body tissues and organs including the brain in particular. The treatment improved the clinical symptoms of the disease in the animals and also increased their survival time. These studies have been invaluable in providing information on the critical time for carrying out bone marrow transplantation before onset of serious disease or CNS lesions.

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Studies on human patients

Bone marrow transplantation has been carried out in over 200 patients with lysosomal storage diseases with mixed success. The largest number of bone marrow transplants has been performed in mucopolysaccharidosis patients, particularly with Hunter and Hurler diseases. Modification of neurological deterioration was achieved but general decline was still evident.

Bone marrow transplantation for Hunter disease appears to have variable results with some features such as hepatosplenomegaly improving but in most cases neurological deterioration continuing (Krivit and Schapiro, 1991).

A review of 38 patients with MPS I (Hurler disease) who received bone marrow transplants over 15 years showed an increased life expectancy and slowing down of disease progression (Vellodi et al., 1997). However, there were still clinical problems particularly concerning the musculoskeletal system. Psychomotor retardation appeared to be slower or arrested in transplanted patients suggesting some correction of brain cells was achieved.

The first transplant for α- mannosidosis was reported in 1987 (Will et al., 1987). The patient died 18 weeks after transplantation. Postmortem studies suggested that the somatic changes of α- mannosidosis had been reversed but that there was no correction of lysosomal storage in the brain tissue.

In a long- term study of bone marrow transplantation for Gaucher disease type 2, with neurological involvement, only one of the six patients rejected the graft (Ringden et al., 1995). The patients had a growth spurt and hepatomegaly was reversed in 1-2 years. Favourable skeletal changes were observed in some patients. No further mental deterioration was seen in patients after bone marrow transplant but this could vary among patients anyway.

Bone marrow transplantation was carried out on an 8 month-old pre-symptomatic boy with fucosidosis (Vellodi et al., 1995). After transplantation, the graft was accepted and there was a normal level of α- L- fucosidase in white blood cells. The pattern of excreted oligosaccharides was also normalised. Neurologically the patient showed mild diplegia but this was arrested. His brother had shown far greater developmental delay at a similar age although it was not certain if this was due to the bone marrow transplantation or

intrafamilial variation. These results are promising and suggest a proportion of cells producing the enzyme might be crossing the blood brain barrier.

There still needs to be long term evaluation of the efficacy of bone marrow transplant for lysosomal storage diseases. There are problems and risks associated with this technique like the difficulty of finding an appropriate donor, the 10-15% transplant failure rate, 10-20% mortality rate and the risk of graft versus host disease.

1.6.5. Gene transfer

Generally 'gene therapy' is defined as the introduction of a normal gene into target cells with a defective copy of the corresponding gene or of genetically modified cells carrying the normal gene into the host to complement the genetic defect and correct the disease phenotype. In recent years immense technological developments have been made in the gene transfer field.

The required gene can be introduced into a cell by simple chemical and physical manipulations but the efficiency of transfer is extremely low. This led to the development of various strategies which have their drawbacks as well as advantages. The techniques exploit three main strategies; a) direct DNA transfer, b) viral- vector aided DNA transfer and c) non- viral vector aided DNA transfer.

1.6.5.1. Direct DNA transfer

Initial experiments demonstrated that when a gene was injected into muscle in the form of naked DNA the expression of the transgene was at a low level. DNA can also be directly introduced into cells by a calcium phosphate precipitation method or by electroporation. However, the efficiency of transfer is extremely low and inconsistent (reviewed by Treco and Selden, 1995).

1.6.5.2. Viral- vector aided DNA transfer

Viruses as vectors are attractive tools since they efficiently infect cells and express their own genes and products. Viruses are manipulated to produce vectors by removing genes involved in viral replication and virulence and replacing them with potential therapeutic genes. Therefore, theoretically the vector transfers the therapeutic gene of interest but does not multiply or cause

disease. The importance of viral vector- aided gene therapy lies in its efficiency. The disadvantage is that the vectors are modified viruses and therefore have the potential to cause disease. They may also be immunogenic.

The five main types of viruses employed in constructing vectors are retroviruses (RV), adenoviruses (AV), adeno-associated viruses (AAV) lenteviruses (LV) and herpes simplex viruses (HSV).

Retroviral (RV) vectors have been studied extensively for gene transfer into dividing and expanding cell populations such as hematopoietic cells. They are efficient, integrate into the host genome, have the potential for long term stable expression of introduced genes. However, these vectors tend to be promiscuous as they deliver genes to a wide variety of cell types which may have an undesirable gene expression effect. Integration into the host genome also has drawbacks since it is random and could possibly disrupt host genes. Developments in RV vectors have included the use of internal promoters to avoid transcriptional silencing, coexpression of selectable markers and the production of vectors which self- inactivate (Reviewed by Morgan, 1995).

Lentiviruses (LV) are a subclass of RV which are able to integrate into quiescent cells (such as neurons and macrophages) as well as dividing cells. They stably integrate and give long term transgene expression without immunogenic effect. The main concern with LV vectors is their safety and further development of LV vectors has concentrated on this issue and on the production of vectors which self- inactivate (Miyoshi et al., 1998; Dull et al., 1998).

Adenoviral (AV) vectors infect a wide range of both dividing and non-dividing cells. Most adenoviruses do not cause serious disease. The AV vectors do not integrate into the genome and hence do not disturb cellular genes. They have a large packaging capacity for foreign genes. Disadvantages of AV vectors include transient expression of introduced genes due to the lack of integration (although expression will be more stable in non- dividing cells) and the response from the host immune system since they are highly immunogenic. They are also fairly non-specific in their targeting of cells. Human and canine gutless AV vectors (high capacity vectors with all viral coding sequences deleted) are being developed to overcome the problem of immunogenicity (review by Perricaudet and Stratford- Perricaudet, 1995).

Adeno- associated viruses (AAV) are apparently not associated with disease in humans. They require proteins derived from other viruses (AV or HSV) in order to complete their life cycle by allowing replication and triggering of the lytic cycle. AAV vectors infect both dividing and non-dividing cells. They have a small cloning capacity of 5kb (review by Samulski, 1995).

The advantages of herpes simplex viral (HSV) vectors are that large inserts can be packaged and they have a natural tropism for neurological tissue and can establish long term latency in neurons. Herpes virus latency is associated with episomal maintenance. Therefore, genetic engineering of these viruses may be suitable for the development of human artificial chromosomes. Long term episomal persistence of the gene avoids random integration of the vector into host DNA. The main concerns with these vectors are of safety and toxicity. Expression is also transient, probably due to promoter shut off or the host immune response (reviews by Vos, 1995; Lachmann and Efstathiou, 1997).

Studies on viral vector- aided DNA transfer for lysosomal storage diseases

There are currently seven human gene therapy trials for lysosomal storage diseases; 3 are for Gaucher disease, 2 for Hunter disease and 2 for Hurler disease. In six of these trials retroviral vectors have been utilised whilst in one trial an adenoviral vector has been used. Since RV vectors only transfect dividing cells, studies have focused on two strategies. The first strategy includes RV transfection of fibroblasts to overexpress and secrete the relevant enzyme for implantation (Moullier et al., 1993a, 1993b, 1995). The second approach has been ex vivo transduction of haematopoietic stem cells such as bone marrow and peripheral blood cells (Fink et al., 1990; Kohn et al., 1991; Nolta et al., 1992; Wolfe et al., 1992; Marechal et al., 1993; Krall et al., 1994; Nimgaonkar et al. 1994; Fairbairn et al., 1996; Whitley et al., 1996; Huang et al., 1997; Schuening et al., 1997). The main drawback of RV- mediated systems has been their inability to transduce quiescent cells. At present these systems have limited efficacy for neuropathic and skeletal forms of lysosomal storage diseases. Studies have also been carried out using AV vectors. Enzymatic correction has been demonstrated in patient cells in culture (Akli et al., 1996;

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Nicolino et al., 1998). *In vivo* studies have concentrated on direct injection of the vectors. Intravitreal or subretinal injections of AV have been shown to produce partial correction of storage in some cell types (Li and Davidson, 1995; Sands et al., 1997).

Gene transfer to the central nervous system

Delivery of lysosomal enzyme genes directly to the central nervous system has been investigated in animal models. Intracerebral implants of genetically modified myoblasts or fibroblasts have been tried previously as a possible therapeutic approach (Gage et al., 1991). Fibroblasts engineered with RV vectors to overproduce β- glucuronidase were transplanted into the brains of MPS VII mice (Taylor and Wolfe, 1997). Storage vesicles were cleared from neuronal and glial cells as far as 2mm away from the grafts. Fibroblast grafts in the brain become isolated from the brain by an astrocytic response, localising the therapeutic effects (Taylor, 1997).

A double copy retroviral vector encoding β- glucuronidase was used to transduce pluripotent mouse neuroglial cells immortalised with v-myc. These cells were transplanted into the MPS VII neonatal mouse brain. The cells migrated, differentiated, secreted β- glucuronidase and corrected the lysosomal storage (Snyder et al., 1995; Taylor and Wolfe, 1997). Transplantation of pluripotent immortalized mouse neuroglial cells transduced with a double copy RV vector encoding β- hexosaminidase A into normal foetal and neonatal mouse brain produced substantial β- hexosaminidase A activity throughout the brain (Lacorazza et al., 1996).

A recombinant HSV-1 virus encoding rat β- glucuronidase has been used to infect MPS VII mice by corneal inoculation. Enzyme- positive neurons were detected in the trigeminal ganglia and brain stem up to 4 months post inoculation (Wolfe et al., 1992). AV and AAV directly infect neurons and glia with high efficiency. Human β- glucuronidase cDNA has been delivered to the eyes of MPS VII mice by intravitreal or subretinal injections of a recombinant AV. β- Glucuronidase activity was observed in the corneal endothelial cells, the reticulopigmented epithelia (RPE) cells and the posterior surface of the iris and it was sustained for up to 3 weeks. (Li and Davidson, 1995). Intracranial injection of AAVs containing β- glucuronidase cDNA into MPS VII mice resulted

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in β- glucuronidase activity above the heterozygote level in the brain. Expression was stable for at least 32 weeks and the enzyme diffused to 10mm from the injection site. At 6 months it appeared that the progression of disease had been stopped by the therapy and vacuolar inclusions had been cleared (Bosch and Heard, 1998). Intracerebral injection of a LV vector has been shown to transduce terminally differentiated neurons. The reporter gene was detectable 3 months after injection and there was no evidence of an immune response (Naldini et al., 1996).

Gene transfer to hematopoietic cell system

Gene transfer to hematopoietic stem cells can directly complement a deficiency which affects the haematopoietic system, such as in Gaucher, or reduce lysosomal storage in non- haematopoietic tissues via secretion and uptake of normal enzyme. This technique has the advantage over bone marrow transplantation in that there is no risk of graft rejection and no need for a matched donor. The main problems facing this strategy are low efficiency, transient expression and selection of stem cells.

In studies using the murine model of Gaucher disease, RV- transduced autologous bone marrow has been shown to produce transgene expression in bone marrow, spleen and thymus for up to 8 months after transplantation and in 20% of microglia as well as correction of the enzyme defect in these cells (Kohn et al., 1991; Krall et al., 1994; Salvetti et al., 1995). Transduction of Gaucher patient bone marrow cells under *in vitro* conditions has produced levels of transgene expression which would be expected to correct the enzyme defect (Fink et al., 1990; Nolta et al., 1992; Xu et al., 1994). Two clinical trials are now being carried out. More recently, RV mediated gene transfer of human α- L-iduronidase cDNA into human hematopoietic progenitor cells has been shown to correct the enzymic defect in Hurler fibroblasts in trans (Huang et al., 1997). Fairbairn et al. (1996) used a RV vector containing human α- L- iduronidase cDNA to directly transduce long term bone marrow cultures from their patients. Expression was demonstrated over 6 weeks and this protocol is being tested in clinical trials. Gene transfer into hematopoietic cells of mucopolysaccharidosis VII mice has resulted in complete correction of the defect in liver and spleen but no reduction in lysosomal storage in any other tissue (Wolfe et al., 1992;

Marechal et al., 1993; Sands et al., 1997). RV -mediated gene transfer of arylsulphatase A cDNA into murine metachromatic leukodystrophy bone marrow cells followed by implantation demonstrated that 6 out of 9 mice were positive for the vector in peripheral white blood cells after 2 months (Naparstek and Turetsky, 1998). RV vectors containing α- L- fucosidase have been used to transduce bone marrow cells of dogs with fucosidosis. There was high level of transduction but the grafts were rejected (Ferrara et al., 1997).

Another approach to transducing hematopoietic progenitor cells is to transduce peripheral blood repopulating cells. Glucocerebrosidase cDNA was cloned into a RV vector and used to transduce peripheral blood $CD34^+$ cells. Approximately 10-30% of the cells were transduced. Transduction resulted in 2.5 –fold increase in the enzyme activity compared with untransduced cells and this activity was sustained for up to 3 weeks of culture (Nimgaonhar et al., 1994)

RV- transformed fibroblasts as neo-organs

Host fibroblasts can be transduced with an appropriate gene and included in collagen lattices with fibroblast growth factor- coated fibres. These structures are implanted back into the peritoneal cavity of the host where they form neo-organs. The neo-organs are usually densely vascularised and they secrete the desired enzyme into the peritoneal cavity. This strategy was developed using fibroblasts transduced with RV containing human β-glucuronidase cDNA in MPS VII mice (Moullier et al., 1993a, 1993b). These neo-organs secreted β- glucuronidase for at least 155 days post- implantation (duration of study). The enzyme was detectable in liver, spleen, lung and brain for at least 90 days. The enzyme activity ranged from 0.5-10% of that in untreated heterozygous mice, depending on when the mice were analysed. The amount of urinary glycosaminoglycans decreased for the duration of the study but not to normal levels. Storage material was reduced in the liver and spleen of the mice but not in any other tissue, despite the presence of enzyme. This procedure was later scaled up for experiments on dogs where β- glucuronidase secreted by neo-organs was detectable in liver up to 1 year after implantation. Therapeutic levels of β- glucuronidase were delivered to liver and spleen (Moullier et al., 1995).

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In other studies fibroblasts have been transduced with RV or AV vectors and enzymatic and phenotypic correction as well as reduction of lysosomal storage material has been demonstrated (Rommerskirch et al., 1991; Peters et al., 1991; Ohashi et al., 1993; Akli et al., 1996; Nicolino et al., 1998). For example, two different retroviral vectors containing α- L- fucosidase cDNA have been used to transduce human and canine fucosidosis fibroblasts. The fibroblasts were corrected enzymatically and also phenotypically with the reduction of accumulated storage products (Occhiodoro et al., 1992).

1.6.5.3. Non- viral vector aided DNA transfer

Non- viral vector aided DNA transfer encompasses any gene therapy system which does not utilise virus vectors, including the direct gene transfer systems described in section 1.6.5.1. The main advantage of non-viral gene transfer is safety since there is no risk of transferring an infectious agent. There is the potential for large scale production of these vectors and the possibility of repeat *in vivo* administrations. The main problems with non- viral gene therapy are the low level and transient expression of the transgene.

DNA-liposome complexes.

Liposomes are minute, artificial spherical vesicles consisting of a lipid bilayer or multiple bilayer enclosing an aqueous solution. Although it is possible to encapsulate macromolecules within liposomes this strategy is not adopted for gene transfer. Instead cationic liposomes are complexed with condensed DNA by charge interactions. The positively charged complex binds to the surface of cells and is internalised by them. The DNA is released from the endosomes and travels to the nucleus. The appeal of this system is its safety *in vivo*, simple preparation and its capacity to deliver large DNA particles.

At least a dozen cationic liposome formulations have been used as vectors in gene transfer. In recent years the efficiency of transfer has been improved and clinical trials have taken place. However, the efficiency is generally still low for most target tissues due to clearance of injected liposomes from the plasma by the reticuloendothelial system and interception by non-target tissue. Since expression is episomal it is only transient, lasting from days to weeks. Therefore, at present the approach is only useful where transient

gene expression can modify disease or where repeated administration is possible (Reviewed by Treco and Selden, 1995; Schofield and Caskey, 1995; Scheule and Cheng, 1998; Felgner, 1997).

Synthetic polymer systems

Synthetic polymers such as poly-lysine and polyethylenimine (PEI) are also being examined as vehicles for gene transfer. Self- assembling systems based on synthetic, linear block copolymers, with each polymeric block designed to fulfill a different vector requirement, such as DNA condensation, are being developed (Wolfert and Seymour, 1996; Wolfert et al., 1996).

The use of a novel fusion protein, GAL4/invasin, is also being investigated for non- viral systems. It consists of the DNA- binding domain of a yeast transcription factor, GAL4, fused to the cell- binding and internalisation domain of *Yersinia pseudotuberculosis* inv gene product, invasin (Paul et al., 1997).

Receptor-mediated gene transfer

In this approach DNA is complexed with a polypeptide which contains a DNA binding element and a ligand for binding to a cell surface receptor. Therefore, the DNA- ligand complex binds to the receptor and is internalised by receptor- mediated endocytosis. The complex then needs to be released from the endosomes into the cytoplasm to avoid delivery to the lysosomes and breakdown, it then enters the nucleus where the DNA can be expressed (reviewed by Schofield and Caskey, 1995).

Receptor- mediated gene transfer allows a certain degree of specificity since ligands may be chosen to direct complexes to selected cell types. However, the selected receptor may be present on several cell types, not all of which are targets. Since the introduced DNA is not integrated and is expressed episomally only transient expression is seen. Methods could be developed to achieve long term expression either by using human artificial chromosomes which replicate or by repeated administration. Other possible problems include immunogenicity of the complex which would lead to its clearance from the circulation. The long- term effects of DNA- protein complexes on specific organ systems must be evaluated e.g. deposition of complexes in the kidney might

have deleterious effect on renal function. It is important that the chemical properties and physical interactions of the constituents of the systems are rigorously characterised.

The DNA- binding element of the polypeptide is generally poly- L- lysine although protamine sulphate (Sorgi et al., 1997), and intercalating agents (Haensler and Szoka, 1993) have also been used. This binding element also condenses the DNA. Poly- lysine domains may also act as nuclear targeting signals since nuclear localisation signals of viral capsid proteins are often lysine- rich.

The ligand targets the complex to cell surface receptors. Various ligands have been used including RGD peptides to target integrins (Hart et al., 1994, 1995, 1998), transferrin to target transferrin receptors (Curiel et al., 1991; Cheng, 1996), asialoglycoprotein to target asialoglycoprotein receptors (Wu and Wu, 1991; Wu et al., 1991), glycans to target lectins (Midoux et al., 1993; Yin and Cheng, 1994) and insulin which targets insulin receptors (Rosenkrantz et al., 1992).

Generally, low levels of expression in the specific cell types targeted have been reported. However, the use of Lipofectin in DNA- ligand complexes has been shown to improve the efficiency of transfection (Cheng, 1996). This is thought to be due to Lipofectin promoting the escape of complexes from endosomes.

Integrin- mediated gene transfer

Structure of integrins

Integrins are a superfamily of about 20 cell adhesion molecules which mediate binding of cells to the extracellular matrix, cell- cell adhesion, motility and signal transduction. They are heterodimers made up of different α- and β- subunits. The α- and β- subunits are transmembrane glycoproteins which are non- covalently associated (Figure 1.4). There are 14 known α- subunits and they vary in molecular mass between 120 and 180 kDa. There are 8 known β- subunits which also vary in molecular mass between 90 and 110 kDa. In mammals several subunits have alternatively spliced cytoplasmic domains which increase the structural versatility of integrins. The extracellular domains of

the subunits associate to form the heterodimers. The integrin globular head comprises both subunits, with each subunit having a separate hydrophobic transmembrane domain and cytoplasmic domain. Both subunits contribute to the ligand binding site and therefore its specificity. Divalent cations are essential for integrin function, as they can affect the affinity and specificity for ligands and are also involved in subunit association. The cytoplasmic domains of the subunits are believed to interact with cytoskeletal proteins and to mediate cellular responses to the binding of extracellular ligands. Different cytoplasmic domains of different subunits mediate different functions (integrin structure reviewed by Chothia and Jones, 1997).
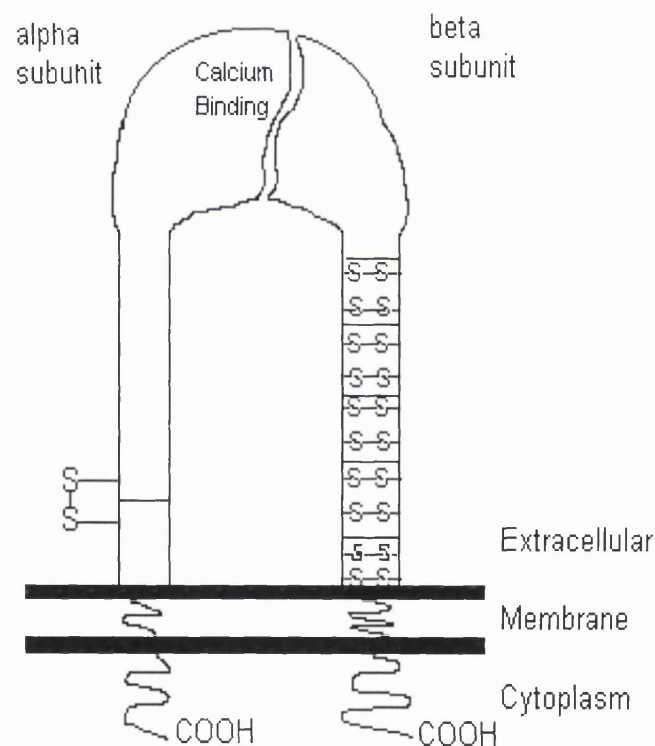
Figure 1.4 Integrin structure.

Binding of calcium ions allows the N- terminal regions of the subunits to associate with each other to form integrin and to bind to the extracellular matrix

Integrin ligands

The polypeptides that bind to integrins include fibronectin, vitronectin, von Willebrand factor and collagen. The specificity of individual integrins is often not restricted and they bind more than one class of ligand. The same ligand can also bind to more than one integrin. The majority of ligands are extracellular matrix proteins involved in cell- substratum adhesion. Some of these proteins, such as vitronectin, mediate cell- cell aggregation whilst other recognise integral immunoglobulin proteins and mediate cell- cell adhesion. Many of these integrin ligands contain a conserved arginine- glycine- aspartic acid sequence (RGD). These include fibronectin and vitronectin. The RGD- binding integrins are closely related and contain cleaved α- subunits. The RGD sequence is recognised by several integrins ($\alpha_5\beta_1$, $\alpha_{II}\beta_3$, and all or most of the $\alpha_v\beta$ integrins) but not by most others which bind different sequences in ligands. The amino acids flanking the RGD motif also affect the specificity and affinity of the peptides (reviewed by Meredith and Schwartz, 1997; Hynes, 1992).

Modulation of integrin activation and affinity

There is increasing evidence that integrins mediate information transfer. This signalling takes two forms, regulation of the affinity and conformation of the receptor (generally from within the cell) and initiation of intracellular events, by ligand binding. Integrins undergo conformational changes between at least two states, inactive and active. They bind ligands while in the active state. The activation of integrins can be modulated by intracellular signals which can change the affinity of some integrins for other ligands. A particular integrin can have different affinities depending on the cell type in which it is expressed. The modulation of integrin function can also be accomplished by the ligands themselves. In an adhesion process, integrin may be activated by stimuli such as soluble mediators (hormone, cytokines etc.) or solid mediators (extracellular matrix proteins or other cells). Intracellular signals also change integrin avidity so that the integrins cluster together and this also increases ligand binding affinity (reviewed by Hynes, 1992; Clark and Brugge, 1995; Richardson and Parsons, 1995; Schlaepfer and Hunter, 1998; Howe et al. 1998; Boudreau and Jones, 1999).

Ligand binding to integrins initiating intracellular events

Ligand binding can trigger intracellular events including tyrosine phosphorylation, activation of lymphocytes and activation of secretion in synovial fibroblasts, monocytes and neutrophils. Tyrosine phosphorylation in adherent cells and platelets could be involved in the association of structural and regulatory proteins into submembranous cytoskeletal structures at the cell-substratum and cell- cell contact points. Adhesion of cell surface integrins on fibroblasts to fibronectin causes an elevation of cytoplasmic pH which correlates with parallel stimulation of spreading and growth. It is hypothesized that integrins work in synergy with receptors for soluble agonists such as growth factors in stimulating these signals. Extracellular matrix proteins have multiple sites for interactions with cell surface receptors, including integrins and proteoglycans. This allows extracellular matrix proteins to link several different cell surface molecules together thereby producing an organised area of submembrane associations. These submembrane interactions can generate cytoskeletal structures and/ or trigger signaling events (reviewed by Hynes, 1992; Clark and Brugge, 1995; Richardson and Parsons, 1995; Schlaepfer and Hunter, 1998; Howe et al., 1998; Boudreau and Jones, 1999).

Application to gene therapy

Integrins mediate cell adhesion and motility on the extracellular matrix, yet they also promote viral attachment and or entry and internalisation of extracellular molecules for degradation. A number of viruses and bacteria exploit integrin- binding in order to enter the cell, including Yersinia pseudotuberculosis and adenovirus. The adenoviral RGD sequences in the penton bases bind to α_V integrins and this induces activation of phosphoinositide- 3- OH kinase (PI3 kinase). PI 3 kinase activation and recruitment of the actin cytoskeleton is required for viral internalisation. It is known to modulate intracellular protein trafficking, although a direct role of PI3 kinase in receptor- mediated endocytosis has not yet been established (Li et al., 1998). Transferrin uptake by the transferrin receptor does not require PI3 kinase activation suggesting that different biochemical pathways may exist for receptor mediated endocytosis.

A receptor- mediated gene delivery system has been developed to exploit cell surface integrins. Short polypeptides containing RGD sequences, or other integrin- binding sequences, have been synthesized and found to bind to cell- surface integrins. The binding affinity of these RGD- containing polypeptides is increased if the RGD region is confined to a cyclised configuration formed by a disulphide bond (Koivunen et al., 1995). An fd filamentous phage displaying a cyclic RGD- containing polypeptide was found to bind and enter epithelial cells in an integrin- mediated manner (Hart et al., 1994). A polypeptide containing an integrin- targeting cyclic RGD domain and a DNA binding domain consisting of 16 lysine residues was complexed with DNA encoding the luciferase gene. This complex was able to bind and enter Caco- 2 cells in an integrin- mediated manner and luciferase expression was demonstrated (Hart et al., 1995). Addition of the liposome formulation Lipofectin to these complexes to form Lipofectin- integrin targeting peptide- DNA (LID) complexes increased the efficiency of gene expression from 1-10% to 50% in three different cell lines (Hart et al., 1998). Addition of Lipofectin to integrin-targeting peptide complexes for in vitro transfection of corneal endothelium also increased transfection efficiency from 0 to 25%. The Lipofectin is believed to aid escape of the complexes from endosomes by destabilisation of the endosomal membrane. Gene transfer with the DNA condensation agent polyethylenimine (PEI) conjugated to an integrin- binding peptide and complexed with the DNA for delivery has also been demonstrated. Transfection increase 10-100 fold compared with PEI alone, even in serum (Boussif et al., 1995).

Route of administration of non-viral vectors

The two main routes of administration are in vivo and ex vivo. A wide range of non- viral systems can be used ex vivo, from the physical and chemical methods of transfection to Lipofection and receptor- mediated transfection. Modified fibroblasts, myoblasts and glial cells could be transplanted into the central nervous system. Modified fibroblasts and keratinocytes could be used for skin engraftment. Bone marrow stem cells and myoblasts modified ex- vivo could then be reimplanted to the sites from which they were removed. Systems used in vivo are limited to naked DNA, liposome-DNA complexes and receptor-mediated systems. These non-viral vectors can be injected directly into the

vasculature or skeletal muscle. DNA-liposome complexes can also be used in an aerosol form to target the lung.

1.7. Aims of this study

The main aim of this project is to initiate an evaluation of integrin-mediated gene transfer as a potential form of gene therapy for the lysosomal storage diseases. It is envisioned that Lipofectin- integrin targeting peptide-DNA (LID) complexes could be injected intravenously, possibly using several integrin-targeting ligands simultaneously, to transfect different cell types. The transfected cells may secrete enzyme, which could be taken up by other cells. Alternatively the LID-complexes could be used to transfect fibroblasts, which could then be implanted as neo-organs to produce a secreted source of the deficient enzyme. Fucosidosis was chosen as a typical lysosomal storage disease in which there is both central nervous system involvement and systemic symptoms. A combination of therapies might be appropriate in fucosidosis in which one form of therapy seeks to correct the central nervous system whereas another, such as integrin- mediated gene transfer or enzyme replacement, targets the other affected organs. Integrin- mediated gene transfer might be useful in treating Fabry disease because the vasculature, which is the main site of lesions in this disease, should be accessible to LID-complexes by direct injection or to enzyme secreted by a neo-organ transfected by LID-complexes.

In this project preliminary experiments will be carried out investigate the transfection of cells in culture by integrin-mediated gene transfer and the subsequent expression of the transfected gene. Ultimately experiments *in vivo* in animal models of lysososmal storage diseases wlil be necessary to establish the range of cells transfected and whether metabolic correction and any clinical improvement can be achieved by this approach.

Plan of Investigation

1. Characterisation of molecular defects in patients with fucosidosis to identify fibroblasts suitable for the gene therapy studies.

2. Optimisation of integrin- mediated gene transfer into normal fibroblasts using the reporter genes, luciferase and β-galactosidase. The composition of the complexes and the conditions of transfection will be systematically varied to determine the best conditions for transfection of fibroblasts.

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3. The optimal system will be used to transfect fibroblasts from patients with Fabry disease and fucosidosis and normal fibroblasts with cDNA encoding the lysosomal enzymes, α-galactosidase and α-fucosidase. The level of expression and distribution of the transfected gene product will be studied.

4. ECV304 cells (transformed human umbilical vein endothelial cells) will also be transfected to investigate whether integrin- mediated gene transfer is applicable to the human endothelium, which is the main site of lesions in Fabry disease.

Materials and methods

2.1. Patient information and material

2.1.1. Fucosidosis patients

Material was collected from patients diagnosed as having fucosidosis on a clinical analysis. Table 2.1 gives the patients' initials, genetic information available at the start of this study and material available for analysis. Further clinical details are in Appendix 2.1. The phenotype was classified as atypical if the symptoms of the patient were limited in their range or if they had higher residual α- L- fucosidase activity than is typical of fucosidosis. Some atypical patients represent misdiagnoses, where the patient has an unidentified neurologic disorder combined with the low α- L- fucosidase activity polymorphism (see Section 1.4.2). In such cases mutation analysis was undertaken to confirm that there was not a mutation in the FUCA1 gene. No detailed clinical information was available for patients D.P., H.A., S.D., Z.T. and A.A., who had been referred to the laboratory for biochemical and/ or genetic analysis only.

Patients' initials	Phenotype	Q/R281 polymorphic phenotype	Mutation in α-fucosidase gene	Material available for analysis
S.B.	Severe	A/A	IVS5+1	Lymphoblastoid cell line and DNA
M.B.	Severe	G/G	E113fs, 10bp deletion	Fibroblast cell line and DNA
C.Lo.	Mild	∇	∇	DNA
C.Le.	Severe	A/G	∇	Lymphoblastoid cell line and DNA
M.M.	Severe	A/G	W183X	DNA
A.M.	Moderate/ severe	A/A	P5R Y330fs 1bp deletion	Fibroblast cell line and DNA
G.S.	Severe	A/A	N329Y	Fibroblast cell line and DNA
D.P.	-	∇	∇	DNA
O.B.	Atypical	∇	∇	DNA
H.A.	-	∇	∇	DNA
S.D.	-	∇	∇	DNA
Z.T.	-	∇	∇	Fibroblast cell line and DNA
A.A	-	∇	∇	DNA
M.C.	Atypical	∇	∇	DNA

Table 2.1 Fucosidosis patients: information from previous studies and material available for analysis.

*In some atypical patients subsequent studies revealed that there was not a mutation in the FUCA1 gene. ∇ denotes that this has not been studied previously.

2.1.2. Fabry patients

Fibroblast cells from a fully characterised, typical Fabry disease patient (P.) were used in this study. This patient had negligible α- galactosidase A activity and mutation 1151 del 2 in exon 7.

2.2. Cell culture

All procedures were carried out under sterile conditions. Cell culture reagents were purchased from Sigma unless otherwise stated. Sterile disposable plasticware such as tissue culture flasks, centrifuge tubes and pipettes were obtained from Falcon.

2.2.1. Tissue culture media

RPMI growth medium

RPMI growth medium was used for the growth of lymphoblastoid cell lines which had been transformed with Epstein-Barr virus (EBV). 500ml of RPMI-1640 medium (Gibco-BRL) was supplemented with 50ml of fetal calf serum (Imperial Laboratories), 73mg of L- glutamine and 5ml of penicillin/streptomycin (1mg/ml) and sterilised by filtration through a 0.22 µm Millex-GV Millipore filter. The medium was stored for up to 1 month at 4°C.

Hams F10 growth medium

Hams F10 growth medium was used for the routine growth of fibroblast cell cultures. 500ml of Hams F10 (Sigma) was supplemented with 70ml of fetal calf serum (FCS, Imperial Laboratories), 73mg of L- glutamine and 5ml of penicillin/streptomycin (1mg/ml) and sterilised by filtration through a 0.22 µm Millex-GV filter (Millipore). The medium was stored for up to 1 month at 4°C.

Dulbecco's modified Eagle's medium (DMEM)

DMEM was used for the growth of ECV304 cells. 500ml of DMEM with Glutamax-1 medium (Gibco-BRL) was supplemented with 50ml of fetal calf serum (Imperial Laboratories), 73mg of L- glutamine and 5ml of penicillin/streptomycin (1mg/ml) and sterilised by filtration through a 0.22 µm Millex-GV filter (Millipore). The medium was stored for up to 1 month at 4°C.

Chang's medium C

Chang's medium C was provided by Irvine Scientific and was used for the initial growth of patient fibroblast cell lines after reconstitution from liquid nitrogen. The lyophilized supplement was allowed to equilibrate to room temperature and was resuspended in 10ml of sterile water. 1ml aliquots were stored at -20°C. Chang's medium C was prepared by adding 9ml basal media to 1ml of supplement, 100ul of 200mM L-glutamine and 100μl of penicillin/ streptomycin (1mg/ml). The medium was sterilised by filtration through a 0.22 μm Millex-GV filter (Millipore). The medium was stored for up to 1 week at 4°C.

2.2.2. Reconstitution of cells stored in liquid nitrogen

To reconstitute a fibroblast, lymphoblastoid or ECV304 cell line, cells were removed from storage in liquid nitrogen and thawed to 0°C. The ampoule containing the cells was then centrifuged at 2 000 x g for 5 min. The supernatant was removed and the cells were resuspended in 1ml of the appropriate growth medium and transferred to a flask with 9ml of additional medium.

2.2.3. Detection of mycoplasma and treatment of infected cell lines

Fibroblast cells were routinely tested for mycoplasma contamination. Cells were grown on a glass coverslip in a cell culture dish with 5ml of medium for 5 days. The cells were fixed in 5ml of methanol:acetic acid (1:3) for 2 min. This was followed by three incubations for 5 min with methanol:acetic acid (1:3). The cells were then left to air- dry before incubation for 30 min with 4', 6-diamidino-2-phentindole (DAPI, 50μg/ml) in phosphate buffered saline (PBS, Sigma). The cells were then rinsed twice with water and mounted on a microscope slide. Mycoplasma were visualised using a fluorescent microscope with a 53/44 barrier filter and a BG-3 exciter filter. Affected flasks were treated for 5 days with Mycoplasma Removal Agent (ICN, 1% (v/v)) in growth medium.

2.2.4. Cell culture conditions

Fibroblasts

Fibroblast cells were supplied either as ampoules of cells in liquid nitrogen or as cultures of growing cells which had been established from a skin biopsy by the Enzyme Laboratory, Institute of Child Health.

Fibroblasts were grown in monolayers in flasks ($25cm^2$, $75cm^2$ or $175cm^2$) or in well plates prior to transfection. They were reconstituted in Changs medium C and after 2 weeks transferred to Hams F10 medium. 4ml, 10ml or 30ml of growth medium were added to $25cm^2$, $75cm^2$ and $175cm^2$ flasks respectively. Flasks were incubated at 37°C in an incubator with a 5% CO_2 atmosphere. The growth medium was changed twice a week. Cells were subcultured, harvested or stored in liquid nitrogen when they reached confluence, as judged by phase contrast microscopy.

To subculture, harvest or store adherent cells, the spent medium was first removed by aspiration. The cell monolayer was washed with PBS and incubated with sterile trypsin solution for 5-10 min at 37°C. The flask was then hit on the side repeatedly to detach the cells. For subculturing, the detached cells were subdivided into 2 new flasks and 4, 10 or 30ml of growth medium added to each flask. For harvesting or storage, growth medium was added to inhibit the action of trypsin. The cells and medium were transferred to a sterile Falcon tube and centrifuged at 2000 x g for 5 min. The medium was removed and the sedimented cells were washed twice in 2ml of PBS. To harvest the cells 50μl of cold water was added after the second wash and the cells were either used immediately or stored at -20°C for subsequent analysis. Cells for long- term storage were resuspended in 1ml of fresh growth medium at 0°C. 1ml of ice cold growth medium containing 10% (v/v) DMSO was added slowly to the cell suspension with mixing at 0°C. 1ml of this suspension was added to each of 2 plastic ampoules (Nalgene Nunc International) at 0°C and they were immediately placed in a Nalgene Cryo 1°C freezing container in a -70°C freezer for one day. This allows the cells to freeze slowly and limits cell damage. The ampoules were finally immersed in liquid nitrogen for storage.

EBV-transformed lymphoblastoid cells

Epstein-Barr virus (EBV)-transformed lymphoblastoid cells, which had been transformed and established previously by Paul Rutland, Institute of Child Health, were available as ampoules of cells in liquid nitrogen. EBV-transformed lymphoblastoid cells were grown in suspension with 10ml of RPMI growth medium in 75cm^2 flasks. They were incubated at 37°C in 5% CO_2. The growth medium was changed approximately twice a week.

The EBV-transformed lymphoblastoid cells were subcultured by transferring half of the cells to a new flask and adding 10ml of fresh RPMI growth medium to each of the flasks. For harvesting and long- term storage lymphoblastoid cells were transferred in RPMI growth medium to a sterile tube and centrifuged at 2000 x g for 10 min. The spent medium was removed and the sedimented cells were washed twice in 2ml of PBS. To harvest the cells 50µl of cold water was added after the second wash. The cells were either used immediately or stored at –20°C. Cells for long- term storage were resuspended in 1ml of fresh RPMI growth medium at 0°C. 1ml of ice cold RPMI growth medium containing 10% (v/v) dimethylsulphoxide (DMSO) was added slowly to the cell suspension with mixing at 0°C. 1ml of this suspension was added to each of 2 plastic ampoules (Nalgene Nunc International) at 0°C and they were immediately placed in a Nalgene Cryo 1°C freezing container in a -70°C freezer for one day. The ampoules were finally immersed in liquid nitrogen for storage.

ECV304 cells

A flask of growing ECV304 cells (spontaneously transformed human umbilical vein endothelial cells) was supplied by Dr. Stephen Hart, Institute of Child Health. ECV304 cells were grown, subcultured, harvested and stored in liquid nitrogen in the same manner as fibroblasts except that the medium used was DMEM.

2.3. Biochemical assays

2.3.1. Determination of protein concentration

Solutions

BSA	Bovine serum albumin protein standard 1mg/ml (Sigma)
BCA	Bicinchoninic acid (Sigma)
Copper sulphate solution	4% (w/v)$CuSO_4.5H_2O$

Protein concentration was measured using a method based on that of Smith et al. (1985). All assays were performed in duplicate. A standard curve was prepared with 5-50µg BSA in 50µl H_2O (0.1-1mg/ml). Samples containing 5 or 10µl of cell extract in 50µl of H_2O were assayed. 1ml of bicinchoninic acid solution was added to the standards and samples and they were incubated at 37°C for 10 min to solubilise the protein. The Copper sulphate solution (20µl) was added and the tubes were incubated further for at least 20 min at 37°C. The absorbance was measured at 562nm using a spectrophotometer (CECIL CE2021) and the protein concentrations calculated in mg/ml from the standard curve.

A modified assay was used to determine the lower protein concentrations obtained when approximately 10^{4-5} cells were used. The assay was carried out as above except that the standard curve was prepared over the range 5-250µg of BSA in 50µl H_2O and the incubation with copper sulphate solution was carried out for at least 20 min at 60°C. Samples contained 20 or 30µl of cell extract or conditioned medium in a total volume of 50µl of H_2O.

2.3.2. Assays of reporter enzymes

2.3.2.1. Lac Z histochemical staining

X-gal solution	5mM $K_4Fe(CN)_6$, 5mM $K_3Fe(CN)_6$, 100µg/ml X-gal (NBI) in N,N-dimethylformamdide

The X-gal solution was prepared, incubated for 10 min at 37°C and filtered through a 0.22µm Millex-GV filter prior to use. Cells were grown in 6-

well plates (approximately 5 µg protein per well), washed with PBS and fixed by incubation in 0.5% (v/v) glutaraldehyde in PBS for 15 min at room temperature. 200µl of X-gal stain buffer was added to well and the plate was incubated at 37°C for 1-5 h to allow colour development. The cells were washed twice with PBS and stored in 0.5ml of 50% (v/v) ethanol in PBS. The cells were viewed using an Olympus 1X70 microscope and photographs taken using an attached Olympus OM10 camera. The percentage of total cells stained for β-galactosidase was estimated and compared to stained control cells transfected with LID complexes that did not contain the pAB11 plasmid.

2.3.2.2. Luciferase assay

Samples were assayed in triplicate. The luciferase assay reagent (Promega) was allowed to equilibrate to room temperature. 20µl of cell extracts were placed in wells of a 96 well plate (Labtech). The plate was assayed in a LKB 1251 luminometer (Labtech) which automatically added 100µl of luciferase assay reagent and measured the total light emission for 60s. The number of Relative Light Units (RLU) per mg protein was calculated for each sample.

2.3.3. Assays of lysosomal enzymes

All assays were performed in duplicate or triplicate, if possible. The lysosomal enzymes α- L- fucosidase, α- galactosidase, and β- hexosaminidase in the intracellular and secreted fractions were assayed using the appropriate, synthetic, fluorogenic 4- methylumbelliferyl- glycoside substrates. The reactions were stopped by the addition of glycine buffer, pH 10.4 and the fluorescence of the released 4-methylumbelliferone was measured at 450nm with an excitation wavelength of 365nm using a Perkin Elmer LS50 fluorimeter. Each assay was validated over the appropriate protein concentration.

The fluorimeter was calibrated with a 1mM solution of 4-methylumbelliferone. A control of buffered substrate and water was included in the assay and its fluorescence was subtracted from all other readings. Enzyme activity was generally expressed as nmol of 4-methylumbelliferone released per mg of total protein per hour. In some transfection experiments the enzyme activity was expressed as nmol of 4-methylumbelliferone released per hour i.e.

units of activity in the cell extract or conditioned media (from 1 well in a 6- or 24-
well plate).

Preparation of material

Cell pellets harvested from flasks (section 2.2.4) were thawed and stored
on ice and cell pellets harvested from well plates were kept on ice (section
2.7.4). The pellets were sonicated for 10 s at an amplitude of 6 microns in a
MSE Soniprep ultrasonicator. The sonicate was vortexed and centrifuged at
2000 x g for 3 min and the pellet of cell debris discarded. Samples of cell
culture medium containing secreted lysosomal enzymes were prepared as
described in section 2.7.4.

Buffers and substrates

Stopping reagent 0.25M glycine adjusted to pH10 with NaOH for α-
galactosidase and β- hexosaminidase assays and to pH
10.4 for α- L- fucosidase assays.

α- L- fucosidase assay solutions

MV4.5   McIlvaine citrate phosphate buffer
                          0.1M citric acid, titrated to pH 4.5 with 0.1M Na_2HPO_4

Substrate   1.6mmol 4-methylumbelliferyl-α-L-fucopyranoside in MV4.5
                          buffer, dissolved by warming to 80°C.

α-galactosidase assay solutions

Acetate buffer   0.1M sodium acetate buffer, pH4.5

Phosphate buffer 20mM sodium phosphate buffer, pH6.4

Substrate   10mmol 4-methylumbelliferyl-α-D-galactopyranoside in
                      sodium acetate buffer, pH4.5

β- hexosaminidase assay solutions

MV4.5   McIlvaine citrate phosphate buffer
                      0.1M citric acid adjusted to pH 4.5 with 0.2M Na_2HPO_4

Substrate   2.5 mmol 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-

glucopyranoside, pH4.5 in MV4.5 buffer

2.3.3.1. α-L-fucosidase assay

To determine the α-L-fucosidase activity in a flask of cells (approximately 250μg protein) 5μl of sonicated cell extract was incubated with 95μl of 0.4% (w/v) human serum albumin in water and 100μl substrate solution for 30 min at 37°C. The reaction was stopped by the addition of 1ml of stopping reagent.

The α- L- fucosidase activity of transfected cells or media conditioned by transfected cells were assayed in duplicate, or triplicate. 20-40μl of cell extract or 50μl of medium was made up to 100μl with 0.4% (w/v) human serum albumin in water. 100μl of substrate solution was then added and the mixture incubated for 3 h at 37°C. The reaction was stopped by the addition of 1ml of stopping reagent and the fluorescence measured.

2.3.3.2. α- galactosidase assay

20-25μl of cell extract or 50μl of medium was made up to 50μl with 20mM sodium phosphate buffer, pH6.4. Two samples were incubated at 4°C while the other 2 were incubated at 50°C to denature the heat labile form of α-galactosidase, α- galactosidase A. All of the samples were then placed on ice for 3 min before brief centrifugation at 2 000 x g. 50μl substrate solution was then added and the mixture was incubated for 45 min at 37°C. The reaction was stopped by the addition of 1ml of stopping reagent and the fluorescence was measured. The total α- galactosidase enzyme activity, heat labile α-galactosidase A enzyme activity and α- galactosidase B enzyme activity were calculated (Total α- galactosidase activity = samples incubated at 37°C, α-galactosidase B activity = samples incubated at 50°C and α- galactosidase A activity = Total α- galactosidase activity - α- galactosidase B activity). The majority (approximately 95%) of total α- galactosidase activity was the A form, therefore, in most experiments only total α- galactosidase activity was assayed in order to use less sample.

2.3.3.3. β-hexosaminidase assay

β- Hexosaminidase is an abundant lysosomal enzyme with a simple, established assay which can be used as a reference enzyme. The activity of β-hexosaminidase can be used to standardise the activity of another lysosomal enzyme by determining the lysosomal enzyme: β- hexosaminidase activity ratio. 5-10μl of cell extract or 50μl of medium was made up to 100μl with 0.2% (w/v) human serum albumin in MV4.5. 100μl of substrate solution was then added and the mixture incubated for 10 min at 37°C. The reaction was stopped by the addition of 1ml of stopping reagent and the fluorescence measured.

2.4. Immunological characterization of α- L- fucosidase

2.4.1. Anti- α- L- fucosidase antibodies

Polyclonal goat anti- (human liver α- L- fucosidase) antibodies (referred to as the polyclonal antibody) and monoclonal anti- (human liver α- L- fucosidase) antibodies (referred to as the monoclonal antibody) were kindly provided by Prof. Jack Alhadeff, of Lehigh University, Bethlehem, U.S.A, (Johnson et al., 1992).

2.4.2. Purification of antibody preparation with protein A- Sepharose

Solutions

Low salt buffer	0.1M NaCl, 0.1M CaCl₂, 0.1mM MnCl₂, 0.1mM MgCl₂ in 10mM sodium phosphate buffer, pH6.8.
High salt buffer	1M NaCl, 0.1M CaCl₂, 0.1mM MnCl₂, 0.1mM MgCl₂ in 10mM sodium phosphate buffer, pH6.8.
Elution buffer	0.5M methyl-α-D-mannopyranoside in the high salt buffer.

Protein A- Sepharose (Pharmacia, 3ml) was washed and equilibrated with low salt buffer. 500μl of the antibody was then added, mixed and allowed to equilibrate for 30 min on a turntable at 4°C so that the glycoprotein antibody could bind the lectin. The suspension was centrifuged and the supernatant discarded. The pellet was washed with 5ml of low salt buffer followed by 5ml of

the high salt buffer. The bound antibody was then eluted with 400μl of cold elution buffer and stored at –20°C for further use.

2.4.3. Immunoprecipitation of α- L- fucosidase activity

The anti- α- fucosidase antibodies were serially diluted in PBS over the range 1/10 to 1/10 000. Human liver supernatant (HLS) was prepared with postmortem tissue which had been frozen at –20°C. Homogenates 1:5 (w/v) were made in 100mM pH6.5 Na_2HPO_4- KH_2PO_4 buffer using a ground glass hand homogenizer. The homogenate was centrifuged for 30min at 23500 x g. 40μl of HLS was diluted to make a total volume of 1000μl in PBS and separated into 25μl aliquots on ice. 25μl of each antibody dilution was added to an aliquot of diluted HLS as antigen. Controls substituting PBS for either the antibody or antigen were also prepared. All of the samples were incubated at 4°C overnight on a rocking platform. 25μl of protein A- Sepharose or protein G- Sepharose (depending on the type of antibody, Sigma) was added to each sample. Controls with both antibody and antigen but with PBS replacing the Sepharose were also prepared. The samples were incubated at 4°C for 1 hour on a rocking platform. The tertiary protein-antibody-antigen complexes were collected by centrifugation at 12 000 x g for 1 min at 4°C. The supernatant was carefully removed and the samples assayed for α-fucosidase activity as described in section 2.3.3.1.

2.4.4. Immunodetecion of α- L- fucosidase protein by Western Blotting

Western blotting was performed according to the method of Johnson et al. (1992), with some modifications, using the Hoefer Scientific Instruments Ltd. System.

Solutions

Sample buffer	2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 0.2M dithiothreitol (DTT), 0.02% (w/v) bromophenol blue, 12.5% (v/v) stacking gel buffer and 5% (v/v) β-mercaptoethanol.
Monomer solution	4M acrylamide, 0.05M bisacrylamide

Resolving gel buffer	1.5M Tris-HCl, pH8.8
Stacking gel buffer	0.5M Tris- HCl, pH6.8
10% resolving gel	10ml of monomer solution, 7.5ml of 4x resolving gel buffer, 0.3ml of 10% (w/v) SDS, 12.1ml of ddH$_2$O, 150ul of 10% (w/v) ammonium persulfate (APS) and 10µl of N, N, N', N'- tetramethylethylenediamine (TEMED).
4% stacking gel	665ul of monomer solution, 1.25ml of stacking gel buffer, 0.05ml of 10% (w/v) SDS, 3.0ml of ddH$_2$O, 25µl of 10% (w/v) APS and 2.5µl of TEMED.
Reservoir buffer	25mM Tris, 0.192M glycine, 0.1% (w/v) SDS, pH8.3
Towbin transfer buffer	25mM Tris, 0.192M glycine, 20% (v/v) methanol, 0.1% (w/v) SDS
TBS	Tris- buffered saline 100mM Tris/HCl, 0.9% (w/v) NaCl
TBS- Tween	TBS with 0.1% Tween-20

Molecular weight markers

A mixture of pre-stained molecular weight markers (BDH) was electrophoresed on the same gel as the test samples to enable the molecular weights of the separated protein to be calculated (see table 2.2). These markers were also transferred onto the nitrocellulose membrane where they were still visible.

Protein	Apparent molecular mass (kDa)
Ovotransferrin	105
Bovine serum albumin	98
Ovalbumin	53
Carbonic anhydrase	33
Soybean trypsin inhibitor	25

Table 2.2 Apparent molecular mass of pre-stained markers used in this study.

The proteins were separated by SDS polyacrylamide gel electrophoresis at 100V for about 1h on a 10% resolving gel with a 4% stacking gel in a Hoefer Scientific Instruments Ltd. SE600 unit containing 3l of reservoir buffer.

Proteins were transferred electrophoretically from the gel to a nitro-cellulose membrane (Biorad) using a sandwich assembly in a Transphor Electroblotting Unit TE50 (Hoefer Scientific Instruments Ltd.), with a current of 20V overnight.

A duplicate identical gel was run at the same time and proteins stained with Coomassie blue to check the specificity of the antibodies. After blotting the gel was stained for protein with Coomassie blue to check the efficiency of the transfer.

Each membrane was briefly washed with TBS-Tween-20 and unoccupied protein- binding sites were blocked by incubation in 5% (w/v) Marvel TBS-Tween-20 for 3 h at room temperature. It was then incubated overnight at 4°C in a 1/2000 dilution of polyclonal goat anti- human liver α-fucosidase antibody in 0.2% (w/v) Marvel TBS-Tween-20. After 3 washes in TBS-Tween-20 the membrane was incubated for 2h at room temperature in a 1/2000 dilution of horse radish peroxidase-conjugated rabbit anti- goat IgG antibody (2ug/ml, DAKO) in 0.2% (w/v) Marvel TBS-Tween-20. The membrane was washed with TBS-Tween-20 and the bound antibody- antigen complex was detected using the Amersham enhanced chemiluminescence (ECL) detection system by incubation with the Luminol substrate solution for 1 min. The membrane was wrapped in cling film and exposed to autoradiography x-ray film (Kodak X-OMAT-AR) which was then developed.

2.5. Molecular biological techniques

2.5.1. Preparation and extraction of genomic DNA from white blood cells, fibroblasts and EBV- transformed lymphoblast cells.

Solutions

Nuclei lysis buffer	10mM Tris-HCl, 400mM NaCl, 2mM Na$_2$-EDTA
Proteinase K solution	2mg/ml of fungal proteinase K (BDH) in 2mM Na$_2$-EDTA, 1% (w/v) SDS
TE buffer	Tris-EDTA buffer, pH 8.0
	10mM Tris-HCl, 1mM EDTA

The cell pellet was lysed with 300µl of nuclei lysis buffer and the resultant suspension thoroughly mixed using a sterile pasteur pipette. 20µl of 10% (w/v) SDS and 60µl of proteinase K solution were added and the sample mixed gently by inversion before incubation at 60°C for 1h or at 37°C overnight. Protein was precipitated from the digested samples by addition of 100µl of saturated ammonium acetate. The samples were vortexed for 15 s and allowed to stand at room temperature for 10-15 min before centrifugation at 1200 x g for 15 min at room temperature. The supernatant was transferred to a separate tube and 2 volumes of absolute ethanol were added and mixed by inversion to precipitate the DNA. The DNA threads were spooled out on the tip of a sealed glass Pasteur pipette and dissolved in 1ml of TE buffer in a 1.5ml microfuge tube. The DNA was stored at –20°C.

2.5.2. Measurement of concentration of DNA and oligonucleotides.

The DNA solution was diluted in ddH$_2$O and its absorbance at 260nm was measured using a GeneQuant spectrophotometer (Pharmacia). The DNA concentration was calculated assuming that DNA contains approximately equal amounts of purine and pyrimidine bases (Sambrook et al., 1989) and therefore

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that 1 absorbance unit is equivalent to 50ng/μl of double stranded DNA or 37ng/μl of single stranded DNA.

2.5.3. Amplification of genomic DNA by the Polymerase Chain Reaction (PCR)

2.5.3.1. Design of oligonucleotide primers

Oligonucleotide primers were designed using the primer program of the HGMP Resource Centre based at the MRC Clinical Research Centre, Harrow, U.K. They were synthesized and purified by Genosys. The DNA was resuspended in TE buffer and stored at -20°C. The intronic primers designed for the amplification of the 8 exons of the α-fucosidase gene are listed in Table 2.3. The PCR conditions are listed in Table 2.4.

Target sequence	Primer	Nucleotide position	Sequence (5'-3')
Exon 1	*FU1+	1044-1062 [a]	TCG TTA GTC AGA GTG GGC G
Exon 1	FU1-	1553-1570 [a]	AGC CCC ACC TCC TGT TTG
Exon 2	*FU2+	133-154 [b]	TCA GGC ATG CTG GGC AAG TTC A
Exon 2	FU2-	384-407 [b]	GAG GAG GTA CAG AAC TCT TGA CAG
Exon 3	*FU3+	537-560 [c]	AAT GCT AGA ACT GAT TTT CCT TAA
Exon 3	FU3-	806-826 [c]	TTA ATG GTA CCC TAT AGG AAG
Exon 4	*FU4+	355-381 [d]	AAT GGT CCA TAA GAT TTT ACT GTG AAC
Exon 4	FU4-	579-599 [d]	ACT CCA GAG TTT GGC TCC TTG
Exon 5	*FU5+	17-34 [e]	GCT GTC CTG TGC ATT GTA
Exon 5	FU5-	537-554 [e]	AGC TTT TGA ACA TTA TAT
Exon 6	*FU6+	68-87 [f]	TAA GCA TGA TGC CAG GCT TG
Exon 6	FU6-	363-382 [f]	AGG AGA TAC CAG TTC CGG AT
Exon 7	*FU7+	443-469 [g]	GGA GGA AAT GTA TAA AGT TGT ATA TCA
Exon 7	FU7-	681-698 [g]	ACC TGG CAG GGA AGG AAG
Exon 8	*FU8+	759-798 [g]	TCC TAC CAT AGT CAG CCT GT
Exon 8	FU8-	996-1015 [g]	AAA CAG TGA GCA GCG CCT CT

Table 2.3 Sequence of oligonucleotide primers used to amplify the 8 exons of the α-fucosidase gene.

*biotinylated when used to prepare samples for manual sequencing. Nucleotide position of primers are numbered according to Genbank database entries M808809[a], M808810[b], M808811[c], M808812[d], M808813[e], M808814[f], M808815[g].

2.5.3.2. Conditions for PCR reaction

All reactions were carried out and reagents prepared under sterile conditions and work was carried out within a designated area of the laboratory. PCR reactions were carried out in a total volume of 50µl in 0.5ml Eppendorf tubes according to the method of Saiki et al (1985). Each reaction mixture contained 100-500ng of DNA template, 20pmoles of each primer, 0.2mmol/L dATP, dCTP, dGTP and dTTP, 1-2mmol/L $MgCl_2$, 4% DMSO, 1 X NH_4 buffer (160mM $(NH_4)_2SO_4$, 0.67M Tris-HCl (pH8.8), 0.1% (v/v) Tween-20, 50mM $MgCl_2$) and 1-2.5 units of Taq polymerase (Bioline). Each sample was overlaid with 40µl of mineral oil to minimise evaporation. Amplification was carried out

on a GeneE (Techne) thermal cycler. A control sample, containing the reaction mix but no DNA template, was used to check for contamination.

The samples were denatured for 10 min at 96°C, with the Taq DNA polymerase enzyme added in a hot start, followed by 30-35 cycles of amplification in 3 stages: 1) denaturation of the double-stranded DNA template at 96°C for 1 min; 2) annealing of the primers to the complementary DNA strands at a temperature, Tm, depending on their composition (Table 2.4) for 45 s 3) extension of the DNA template copy by the 5' to 3' activity of the Taq DNA polymerase at 72°C for 45s. Finally, after cycling a step of 72°C for 10 min ensured elongation was complete.

Exon number	PCR product size (bp)	Annealing temp (Tm). (°C)		$MgCl_2$ conc. (mM)	
		Unmodified primers	M13 modified primers	Unmodified primers	M13 modified primers
1	527	60	59	2	1.5
2	275	57	57	1.5	1.5
3	290	48	57	1.5	1.5
4	245	54	58	1.5	1.5
5	538	47	57	1.5	1.5
6	315	57	55	1.5	1.5
7	256	57	57	1.5	1.5
8	257	61	58	1.5	1.5

Table 2.4 Conditions for PCRs

2.5.3.3. Analysis of PCR- products by agarose gel electrophoresis

Solutions

TBE buffer   Tris-Borate EDTA buffer, pH8.0 consisting of
                        45mM Tris-HCl, 45mM boric acid, 10mM Na_2-EDTA

Loading dye   0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene-cyanol,
                        0.1M EDTA, pH8.0 in 50% (v/v) glycerol/ water

The PCR- products were analysed by electrophoresis to check the specificity of amplification and the size of the product. A 2% (w/v) agarose gel was prepared in 100ml of TBE containing 1mg/ml ethidium bromide. The gel was poured in an 8 x 10cm minigel tray (GNA-100, Pharmacia) and allowed to

set at room temperature for 1-2 h. The gel tray was placed in a gel tank (Pharmacia) containing TBE buffer.

5μl of PCR- product were mixed with 2.5μl of loading dye and loaded into the submerged wells. A lambda 100bp ladder (Gibco-BRL) was also loaded as a molecular weight marker. Electrophoresis was carried out at 100V for 30 min-1 h depending on the size of the PCR- product. The DNA bands were visualised using an ultra-violet (UV) transilluminator and photographed using a Mitsubishi videocopy processor.

Each PCR reaction was optimised with regards to magnesium concentration (titration from 0.5-5mmol/L), the addition of 5-10% (v/v) DMSO and Tm. When non-specific amplification of template DNA was observed the following strategies were employed; 1) increasing the Tm of the reaction, 2) reducing the concentration of Taq Polymerase in the reaction mixture, 3) diluting the DNA samples in the reaction mixture, and 4) redesigning primers to amplify a more specific product. If non-specific products were consistently observed then, after electophoresis on an agarose gel, the band corresponding to the expected size of target DNA was excised and purified using the protocol described in section 2.5.3.4.

2.5.3.4. Purification of PCR- products

Purification of PCR- products from an agarose gel

Solutions

TE buffer   10mM tris-HCl, 1mM EDTA, pH8.5

The QIAquick gel extraction kit (Qiagen) was used. The target DNA band was excised from the gel with a scalpel under illumination the low setting of an U.V. transilluminator. The gel slice was cut into $2mm^3$ cubes, weighed and placed in a 1.5ml microfuge tube, 3 volumes of buffer QX1 were added to 1 volume of gel (where 100mg is equivalent to 100μl) and the sample incubated at 50°C for 10 min with vortexing every 2 min. If all of the gel slices had not dissolved after this time the suspension was thoroughly mixed and incubated for a further 2 min. If the colour of the solution had changed to orange or violet, the pH was readjusted to pH7.5 by adding 10μl of 3M sodium acetate. 1 gel

volume of isopropanol was added and the solution was mixed and applied to a Qiaquick column seated in a collection tube. The column was centrifuged at 10 000 x g for 1 min forcing the solution through the column so that the DNA could bind. The flow-through was discarded. 0.5ml of buffer QX1 was applied to the column and the column was centrifuged at 10 000 x g for 1 min to remove any remaining agarose. 0.75ml of buffer PE was applied to the column, which was then left to stand for 5 min before centrifugation at 10 000 x g for 1 min to wash the column. The flow-through was discarded and the column centrifuged again to remove any residual ethanol from buffer PE. The QIAquick column was placed in a clean Eppendorf tube. The DNA was eluted from the column by adding TE buffer, pH8.5, to the centre of the column, which was left to stand for 1 min before centrifuging at 12 000 x g for 1 min.

Purification of PCR- products directly

Spin column chromatography was used to purify DNA from PCR reaction mixtures. One Microcon-100 column (Amicon) was inserted into a sample reservoir, for each sample. 15µl of the PCR reaction mixture and 420µl of water were mixed and pipetted onto the column. The column was centrifuged at 500 x g until all the solution had passed through the column allowing DNA to bind but not excess primers, salts and deoxynucleotides. 400µl of Microcon water (Amicon) was applied to the column, which was then centrifuged at 3 000 x g for 20 min. The column was then inverted and placed in a new collection tube. The column was centrifuged at 1 000 x g for 3 min to transfer the concentrate to the vial. The volume of the concentrated solution was made up to 10µl with Microcon water. 1µl of the concentrate was mixed with 4µl of water and 2µl of loading dye and electrophoresed on a 1.5% agarose gel to assess the recovery of DNA (see section 2.5.3.3).

2.5.4. Detection of specific mutations and polymorphisms by restriction enzyme digestion of PCR amplified products

Two mutations of the α- L- fucosidase gene, G60D and Q422X, are present in several patients with fucosidosis. Both of these mutations and the Q/R281 polymorphism alter different restriction enzyme sites and can therefore be

detected by restriction enzyme digestion of PCR products (band patterns in section 3.4.2.1). 10µl of the PCR- product for the appropriate exon was digested with the appropriate restriction enzyme in a total volume of 30µl, according to the manufacturers instructions (Table 2.5).

30µl of the digested product was mixed with 3µl of loading buffer and electrophoresed on a 2% agarose gel for detection of mutations or a nusieve-GTG-agarose gel (1:1)) with 5% (v/v) ethidium bromide for detection of the polymorphism, at 100V for 1-3 h. A lambda 100bp ladder was also loaded as a molecular weight marker. The digestion products were visualised by U.V. transillumination and photographed using a Mitsubishi videocopy processor.

Polymorphism/ mutation	Exon	Restriction enzyme	Incubation time (h)	Incubation temperature (°C)
G60D	1	Afl III	2	37°C
Q422X	8	ECOR1	2	37°C
Q/R281	5	BSAJ1	3	60°C

Table 2.5 Conditions for detection of Q/R281 polymorphism and G60D and Q422X mutations by restriction enzyme analysis.

2.5.5. Single Strand Conformation Polymorphism (SSCP) analysis

SSCP analysis was used to detect changes in the sequence of DNA from patients. An altered mobility during electrophoresis in a non-denaturing polyacrylamide gel of single- stranded DNA from patients compared with the wild type can be attributed to the altered folding of the chain resulting from a sequence change (Orita et al., 1989; Hayashi et al., 1993).

2.5.5.1. Sample preparation

Solutions

Denaturing solution   95% (w/v) deionised formamide, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol and 20mM EDTA

The 8 exons of the α- L- fucosidase gene were amplified from genomic DNA as described in section 2.5.17. Since the PCR product for exon 1 was larger than 500bp it was digested with Not 1 in the same way as describes in (section 2.5.4) to give smaller fragments of 194 and 319bp, increasing the probability of detecting a conformational change (Hayashi et al., 1993).

Prior to electrophoresis, 5μl of PCR product was mixed with 3μl of denaturing solution and centrifuged at 3 000 x g for 5 s. The samples were denatured for 3 min at 94°C and immediately placed on ice before loading on the gel.

2.5.5.2. Preparation of polyacrylamide gels

Two glass plates (42 x 33cm and 39 x 33cm) were washed with 2% Micro detergent (International Products Corp), rinsed with tap water and wiped with 70% (v/v) ethanol to dry the plates. One side of the smaller plate was coated with Sigmacote siliconising fluid (Sigma), to ensure the gel remained on the large plate during plate separation, in a fume hood where it was allowed to air dry for 10 min. The plates were placed together with 0.4mm spacers separating them at either side. The sides of the plates were clamped with bulldog clips.

A non-denaturing polyacrylamide gel mixture was prepared by mixing 35ml of 2 x Hydrolink MDE Gel (Baker), 7ml of 5 x TBE, 8.2ml 87% (v/v) glycerol (optional) and water to make a total volume of 70ml. 350μl of 10% (w/v) ammonium persulphate and 35μl TEMED were added to initiate polymerisation and the gel was poured between the 2 horizontal plates using a 50ml syringe. The straight edge of a sharks tooth comb (well width 1cm) was inserted at the top of the gel and clamped into position. The gel was left to polymerise for 2h before use.

2.5.5.3. Gel electrophoresis

Electrophoresis was carried out in a S2 sequencing gel system (Gibco-BRL). The bottom reservoir of the apparatus was filled with 500ml of 0.5 x TBE. The bulldog clips and plastic spacer strips were removed from the plates and the plates clamped vertically into the gel tank. The upper reservoir was filled with 500ml of 0.5 x TBE. A syringe needle was used to remove air bubbles

from below the plates. The comb was removed from the top of the plates and the top of the gel washed using a plastic pasteur pipette to remove any unpolymerised acrylamide. The sharks tooth comb was inserted to form wells and the wells were washed with 0.5 x TBE. 5μl of each sample was analysed and electrophoresis was carried out at 4°C or 37°C, at 15W power overnight (45W for gels containing glycerol).

2.5.5.4. Silver staining

The protocol is as described by Bassam et al. (1991). All solutions were removed from the tray using a water pump.

After electrophoresis the plates were separated and the gel transferred from the large plate to 3MM Whatman paper, which was then placed in a tray containing 10% (v/v) ethanol for 5 min to fix the DNA. The solution was removed and the gel was oxidised by addition of 1% (v/v) nitric acid for 3 min. The oxidising solution was removed and the gel rinsed with double distilled water for a few seconds before being impregnated with 0.012M AgNO$_3$ for 20 min. The solution was removed and the gel rinsed with double distilled water. The gel was developed in 0.28M sodium carbonate/ 0.019% (v/v) formaldehyde until the bands appeared. When the bands were dark enough but the background was still clear, development was stopped by immersion in 10% (v/v) acetic acid for 2 min. The gel was rinsed with double distilled water and shrunk back with 50% (v/v) ethanol for 15-30 min. The gel was then transferred to 3MM Whatman paper, covered with cling film and dried under a vacuum at 80°C for 1-2h.

2.5.6. Sequencing

2.5.6.1. Direct manual sequencing of PCR- products

Preparation of samples

PCR reactions were performed as described in section 2.5.17 with the 5' primer biotinylated at the 5' terminus. The 3' non-biotinylated primer was used for sequencing each amplified exon initially and the 5' biotinylated primer used to sequence in the other direction in order to confirm sequence changes.

Preparation of single-stranded template for sequencing using magnetic Dynabeads

Solutions

TES   10mM Tris-HCl (pH8), 1mM EDTA, 100mM NaCl

A 0.5ml Eppendorf tube containing 30µl of a suspension of the magnetic M-280 streptavidin coated Dynabeads (Dynal) was placed in a magnetic separation device (Dynal MPC-E magnet, Promega) for 30 sec. This allows the magnetic beads to be drawn towards the magnet and the supernatant to be removed. This was repeated each time a new solution was added. The beads were prewashed twice with 100µl of TES to remove preservatives. 50µl of the PCR product was added to the washed beads, mixed and left for 15 min to enable DNA to adsorb onto the beads. The beads with bound DNA were washed twice with 100µl of TES before adding 100µl of freshly prepared 0.1M NaOH and incubated for 5 min to denature the double-stranded DNA. The supernatant containing the non-biotinylated single strand was removed. The pellet containing the single-stranded product adhered to the beads was washed once with TES and once with water. It was reconstituted in 7µl of distilled water for the sequencing reaction.

Sequencing reaction

Solutions

Sequenase annealing buffer (x5)	200mM Tris-HCl pH7.5, 100mM $MgCl_2$, 250mM NaCl
Labelling mix (x5) for dGTP	7.5µM dGTP, 7.5µM dCTP, 7.5µM dTTP
4 termination mixtures	Each have 80µM of dGTP, dATP, dTTP and dCTP with 50mM NaCl and in addition each has one of the four dideoxynucleoside triphosphates (ddGTP, ddATP, ddTTP and ddCTP)
Enzyme dilution buffer	10mM Tris-HCl pH 7.5, 5mM DTT, 0.5mg/ml bovine serum albumin
Stop solution	95% (v/v) formamide, 20mM EDTA, 0.05% (v/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF

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The sequencing reaction was performed using a protocol based on the chain termination method (Sanger et al., 1992 original article 1977) with the USB Sequenase 2 kit. The first step consisted of annealing the non-biotinylated primer to the single-stranded PCR template. 2μl of 5 x Sequenase annealing buffer, 1-20 pmoles of primer and 7μl of the bead-DNA preparation were incubated at 65°C for 2 min. The reaction mixture was cooled on ice and allowed to stand for 30 min to 4 h. The primers were extended using DNA polymerase with limiting concentrations of deoxynucleoside 5'-triphosphates (dNTPs) including radiolabeled α-^{35}S dATP (Amersham International plc.) to allow detection by autoradiography. 1μl of 0.1M dithiothreitol (DTT), 2μl of diluted labelling mix, 1μl of α-^{35}S dATP (1000Ci/mmol) and 2μl of Sequenase 2 enzyme diluted 1:8 in ice cold enzyme dilution buffer were added to the annealed primer template and the reaction was incubated for 2 min at room temperature.

In the termination step four tubes each containing 2.5μl of one of the four termination mixtures were pre-warmed at 42°C for 1-5 min before adding 3.5μl of the extension reaction to each tube and incubating for 5 min at 42°C. The reactions were stopped by adding 4μl of formamide stop solution and stored at –20°C.

Denaturing Polyacrylamide Gel Electrophoresis

The plates were prepared and the gels cast as in section 2.5.19.2 except for the composition of the gel mix. A denaturing polyacrylamide gel was prepared with ACCUGEL 40 (National Diagnostics) which contains acrylamide and bisacrylamide in a ratio of 19:1. To prepare a gel with a final concentration of 6% polyacrylamide 150ml of ACCUGEL solution was mixed with 420g of urea, 200ml of 5 x TBE buffer and water to give a final volume of 1l (stored in the dark at 4°C). Prior to pouring the gel, 350μl of 10% (w/v) ammonium persulphate and 80μl of TEMED were added to 80ml of the mix to induce polymerisation.

The electrophoresis system was prepared as in section 2.5.5.3, except that 1 x TBE was used. Electrophoresis was carried out for 30 min at 60W prior to applying the samples.

The sequencing reaction mixture was denatured at 94°C for 3 min, immediately placed on ice. 3μl was loaded into 0.5cm-wide wells. Electrophoresis was carried out for 1-3 h at 60W. After electrophoresis the plates were separated and the gel transferred from the larger plate to 3MM Whatman paper, covered with cling film and dried under a vacuum at 80°C for 30 min. After the cling film had been removed the gel was exposed to X-ray film (Kodak-X-OMAT-AR) for 1-3 days at –70°C. The film was developed using a Fuji Photo Film developer.

2.5.6.2. Cycle sequencing of PCR amplified products

Preparation of samples

PCR reactions were performed as described in section 2.5.17 and the resultant products were purified as described in section 2.5.17.4.2.

5'- end labelling of primer with γ^{32}P- dATP

10x T4 polynucleotide kinase buffer   500mM Tris- HCL pH7.6, 100mM MgCl$_2$, 50mM DTT

30μl containing 10 pmole of primer, 7μl of γ^{32}P- dATP, 2.5μl of T4 polynucleotide kinase buffer, 1μl (10 units) of T4 polynucleotide kinase and 13.5μl of water were mixed in a 0.5ml microcentrifuge tube and incubated for 30 min at 37°C. The reaction was terminated by heating at 95°C for 5 min and the labelled primer was stored at –20°C.

Cycle sequencing reactions

Solutions

Stop solution   95% (v/v) formamide, 20mM EDTA, 0.05% (v/v) bromophenol blue, 0.05% (w/v) xylene cyanol

3μl of each of the Circumvent deoxy/dideoxy sequencing mixes (each containing all four dNTPs and one of the ddNTPs) was added to 4 different eppendorf tubes labelled A, C, G and T. 0.01 pmol of double-stranded template DNA, 1.2 pmol of end- labelled primer, 1.5μl of 10 x Circumvent sequencing buffer (New England Biolabs), 1μl of 30 x Triton X-100 solution and distilled water to a total volume of 14μl were mixed gently in a 0.5ml microcentrifuge tube. 2 Units of Vent$_R$ (exo⁻) DNA polymerase were added and the solution

gently mixed. 3.2μl of the reaction mixture was aliquoted into each of the 4 eppendorf tubes containing the Circumvent deoxy/dideoxy sequencing mixes and mixed. The reactions were overlaid with mineral oil and cycled 25 times through the same temperature profile that was used in the original PCR. Following the last cycle 4μl of stop solution was added below the mineral oil. The samples were electrophoresed as described for samples prepared for manual direct sequencing.

2.5.6.3. Direct automatic sequencing of PCR- products

Preparation of samples using primers with M13 tails

PCR reactions were performed as described in section 2.5.17 with primers (sequence shown in Table 2.3) to which were added M13 forward and reverse at the 5' ends.

Forward primer: -21M13   5'-TGTAAAACGACGGCCAGT

Reverse primer: M13 Rev   5'-CAGGAAACAGCTATGACC

The DNA was then purified as described in section 2.5.3.4.

Sequencing reactions using the –21M13 and M13 Rev sequencing dye primers

1-6μl of DNA template was made up to a total volume of 6μl with water. Four 0.5μl microfuge tubes were labelled A, C, G and T respectively and were placed on ice. 1μl of diluted DNA was added to each of the tubes. 4μl of forward or reverse Big Dye Primer Cycle Sequencing Ready Reactions A, T, C and G (Perkin Elmer Applied Biosystems) were added to tubes A, T, C and G respectively. The reactions were then overlaid with 40μl of mineral oil. The thermal cycler (GeneE, Techne) was pre-heated to 95°C and the reactions were taken through the cycling programme of 95°C for 1min; 15 cycles of 95°C for 30s, 52°C for 30s, 70°C for 1min; 15 cycles of 95°C for 30s, 70°C for 1min; 35°C for 30min. The reaction mixes were separated from the mineral oil by allowing them to run out on Parafilm until the phases separated. The mixes were then pooled for each template and the DNA was precipitated by addition of 53μl of 95% (v/v) ethanol, followed by an incubation at 4°C for 30 min. The solution was centrifuged for 20 min at 12 000 x g and the supernatant removed. The DNA pellet was washed with 70% (v/v) ethanol and stored at –20°C.

Polyacrylamide Gel Electrophoresis in ABI Prism™ Sequencer

Solutions

Loading dye   20μl 0.05% (v/v) bromophenol blue dye and 100μl
                     formamide

Glass ABI Prism™ plates were washed with Alconox, rinsed with MilliQ water and left to air- dry. The plates were separated by spacers, placed in the cassette and clamped into position. To prepare a gel with a final concentration of 4.1% polyacrylamide, 5.2ml of acrylamide: bisacrylamide (19:1, Amresco) was mixed with 18g Urea, 0.5g Amberlite MB-150 (Sigma) and 25ml of water. This mixture was then filtered through a 0.2μM cellulose nitrate filter. The total volume of the solution was made up to 50ml with MilliQ water. 250μl of 10% (w/v) ammonium persulphate and 35μl of TEMED were added to initiate polymerisation. The solution was mixed and poured between the gel plates using a 50ml syringe. The straight edge of a sharks tooth comb was inserted at the top of the gel and clamped in position. The gel was left to polymerise for at least 2 hours before use.

The gel was placed in the ABI Prism™ 377 DNA Sequencer (Perkin Elmer Applied Biosystems) and pre-run to assess the level of background. The comb was taken out of the top of the gel, washed and reinserted to form wells. The buffer trays were attached and TBE was poured into the chambers. A hot plate was fixed to the front of the gel plate. The wells were washed out and the system was pre- run at 1kV until it reached 51°C.

4μl of loading dye was added to the DNA samples and they were denatured for 2 min at 91°C before being cooled on ice. The wells were washed again and 1.5μl of each sample was loaded. Electrophoresis was carried out at 1.6kV for 7 hours. The data were analysed using Sequence Navigator™, version 1.01.

2.6. Preparation of the components of LID transfection complexes

LID complexes consist of Lipofectin, integrin- binding peptide and DNA. The basic protocol for preparing LID complexes and using them to transfect cells used initially in this study had been developed for three different cell lines,

COS7, A375M and ECV304. In this study it was used, for the first time, to transfect cultured fibroblast cells. In the first part of the study LID complexes containing lac Z or luciferase cDNA were used to optimise the transfection for delivery of genes to fibroblasts. The expression of the reporter cDNA was used as an index of expression of the introduced cDNA. The system was optimised by varying parameters such as incubation time which is described in Chapter 4. The optimised system was then used to transfect cells with LID complexes containing the lysosomal cDNAs, α- L- fucosidase and α- galactosidase A, and the expression of these enzymes was then studied (the complexes were prepared in an identical manner for all cDNAs used).

2.6.1. Plasmid vectors containing cDNA for reporter or test enzymes

pAB-11 lac Z reporter expression vector

pAB-11 is an adeno-associated viral vector expressing the lac Z reporter gene from a Cytomegalovirus (CMV) promotor which was kindly provided by Adrian Thrasher, Institute of Child Health. The vector also contains an ampicillin resistance gene.

pGL2 luciferase reporter expression vector

The pGL2 vector (Promega) contains a Simian Virus 40 (SV40) promotor and enhancer driving the firefly luciferase reporter gene within a pUC19 backbone. The vector also contains an ampicillin resistance gene.

pF1.4, pLF, pLNCF and pLFSN α- L- fucosidase expression vectors

The plasmids containing α-fucosidase cDNA (pF1.4, pLF, pLNCF, pLFSN) (Occhiodoro et al., 1992), were kindly provided by Dr. Don Anson, of the Department of Chemical Pathology, Women's and Children's Hospital, Adelaide, South Australia.

pF1.4 is the full length α- L- fucosidase cDNA clone, consisting of 11 base pairs (bp) of 5'- untranslated sequence, the signal peptide sequence, the full protein coding sequence and 11bp of 3'- untranslated sequence (Occhiodoro et al., 1989), in a pUC backbone. The vector also contains an

ampicillin resistance gene. The cDNA fragment was cloned into the EcoR1 site. An internal EcoR1 site in the coding sequence means that complete digestion with EcoR1 releases 1.2 and 0.2 kb fragments. A partial digest releases the 1.4Kb fragment which was subcloned to produce the pLF, pLNCF and pLFSN retroviral constructs .

pLF (Figure 2.1) is a simple retroviral construct with human α- L- fucosidase cDNA described above driven by the long terminal repeat (LTR) within a pLX backbone. The vector also contains an ampicillin resistance gene.

pLNCF (Figure 2.1) is a retroviral construct with human α- L- fucosidase cDNA, described above, cloned into pLNCXE (Miller and Rosman., 1989). The human α- L- fucosidase cDNA is driven by an internal CMV immediate early promotor. A LTR drives the neomycin resistance gene. The vector also contains an ampicillin resistance gene.

pLFSN (Figure 2.1) is a retroviral construct formed by cloning human α- L- fucosidase cDNA, described above, into pLXSN (Miller and Rosman., 1989). The α- L- fucosidase is driven by a retroviral LTR which is followed by the SV40 promotor and neomycin resistance gene. The vector also contains an ampicillin resistance gene.

pCGN α- galactosidase A expression vector

pCGN (Figure 2.1) is the full length α-galactosidase A cDNA cloned into the ECOR1 site of pcDNA3 (Invitrogen). The α-galactosidase A cDNA is driven by a CMV promotor and SV40 drives a neomycin resistance gene. In the vector there is also an ampicillin resistance gene.

pLF   LTR —ᵂᵗ—[fuc]——— $(A)_n$ LTR

pLNCF   LTR —ᵂᵗ—[neo | CMV | fuc]——— $(A)_n$ LTR

pLFSN   LTR —ᵂᵗ—[fuc | SV | neo]——— $(A)_n$ LTR

pCGN   CMV———[gal | SV | neo]
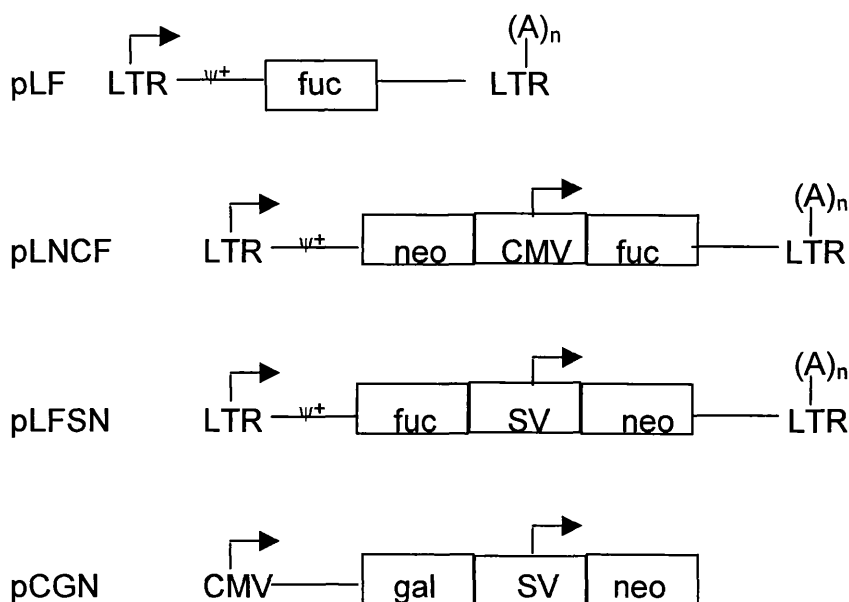
Figure 2.1 Structures of constructs used in this study within plasmids containing the ampicillin resistance gene

LTR, long terminal repeat; ψ^+, retroviral packaging signal; fuc, α- L- fucosidase cDNA; gal, α-galactosidase A cDNA; neo, neomycin resistance gene; CMV, human cytomegalovirus immediate early promotor; SV40, SV40early promotor; $(A)_n$, polyadenylation site.

2.6.1.1. Preparation, purification and characterisation of plasmids containing reporter or test gene cDNA

Plasmid DNA, containing reporter gene or test gene cDNA, was used to transform competent E. Coli cells to produce large amounts of the plasmid DNA for transfection. Transformants were isolated on the basis of the antibiotic resistance (ampicillin) conferred on the bacteria by the plasmid DNA which allowed the cells that had taken up plasmid DNA to form white colonies in the presence of ampicillin.

2.6.1.2. Production of competent E. Coli cells and their transformation

Solutions

LB broth   Luria- Bertaini (LB) growth broth

10g/l bacto-tryptane (Gibco BRL), 5g/l bacto-yeast extract (Gibco BRL), 10g/L NaCl, pH7.5

TSB   Transformation and Storage buffer

2g PEG-4000, 1ml DMSO, 0.1ml 2M $MgCl_2$, 0.1ml 2M $MgSO_4$ and 18.8 ml LB growth broth

The method used for production of competent cells was that of Chung and Miller (1988). Bacterial cells were grown in LB broth to the early log phase when the optical density (OD) was between 0.3 and 0.6. The cells were then centrifuged at 1 000 x g for 10 min at 4°C. The bacterial pellet was resuspended in 1/10th volume of TSB and incubated on ice for 10 min. 100µl aliquots of these cells were placed in cold polyprolene tubes and used to perform the transformation.

100pg of plasmid was mixed with the aliquot of competent cells and returned to ice for 5-30 min. The total volume was made up to 1ml with TSB containing 20mM glucose and grown at 37°C for 1 h to permit expression of the antibiotic resistance gene. 100µl of the cells were spread on to agar Petri dishes containing 50µg/ml ampicillin, 50µl of a 4% solution of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Promega) and 50µl of 0.1M IPTG (isopropyl β-D-thiogalactopyranoside; Promega). The plates were dried at room temperature and incubated at 37°C overnight.

At least three single white colonies were picked from each plate and used to inoculate 5ml of LB broth containing 50µg/ml ampicillin. The cultures were incubated in an orbital shaking incubator overnight and used to make glycerol stocks, to determine the presence and orientation of the α- L-fucosidase or α- galactosidase A insert and to grow up large amounts of plasmid for use in transfection experiments.

2.6.1.3. Small scale growth of bacterial culture and extraction of plasmid DNA (Miniprep)

Solutions

P1	50mM Tris-HCl, 10mM EDTA, 100μl RNAse A (100μg/ml), pH8
P2	200mM NaOH, 1% (w/v) SDS
P3	2.55mM Potassium acetate, pH4.8
QBT	750mM NaCl, 50mM 3-[N-morpholino] propane sulphonic acid (MOPS), 15% (v/v) ethanol and 0.15% (v/v) Triton X-100, pH7
QC	1M NaCl, 50mM MOPS and 15% (v/v) ethanol, pH7
QF	1.25M NaCl, 50mM MOPS and 15% (v/v) ethanol, pH8.2

Minipreparations of plasmid DNA were obtained by the alkaline lysis method whereby bacterial cells are lysed and the plasmid DNA adsorbed on to an ion-exchange column from which the plasmid DNA was eluted specifically.

A 5ml culture of cells containing the relevant plasmid, as prepared in section 2.6.1.2, was chilled on ice and then pelleted by centrifugation at 5000 x g for 20 min at 4°C, in an IEC CENTRA-7R refrigerated centrifuge. The supernatant was discarded and the pellet resuspended in 0.3ml of buffer P1 and transferred to a Falcon tube. 0.3ml of buffer P2 was added, mixed gently and left at room temperature for 5 min. 0.3ml of buffer P3 was added, mixed gently and centrifuged at 30 000 x g for 15 min. The supernatant was applied to a Qiagen- tip 20 equilibrated with 1ml of buffer QBT. The column was washed twice with 1ml of buffer QC. The DNA was eluted with 0.8ml of buffer QF. The DNA was precipitated with 0.8 volumes of isopropanol and after centrifugation at 15 000 x g for 30 min the supernatant was discarded and the DNA pellet washed with 70% (v/v) ethanol. The pellet was air- dried and resuspended in 50μl of TE buffer.

2.6.1.4. Large scale growth of bacterial culture and extraction of plasmid DNA (Maxiprep)

A 5ml culture of cells containing the relevant plasmid, as prepared in section 2.6.1.2, was used to inoculate 200-300ml of LB broth containing ampicillin (50μg/μl). The cells were pelleted by centrifugation at 6 000 x g for 10 min at 4°C, in a Sorvall refrigerated centrifuge. The solutions and protocol used were provided by the manufacturer of the Qiagen Maxi-prep kit (Qiagen). The

cell pellet was resuspended in 10ml of cold buffer P1. 10ml of buffer P2 was added, mixed gently by inversion and the mixture incubated at room temperature for 5 min. 10ml of chilled buffer P3 was added, the solution was mixed gently by inversion and incubated for 20 min on ice. The solution was centrifuged at 30 000 x g for 30 min at 4°C. The supernatant was removed promptly and the centrifugation step repeated to produce a particle-free solution.

The supernatant was applied to a Qiagen- tip 500 (Qiagen) equilibrated with 10ml of buffer QBT. The column was washed twice with 30ml of buffer QC and the DNA eluted with 15ml of buffer QF. The DNA was precipitated with 0.7 volumes of isopropanol and after centrifugation at 15 000 x g for 30 min the supernatant was discarded and the DNA pellet washed with 70% ethanol. The pellet was air- dried and resuspended in 500µl of TE buffer.

2.6.1.5. Restriction enzyme digestion to determine the presence and orientation of the plasmid insert.

Plasmids were digested with a variety of restriction enzymes (Table 2.6 and section 2.5.4) to establish that the insert was present and in the correct orientation.

Plasmid	Restriction enzyme	Size of digestion products if cDNA insert present (base pairs)	Size of digestion products if cDNA insert in correct orientation (base pairs)
pF1.4	EcoR1*	140**, 1260**, 1408 and 3000.	
pLF	EcoR1*	140**, 1260**, 1408 and 1000.	
pLNCF	EcoR1*	140**, 1260**, 1408 and 6620.	
pLFSN	EcoR1*	140**, 1260**, 1408 and 5874.	
pLNCF	Bgl II and Cla I		1286 and 6670.
pLFSN	Xho I and Not I		1146 and 5888.
pCGN	EcoR I	5400 and 1350.	

Table 2.6 Restriction enzymes used to determine the presence and orientation of expression vector inserts and their diagnostic digestion products.

*Partial digest at 37°C for 15 min needed due to internal site in the insert. ** Fragments of insert due to digestion at internal restriction site.

2.6.1.6. Isolation of DNA by ethanol precipitation

A tenth of the volume of 3M sodium acetate, pH5.2 and 2 volumes of 100% ethanol were added to the DNA solution and mixed. The solution was incubated at −70°C for 1 h. The DNA was pelleted by centrifugation at 2 000 x g for 20 min at 4°C. The supernatant was discarded and the pellet washed with 70% ethanol to remove co-precipitated salt. The pellet was air- dried and then resuspended in TE buffer or water as required to the appropriate concentration.

2.6.2. Peptide component of LID complexes

2.6.2.1. Peptide synthesis and purification

Peptides containing an RGD sequence and a polylysine domain were used in LID complexes for transfection. The RGD domain binds to integrins on the cell surface and mediates internalisation of the complex. The poly-lysine domain binds to DNA by electrostatic interaction of the positive lysines with the negative phosphate backbone of DNA. This might also allow a degree of condensation of the DNA.

Peptide K16 was a gift from Dr. A. Miller, Department of Chemistry, Imperial College, London. Peptide 6 was synthesized and purified in the Peptide Synthesis Laboratory of the Imperial Cancer Research Fund, London as described by Hart et al., (1998). Peptide 1, peptide 5 peptide 9 and 11 were synthesized and purified by Zinsser Analytic. Details of the peptides are in Table 2.7.

The peptides were diluted in OptiMEM medium (Gibco-BRL) to 0.1mg/ml and incubated overnight at 4°C, exposed to air to allow oxidation of cysteine residues to cyclise the RGD domain. The solution was then stored at −70°C in 1.5ml aliquots prior to use for transfections.

Peptide name	Peptide sequence	Molecular weight	Net charge	Reference
K16	$[K]_{16}$	2079	+16	
Peptide 1	$[K]_{16}$GACRGDMFGCA	3124	+16	Hart et al., 1994,1995
Peptide 5	$[K]_{16}$GACDCRGDCFCA	3271	+15	Koivunen et al., 1995; Hart et al., 1997
Peptide 6	$[K]_{16}$GACRRETAWACG	3331	+17	Koivunen et al., 1995
Peptide 7	$[K]_{16}$GAGPEILDVPST	3206	+14	
Peptide 9	$[K]_{15}$GACRRETAWACG K GACRRETAWACG	4594	+18	
Peptide 11	$[K]_{16}$GACRGEMFGCA	3151	+16	

Table 2.7 Details of peptide sequences, their weight and net charge.

Calculations of charge and molecular composition of transfection complex

The charge ratios of LID complexes were calculated using the data in Table 2.8. The number of moles of negative charge per mole of plasmid DNA was calculated on the basis of 1 negatively charged phosphate group being attached to each base, multiplied by 2 to take into account both strands of DNA e.g. 1µg of pGL2 plasmid DNA (6 046bp, Mr=3.92 x 10^6) contains 0.26 pmol of plasmid and 3.1nmol (0.26 x 10^{-12} x 12 092) of negative charge. In the peptides aspartic acid and glutamic acids contribute one negative charge whereas lysine and arginine each contribute one positive charge e.g. each mole of peptide 1 contributes 16mol of positive charge.

Molecule	Molecular weight	Net charge
Peptide K16	2 079.84	+16
Peptide 1	3 124.45	+16
Peptide 5	3 271.17	+15
Peptide 6	3 331.5	+17
Peptide 7	3 331.5	+14
Peptide 11	3151.3	+16
N-[1-((2,3,-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA)	669.5	+1
Dioleoyl phosphatidylethanolamine (DOPE)	744	0
2,3-dioleyloxy-N(sperminecarboxamido)ethyl)-N, N-dimethyl-1-propanaminium trifluoroacetate (DOSPA)	1461	+5
(Tfx)	1048.5	0
pGL2 plasmid (6 046bp)	3.92 x 10^6	-12 092

Table 2.8 Formula weight and charge of components used in transfection complexes.

2.6.3. Liposome component of LID complexes

The liposomes used routinely in this study were Lipofectin (Life Technologies), Lipofectamine (Life Technologies) and Tfx (Mirus). Lipofectin is a formulation of 1:1 N- [1-((2,3,-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE).

Lipofectamine is a formulation of 2,3-dioleyloxy-N(sperminecarboxamido)ethyl)-N, N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and DOPE. These formulations were stored at 4°C. It has been proposed that the liposome destabilises endosomes containing the LID complexes, allowing them to escape into the cytosol.

2.7. Transfection of cells with LID complexes

2.7.1. Preparation of cells for transfection with LID complexes

Two normal cell lines were used in experiments to optimise the transfection system for delivery of genes to fibroblasts. Optimisation experiments were generally repeated on the second cell line to verify the results.

LID complexes containing α- L- fucosidase cDNA were used to transfect cells from fucosidosis and Fabry patients as well as normal controls. LID complexes containing α- galactosidase A cDNA were used to transfect cell lines from Fabry patients, normal controls and ECV304 cells.

Fibroblasts and ECV304 cells to be transfected with LID complexes containing reporter cDNA were seeded into 24- well plates (Helena Biosciences) at a density of 5 x10^4 cells per well for transfection with LID complexes containing lysosomal cDNA the fibroblasts or ECV304 cells were seeded into 6- well plates (Helena Biosciences) at a density of 25 x10^4 cells per well. The cells were allowed to grow overnight at 37°C in an atmosphere of 5% CO_2.

2.7.2. Preparation of LID complexes

The plasmid was diluted in an Eppendorf tube so that there was 1µg of plasmid in 100µl or 5µg of plasmid in 500µl of OptiMEM serum- free medium (Gibco life technologies) per well of cells for transfection with the reporter cDNA and lysosomal cDNA, respectively. For reporter cDNA transfections, the transfection complex was prepared by putting 0.75µl Lipofectin in an eppendorf tube to which was added 40µl of peptide solution followed by 100µl of plasmid DNA per well to be transfected. The solutions were mixed thoroughly after each addition. The ratio of the components was 1µg plasmid: 4µg peptide: 0.75µg Lipofectin. For lysosomal cDNA transfections, the transfection complex was

prepared by adding first 3.75µl Lipofectin then 200µl peptide solution and finally 500µl of plasmid DNA solution per well to be transfected to an Eppendorf tube, with mixing at each step. The ratio of the components is the same in all experiments. The complexes were incubated at room temperature for 30 min. The volume was then made up to 500µl (reporter cDNA transfections) or 2500µl (lysosomal cDNA transfections) per well to be transfected with OptiMEM and the solution mixed gently.

2.7.3. Transfection

The culture medium was removed from the cells to be transfected and 500µl (reporter cDNA transfections) or 2 500µl (test cDNA transfections) of transfection complex solution was added to each well. The plates were then incubated for 6 h at 37°C in an atmosphere of 5% CO_2. The transfection medium was removed and replaced with 0.5ml normal growth medium (reporter cDNA transfections) or 2ml OptiMEM (Gibco life technologies) low serum medium (lysosomal cDNA transfections) 0.25ml or 1ml of the appropriate medium was added to the medium already in the well every 2 days until the cells were harvested.

2.7.4. Analysis of transfected cells and medium

LAC Z histochemical staining

The medium was removed from the cells and they were washed twice with PBS, before fixing by incubation in 0.5% (v/v) glutaraldehyde in PBS for 15 min at room temperature. They were then washed 3 times for 5 min with PBS at room temperature. β- Galactosidase activity in cultured cells was assessed by staining with X-gal solution (Section 2.3.2.1).

Luciferase assays

Reporter Lysis Buffer (RLB, Promega) was diluted to make a 1 x stock. Cells were grown in 24- well plates (approximately 5µg protein per well). Growth medium was removed from the cells to be assayed and the cells were washed twice with PBS. 100µl of RLB was added to each well and incubated at 4°C for 15 min to lyse the cells. The cells were then scraped off the cell surface and the

dish tilted to concentrate the cell suspension at the lower edge of the well

(taking care to scrape off all visible cell debris). The cell lysate was transferred

to an Eppendorf tube, vortexed briefly and incubated at −70°C for 90 min. The

samples were then thawed to room temperature, vortexed briefly and

centrifuged at 12 000 x g for 15 min at 4°C. The supernatant was transferred to

a fresh tube and allowed to equilibrate at room temperature before being

assayed (Section 2.3.2.2).

Lysosomal enzyme assays

Cells were trypsinized in initial experiments but gently scraping the cells

off the dish was found to be more efficient. The cells were scraped gently off

the dish in 200µl of sterile PBS using a Gilson tip. The dish was tilted to decant

the released cells to the lower edge of the well. The cell extract was transferred

to an Eppendorf tube, vortexed briefly and centrifuged at 12 000 x g for 15 min.

The cells were then washed in 200µl of sterile PBS and sonicated in 100µl of

water for 10s at an amplitude of 6 micons in an MSE ultrasonicator. The

sonicate was vortexed and centrifuged at 2000 x g for 3 min and the pellet of

cell debris discarded. Cell extracts were stored on ice and the lysosomal

enzymes were assayed (section 2.3.3).

After transfection (see section 2.7.3) cells were grown in OptiMEM low

serum medium (Gibco life technologies) and fresh medium was added to that

already in the well every 2 days. At certain time points over a week, the spent

medium was removed and centrifuged at 1 000 x g for 5 min and the cells were

harvested as described above. The supernatant was placed on ice and the

lysosomal enzymes were assayed as described in section 2.3.3.

Biochemical and genetic characterisation of fucosidosis patients

3.1. Introduction

The severity and progression of a genetic disease, as assessed by the clinician, are the primary criteria for the selection of a patient for gene therapy but the biochemical and genetic characterisation of the underlying defect is also essential. Therefore, this study starts with characterisation of the fucosidosis patient material available for analysis (see Section 2.1.1).

The residual α- L- fucosidase activity was measured in patients and family members, where available, to confirm the diagnosis and to provide information on the enzyme replenishment required. Measurement of the concentration of cross- reacting immunological material (CRIM) was important since low levels of CRIM should not interfere with the production of active introduced enzyme and should also ensure that this exogenous enzyme does not evoke an immune response in patients. The haplotype of patients for the Q/R281 polymorphism (Section 1.4.2) was determined as this might provide a method of distinguishing between native and introduced α- L- fucosidase DNA and polypeptide. The disease- causing mutations were identified and used to assess any possible correlation between the genotype and the biochemical and clinical phenotype. These analyses fully characterised the patients which could be used in further studies on gene therapy. This information was used to select patient cells for use in the gene transfer studies described in this thesis. Ideally these cells would have negligible α- L- fucosidase activity and low levels of CRIM.

3.2. Residual α- L- fucosidase activity in fibroblasts and lymphoblastoid cells from fucosidosis patients

α- L- fucosidase can be assayed rapidly and conveniently by the hydrolysis of the synthetic fluorigenic substrate, 4-methylumbelliferyl- α- L- fucopyranoside (Section 2.3.3).

The specific α- L- fucosidase activity was measured in cells or blood of 12 patients and some members of their families (Table 3.1.).

Patient	α- L- fucosidase
Fibroblasts	*nmol/h/mg protein (normal range 27-229)*
M.B	0.4
C.Lo.	0.3
A.M.	1
G.S.	0.3
Z.T.	0.3
Lymphoblastoid cells	*nmol/h/mg protein (normal range 30-253)*
S.B	0.2
C.Le.	0.3
Leukocytes	*nmol/h/mg protein (normal range 30-189)*
H.A.	0 [a]
Father of H.A.	11 [a]
M.C.	15 [a]
Father of M.C.	25 [a]
Mother of M.C.	33 [a]
S.D.	0 [a]
Father of S.D.	40 [a]
Mother of S.D.	55 [a]
Sibling of S.D.	32 [a]
D.P.	"low"[b]
Plasma	*nmol/h/ml (normal range 170-500)*
O.B.	75 [a]
M.C.	49 [a]
Father of M.C.	46 [a]
Mother of M.C.	61 [a]

Table 3.1 α- L- fucosidase activity in fibroblasts, lymphoblastoid cells, plasma or leukocytes of fucosidosis patients and family members.

[a] assay carried out by the Enzyme Laboratory, Institute of Child Health. [b] no further information

10/12 of the patients had negligible α- L- fucosidase activity confirming diagnosis of classic fucosidosis. The father of H.A., the parents of M.C and parents and a sibling of S.D. were found to have enzyme activities consistent with heterozygote status.

Two patients, M.C. and O.B., were atypical patients biochemically. Patient M.C. had intermediate levels of α- L- fucosidase activity in plasma and leukocytes of 49nmol/h/ml and 15nmol/h/mg protein, respectively. These values are typical of a heterozygote for fucosidosis and are unlikely to be due to the pseudodeficiency of α- L- fucosidase because this is not expressed in leukocytes. The parents of M.C. also had enzyme activities consistent with heterozygote status. Therefore, genomic DNA of M.C. was subjected to mutation analysis (Section 3.4.2). Patient O.B. had an intermediate level of α- L- fucosidase activity in plasma of 75nmol/h/ml. This value is higher than activities typically present in plasma of fucosidosis patients but lower than normals and could be due to the low plasma α- L- fucosidase activity polymorphism (Section 1.4.2) or heterozygosity for a mutation in the α- L- fucosidase gene. Since the only other sample available from this patient was DNA this was also subjected to mutation analysis (Section 3.4.2).

3.3. Immunological determination of α- L- fucosidase protein in fibroblast and lymphoblastoid cells

The deficiency of α- L- fucosidase in the fucosidosis patients could be due to the production of an enzymic protein that is partially or totally inactive, a decreased amount of the normal enzyme or a combination of both. To investigate these possibilities, the amount of α- L- fucosidase protein cross-reacting (CRIM) with anti- serum raised against the normal enzyme was measured in cell extracts. Polyclonal goat anti- human liver α- L- fucosidase antibody (referred to as the polyclonal antibody) and monoclonal anti- human liver α- L- fucosidase antibody (referred to as the monoclonal antibody) were available for this study. They were characterised by immunoprecipitation of human liver α- L- fucosidase before being used to measure the amount of cross- reacting material in patient cell extracts.

Direct immunoprecipitation

The ability of the polyclonal and monoclonal antibodies to immunoprecipitate α- L- fucosidase from human liver supernatant was investigated (Section 2.4.3). The polyclonal antibody precipitated about 94% of

total enzyme activity whereas the monoclonal antibody only precipitated 20% of the enzyme activity. This would be expected since the monoclonal antibody recognises a single antigen which is only likely to occur once within a protein molecule therefore, it is unable to form large immunoprecipitates. This experiment confirmed the specificity of the antibodies.

ELISA

A triple antibody enzyme- linked immunoabsorbant assay (ELISA) was developed to estimate the amount of α- L- fucosidase protein present in cell lines. The polyclonal antibody was bound to well plates and cell extracts were added followed by the monoclonal antibody and finally horse radish peroxidase conjugated- goat anti- (rat IgG) antiserum. Initial results demonstrated that the monoclonal antibody was contaminated with α- L- fucosidase activity. The antibody preparations were purified using protein A- Sepharose to remove the α- L- fucosidase (see Section 2.4.2). The purified monoclonal antiserum was able to immunoprecipitate 40% of the total enzyme activity. Further studies demonstrated that known concentrations of α- L- fucosidase did not correlate with the ELISA measurement suggesting that there could be non- specific binding between ELISA components. The use of an antibody which only precipitated 40% of total activity also contributed to the inaccuracy of this method.

Western blotting

Western blotting of proteins separated by SDS- PAGE was developed (see Section 2.4.4). The Western blots were immunostained using the polyclonal antiserum as the primary antibody and horseradish peroxidase- conjugated rabbit anti- (goat IgG) antibody as the secondary antibody with a chemiluminescent detection system. The appropriate amount of polyclonal antibody to be used as a primary antibody was determined by varying its dilution. A dilution of 1/2000 gave the best specific binding with the lowest background. The amount of horseradish peroxidase- conjugated rabbit anti- (goat IgG) antibody to be used as a secondary antibody was also investigated and a dilution of 1/2000 was found to be satisfactory.

The experiment was repeated several times to assess the consistency of the method. A typical Western blot is shown in Figure 3.1.
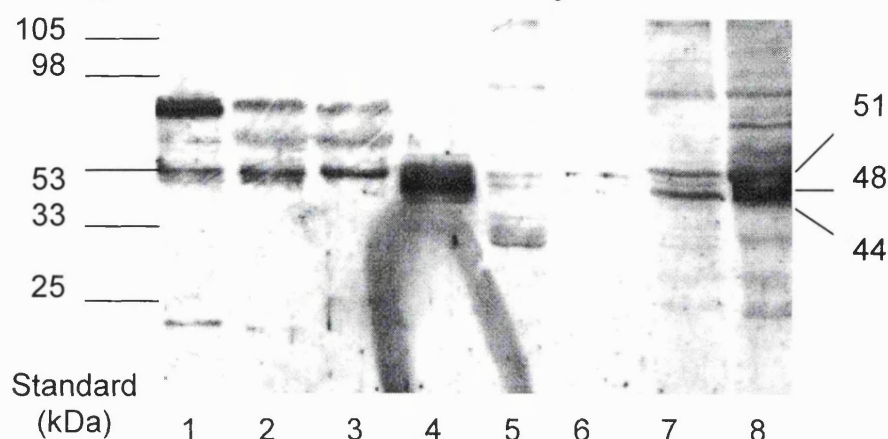
Figure 3.1 Western blotting of α- L- fucosidase in control and patient cell lines
Lane 1, C.Le lymphoblastoid cells. Lane 2, control lymphoblastoid cells. Lane 3, S.B lymphoblastoid cells. Lane 4, human liver supernatant. Lane 5, G.S fibroblasts. Lane 6, A.M. fibroblasts. Lane 7, M.B. fibroblasts. Lane 8, control fibroblast cells.
Comparable amounts of protein were loaded into each well on SDS- PAGE. Immunostaining was carried out using a primary polyclonal goat anti- (human liver α- L- fucosidase) antibody, with secondary horseradish peroxidase- conjugated rabbit anti- (goat IgG) antibody and a chemiluminescent detection system.

Only two major bands with molecular masses of 44 and 51 kDa were detected in human liver supernatant (Figure 3.1, lane 4). This pattern of bands is the same as that reported previously for human α- L- fucosidase in human liver using the same anti- serum and comparable amounts of material (Johnson et al., 1992). This showed that the technique was specifically detecting α- L- fucosidase protein with an appropriate sensitivity.

Two predominant bands of Mr 51 and 44 kDa were also detected in the normal fibroblasts (Figure 3.1, lane 8) but another distinct band of 48 kDa was also present. These bands were also detected in extracts of fibroblasts from three patients (Figure 3.1, lanes 5-8) but at lower concentrations.

Interestingly, the patterns of protein bands seen by Coomassie blue staining and Western blotting in lymphoblastoid cell lines were different from those seen in fibroblasts. The main bands on Western blotting of normal lymphoblastoid cells had molecular masses of 51, 62 and 77 kDa (Figure 3.1, lane 2). The protein band of molecular mass 51 kDa probably corresponds to

the mature α- L- fucosidase protein seen in liver and fibroblasts. The isoforms of 44 and 48 kDa were not detected. The 51kDa band was less intense in both patient samples (Figure 3.1, lane 1). The protein bands with molecular masses 62 and 77 kDa could be alternatively spliced forms of α- L- fucosidase, precursors not previously identified or material cross- reacting non- specifically with the antibody. The band of Mr 77kDa has a higher intensity in patient C.Le (Figure 3.1, lane 1). The major bands detected by Western blotting in both cell types were not observed on Coomassie blue staining, suggesting they were minor components, which cross- reacted specifically with the anti-α- L- fucosidase polyclonal antibody.

The Western blots were analysed with computer software to determine the relative amounts of the main components (Table 3.2).

Patient	Cell type	Main bands	Relative intensity of bands in extracts of patient cells*
M.B.	Fibroblast	51	72%
		48	73%
		44	97%
A.M.	Fibroblast	51	5%
		48	32%
		44	0%
G.S.	Fibroblast	51	8%
		48	53%
		44	37%
S.B.	Lymphoblastoid	76	99%
		62	99%
		50	86%
C.Le.	Lymphoblastoid	76	177%
		62	76%
		50	64%

Table 3.2 Main bands seen on Western blotting and their relative intensities.
*Intensities were relative to the same band in a normal cell extract on the same gel.

The three main bands in the fibroblasts of patient M.B., that are presumed to correspond to α- L- fucosidase, had relatively high intensities suggesting the presence of a catalytically inactive protein. Patient A.M. only had

one major band, which had a relatively low intensity and corresponded to the α-
L- fucosidase band of 48kDa. Patient G.S. had two major bands corresponding
to α- L- fucosidase which were less intense compared with the corresponding
bands from the control. In patient M.B. the three main α- L- fucosidase bands in
fibroblasts were less compared with the corresponding bands from the control.
The 50kDa band corresponding to α- L- fucosidase was 86% of the intensity of
the control in lymphoblastoid cells from patient S.B., and 64% for C.Le. It is
interesting to note that in patient C.Le. the intensity of the 76kDa band was
consistently greater than that observed in normal cells. This suggests that an
alternatively processed form of α- L- fucosidase is building up.

3.4. Molecular genetic analysis of fucosidosis patients

3.4.1. Identification of Q/R281 polymorphism in fucosidosis patient DNA

The Q/R281 polymorphism is due to an A to G transition at nucleotide 861
in exon 5 of the FUCA1 gene. This causes the substitution of an arginine for a
glutamine at codon 281 and creates a new restriction site for the enzymes
BsaJ1 and Dsa1 (Cragg et al., 1994, see section 1.4.2 and Figure 3.2).
Information on the presence of the Q/R281 polymorphism of patients would be
invaluable in determining the presence of transfected α- L- fucosidase cDNA in
host cells if the endogenous DNA had a different phenotype. Since the different
allelic variations at Q281 would produce different migration patterns on SSCP it
is also necessary to determine the genotype of patients in order to assess if
different band patterns on SSCP are due to this polymorphism. Samples were
analysed for this polymorphism by amplification of exon 5 by PCR followed by
restriction enzyme digestion with BsaJ1 (See section 2.5.4). A schematic
representation of the BsaJ1 digestion of exon 5 is shown in Figure 3.2 and the
BSAJ1 digestion of exon 5 of patient and control samples is shown in Figure
3.3, with a summary of results in Table 3.3.
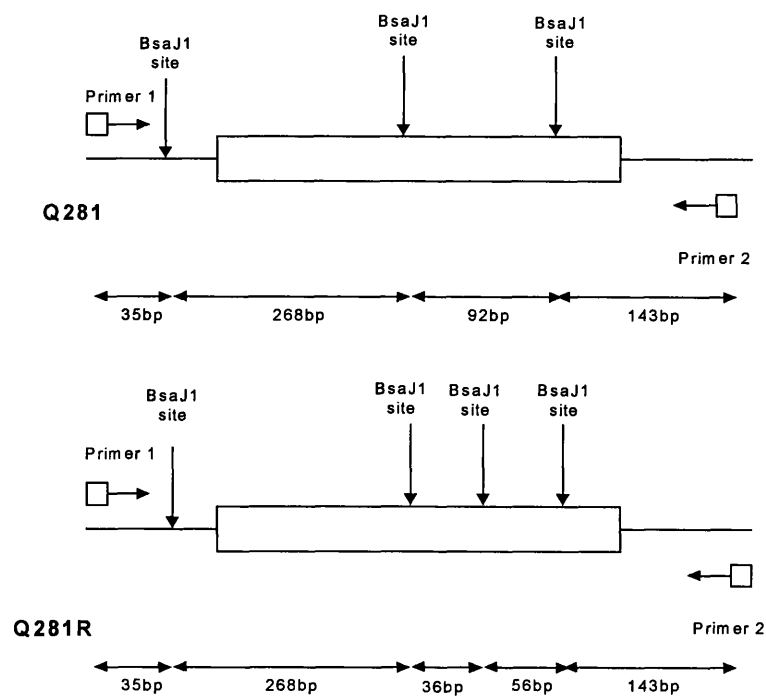
Figure 3.2 Schematic representation of digestion of exon 5 PCR products to determine the genotype at nucleotide 861.

Gel A
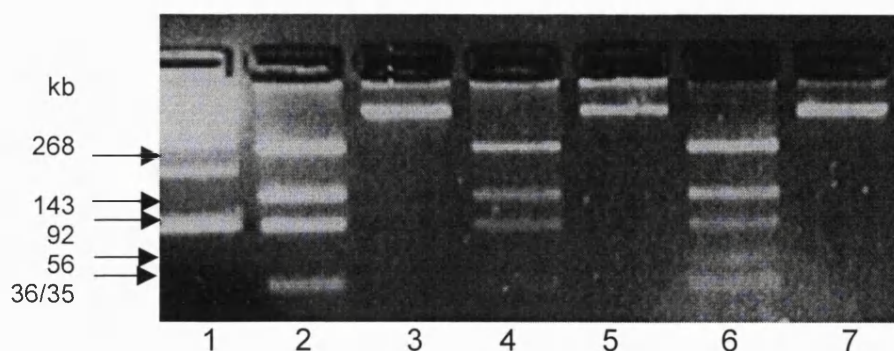
Gel B
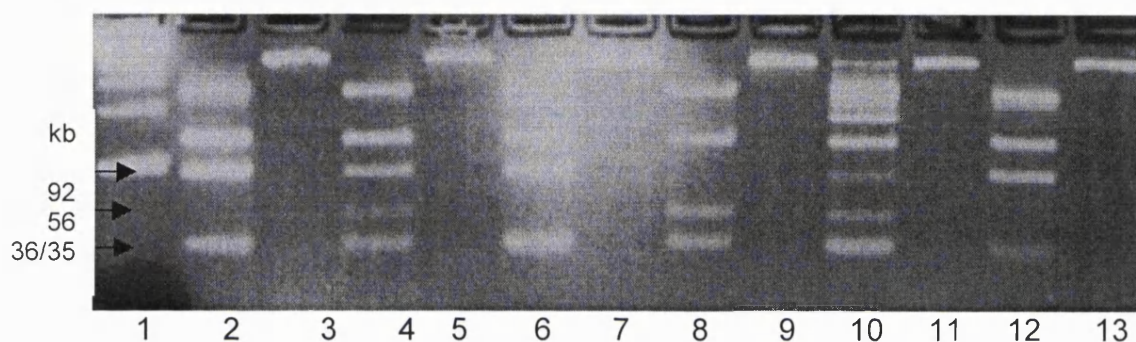
Gel C
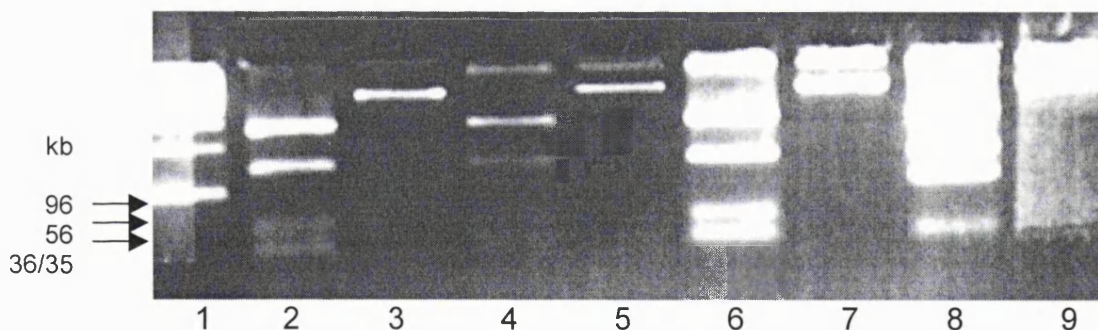
Figure 3.3 Determination of Q/R281 polymorphism by restriction enzyme digestion

Gel A: Lane 1, 100bp ladder. Lane 2, S.D. digested DNA. Lane 3, S.D. undigested DNA. Lane 4, A.A. digested DNA. Lane 5 A.A. undigested DNA. Lane 6, C.Le. digested DNA. Lane 7 C.Le undigested DNA.

Gel B: Lane 1, 100bp ladder. Lane 2, H.A. digested DNA. Lane 3, H.A. undigested DNA. Lanes 4 and 10, C.Le. digested DNA. Lane 5 and 11, C.Le undigested DNA. Lane 6, A.M. digested DNA. Lane 7, A.M. undigested DNA. Lane 8, Z.T. digested DNA. Lane 9, Z.T. undigested DNA. Lane 12, O.B. digested DNA. Lane 13 O.B. undigested DNA.

Gel C: Lane 1, 100bp ladder. Lanes 2 and 4, C.Lo. digested DNA. Lanes 3 and 5, C.Lo. undigested DNA. Lane 6, D.P. digested DNA. Lane 7, D.P. undigested DNA. Lane 8, S.D. digested DNA. Lane 9, S.D. undigested DNA.

PATIENT	Nucleotide at position 861
C.Le.	A/G
A.M.	A/A
C.Lo.	G/G
O.B.	A/A
H.A.	A/A
S.D.	A/A
A.A.	A/A
Z.T.	G/G
D.P.	G/G

Table 3.3 The genotype of patients at the polymorphic nucleotide 861. Where the nucleotide is A then amino acid 281 is Q whereas if the nucleotide is G the amino acid is R.

Five out of nine patients had an A for both alleles (A/A) which is the predominant sequence in the general population (Cragg et al., 1994). Two patients were homozygous for G and one was a compound heterozygote (A/G).

3.4.2. Mutation analysis of patients with fucosidosis

Mutation analysis was carried out on six patients diagnosed clinically and/ or enzymatically as suffering with fucosidosis. This involved 1) screening for commonly occurring mutations, 2) screening for mutations in the 8 exons of the FUCA1 gene by single strand conformation polymorphism analysis (SSCP) and 3) sequencing specific exons to identify the mutations.

As the mutation analysis methods were all PCR- based, it was important initially to optimise the PCR protocols for the amplification of each exon. The optimal protocols are given in Section 2.5.3.

3.4.2.1. Detection of recurrent mutations in FUCA 1

The mutations G60D and Q422X have been identified previously in several patients (O'Brien et al., 1987; Willems et al., 1988d; Kretz et al., 1989; Willems et al., 1991; Yang et al., 1992; Seo et al., 1993; Gordon et al., 1995). One of these mutations is present in heterozygous or homozygous form in 10 out of the 34 families that have been analysed world- wide. Both of these

mutations change a restriction enzyme site. Therefore the patients available at the start of the study were screened for the presence of these mutations using restriction enzyme digestion. Samples which became available during the study were not analysed for these mutations because of their ethnic origins and the introduction of automated sequencing.

Detection of mutation G60D

G60D results from a change of G to A at nucleotide 179 which creates an Afl III restriction enzyme site and has been found in patients of Italian origin. Exon 1 was amplified from the genomic DNA of patients C.Le., D.P. and O.B. Afl III did not digest the products of normal controls as was expected (Figure 3.4, lanes 2-5). The DNA from the patients also remained undigested indicating that they did not have the point mutation, G60D (Figure 3.4, lanes 6-11). There were no positive controls available.
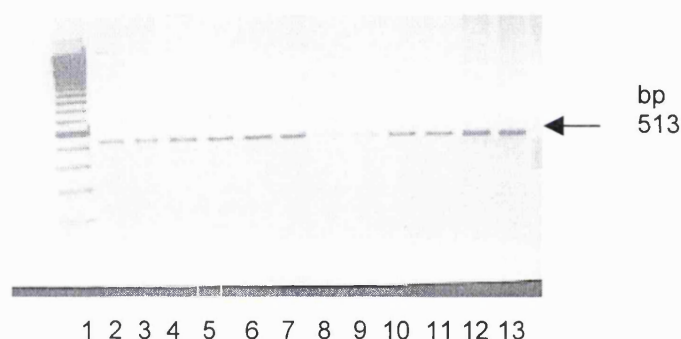
Figure 3.4 Digestion of amplified exon 1 with Afl III to detect G60D mutation.
Lane1, 100bp ladder. Lane 2, digested control 1 DNA. Lane 3, undigested control 1 DNA. Lane 4, digested control 2 DNA. Lane 5, undigested control 2 DNA. Lane 6, C.Le. digested DNA. Lane 7, C.Le. undigested DNA. Lane 8, D.P. digested DNA. Lane 9, D.P. undigested DNA. Lane 10, O.B. digested DNA. Lane 11, O.B. undigested DNA.

Detection of mutation Q422X

This point mutation alters the last base of an EcoR1 restriction site causing its abolition. It has been found in patients from France, Italy and Cuba. Exon 8 was amplified from DNA of patients C.Le., D.P. and O.B. using primers FU8+ and FU8- (Table 2.3). Digestion of this product from normal controls with the EcoR1 restriction enzyme resulted in the production of a visible band of 190 bp (Figure 3.5 lanes 2,4 and 8). Digestion of the products from patients C.Le., D.P. and O.B. also resulted in the production of a band of 190bp (Figure 3.5

lanes 6, 10 and 12), excluding the presence of the point mutation Q422X in these patients.
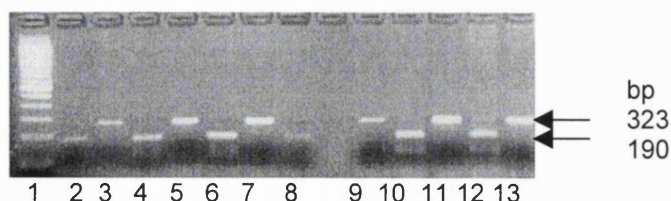
Figure 3.5 Restriction enzyme digestion of exon 8 PCR products with EcoR1 to detect Q422X mutation.

Lane 1, 100bp ladder, Lane 2, digested control 1 DNA. Lane 3, undigested control 1 DNA. Lane 4, digested control 2 DNA. Lane 5, undigested control 2 DNA. Lane 6, C.Le. digested DNA. Lane 7, C.Le. undigested DNA. Lane 8, digested control 3 DNA. Lane 9, undigested control 3 DNA. Lane 10, D.P. digested DNA. Lane 11, D.P. undigested DNA. Lane 12, O.B. digested DNA. Lane 13, O.B. undigested DNA.

3.4.2.2. Identification of mutations by single-stranded conformational polymorphism (SSCP) analysis and sequencing

SSCP analysis was performed on all eight exons and their exon/ intron boundaries for five typical fucosidosis patients, one atypical patient and a number of controls (Section 2.5.5). Examples of known mutations were included where possible as positive controls. The optimal size of PCR products to be analysed by SSCP is reported to be 200-250bp (Orita et al., 1989). Therefore, amplified exon 1 (513bp) was digested with Not 1 before analysis to give products of the desired size. SSCP analysis of exon 5 included controls for all the allelic variations for the polymorphism Q/R281. Where no changes in migration were detected under the standard conditions the samples were analysed under different SSCP conditions (with or without glycerol) to maximise the possibility of detecting a conformational change. A summary of the results is presented in Table 3.4. Conformational changes were only detected in exons 4 and 7.

PATIENT	EXON 1	EXON 2	EXON 3	EXON 4	EXON 5	EXON 6	EXON 7	EXON 8
C.Lo.	–	–	–	–	–	–	+	–
C.Le.	–	–	–	–	–	–	–	–
D.P.	–	–	–	+	–	–	–	–
O.B.	–	–	–	–	–	–	–	–
H.A.	–	–	–	–	–	–	–	–
S.D.	–	–	–	+	–	–	–	–

Table 3.4 Summary of the SSCP analysis of all eight exons for patients C.Lo., C.Le., D.P., O.B., H.A. and S.D.

- indicates no conformational changes observed at room temperature or 4°C

+ indicates that a conformational change was identified in the exon

The exons in which conformational changes were observed were sequenced to identify the sequence changes. In classic fucosidosis patients where no conformational changes were observed on SSCP each exon was analysed by sequencing. Several different sequencing methods were utilised including sequencing products amplified using a biotinylated primer (Section 2.5.6.1), cycle sequencing (Section 2.5.6.2) and automated sequencing using dye-primer technology on an ABI 377 instrument (Section 2.5.6.3). The identification of a sequence alteration, regardless of the sequencing method employed, was always carried out at least twice, once in the forward direction and once in the reverse direction. In cases where the sequence alteration identified affected a restriction enzyme site, confirmation of the mutation was obtained by the digestion of PCR- amplified DNA from a patient and a control with the appropriate enzyme.

Patient D.P.

Exon 4 and its flanking regions were amplified using primers FU4+ and FU4- (Table 2.3). Patient D.P. (Figure 3.6, lanes 1 and 5) showed a different migration pattern from controls. The shift was only seen in one band suggesting that the mutation might be heterozygous. No other conformational changes were seen in the SSCP patterns for the other exons in this patient.
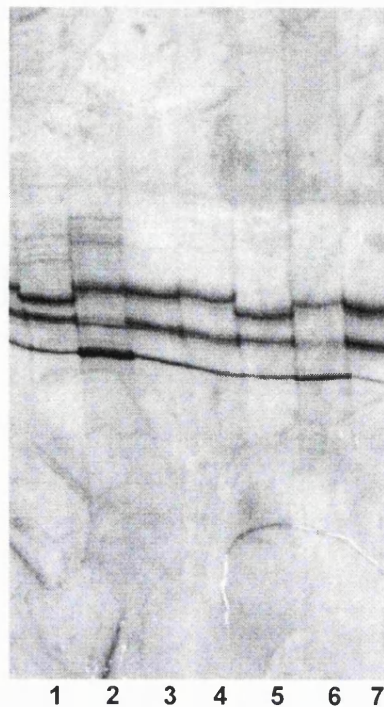
Figure 3.6 SSCP analysis of exon 4.

Lanes 1 and 5, D.P. Lane 2, O.B. Lanes 3, 6 and 7, controls. Lane 4, C.Le.

Sequencing of exon 4 identified the mutation in D.P. as a homozygous G → A transition at nucleotide 703 in codon 228 causing substitution of a tryptophan codon TGG by a stop codon TGA, W228X (Figure 3.7).

The possible effects of this nonsense mutation would be the formation of a truncated protein (which might be degraded in the E.R.), severe reduction in the levels of mRNA, exon skipping or production of a mutant read- through polypeptide. Skipping of exon 4 would cause an in- frame deletion. Read- through polypeptides can be produced when stop codons are recognised by an aminoacyl- tRNA with subsequent insertion of an amino acid (Valle and Morch, 1988). Yang et al. (1992, 1993) have described this phenomenon in fucosidosis where 3 different premature stop codons were shown to allow read- through leading to synthesis of full length, inactive α- L- fucosidase polypeptides. This phenomenon is probably related to the nature of the DNA in the regions flanking the stop codon.
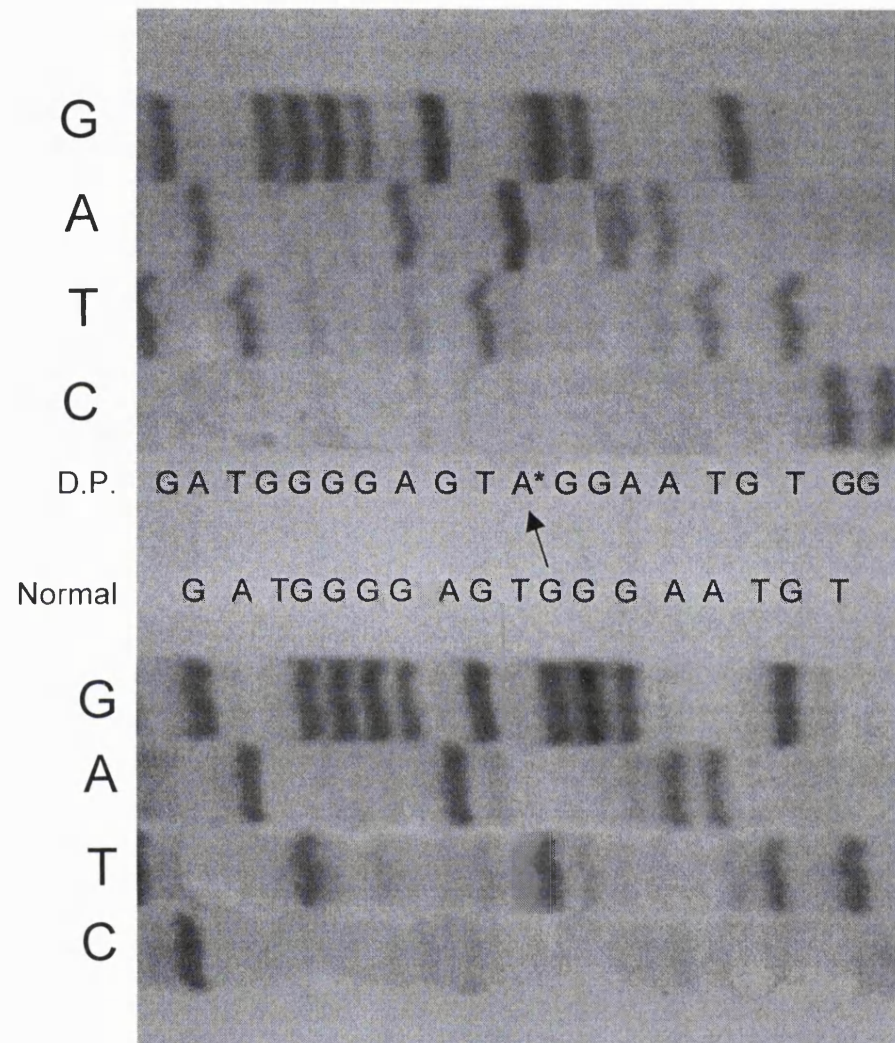
Figure 3.7 Sequencing of exon 4 from a control and patient D.P.

A homozygous G → A transition is present in patient D.P. (marked *).

Patient S.D.

SSCP analysis for patient S.D. and his parents revealed a different migration pattern from controls in exon 4 (Figure 3.8). The only band present had shifted suggesting the mutation was homozygous (Figure 3.8 lane 6). In the parents both the normal and mutated bands were present suggesting they were both heterozygotes for the mutation (Figure 3.8, lanes 7 and 8). The pattern of bands in controls differs from that for exon 4 of D.P. since different SSCP conditions were employed. No conformational changes were observed in the remaining exons.
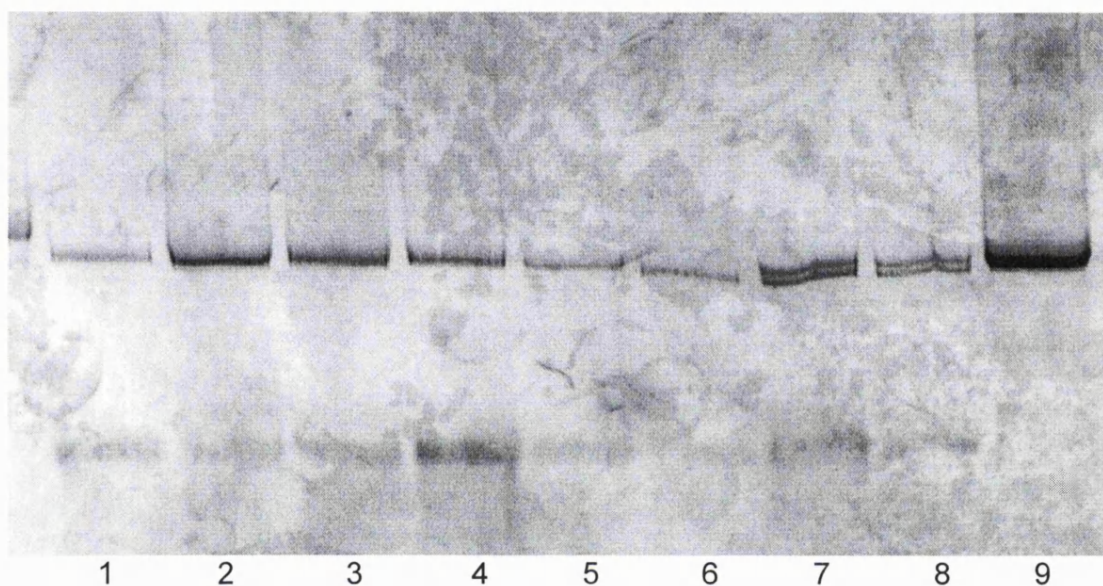
Figure 3.8 SSCP analysis of exon 4 from patient S.D.

Lane 1,2 and 9, controls. Lane 3, C.Le. Lane 4, mother of H.A. Lane 5, father of H.A. Lane 6, S.D. Lane 7, mother of S.D. Lane 8, father of S.D.

Subsequently sequencing identified a homozygous 10bp deletion in patient S.D. (Figure 3.9). The deletion begins at nucleotide 686 in codon 222 and results in a frameshift, which changes the amino acid sequence from PDLIWSDGQWQCP in the normal sequence to PDLMGSGNVLILT in patient S.D. As a result of the deletion a novel, TAG, stop codon is created, 46 amino acids downstream of the deletion. The 10 bp deleted in this patient are preceded by 10bp that are identical apart from 1 base, which probably caused the mutation by replication slippage. The effects of this mutation could be the same as those discussed previously for the nonsense mutation W228X. Sequencing of DNA from the parents of S.D. (H.D. and M.D.) and a sibling

(R.D.) revealed that all three were heterozygous carriers of this deletion supporting the conclusions from their enzyme activities (Figure 3.9).
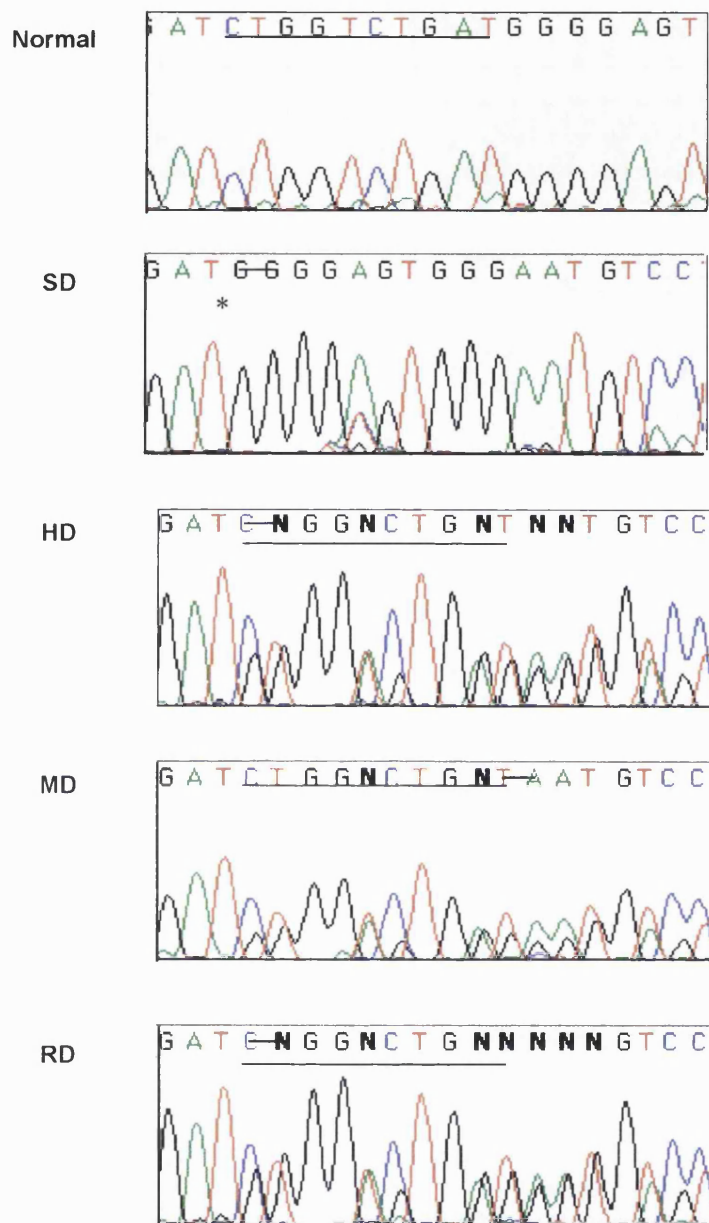
Figure 3.9 Sequencing of exon 4 from a control, patient S.D., his parents (H.D. and M.D.) and his sibling (R.D.)

A homozygous 10bp deletion (marked *) compared with the normal (underlined) was identified in S.D. His parents and sibling were identified as heterozygous for this mutation (underlined).

The samples for S.D., H.D. and M.D. were analysed by both cycle- and automated sequencing. Automated sequencing was more precise and reliable, mainly because a better signal to noise ratio was achieved.

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Patient C.Lo.

Exon 7 and its flanking regions were amplified using primers FU7+ and FU7- (Table 2.3) producing a product of 249bp. Patient C.Lo. showed a different migration pattern from controls and the other patients (Figure 3.10). Two bands were present and both had shifted for patient C.Lo. indicating a homozygous mutation (Figure 3.10 lane 1).

1   2 3   4

Figure 3.10 SSCP analysis of exon 7.

Lane 1, C.Lo. Lane 2, C.Le. Lanes 3 and 4, controls.

Sequencing of exon 7 revealed a homozygous mutation, T → G transversion, at nucleotide 1235 in codon 405, which changes the amino acid from leucine (CTT) to arginine (CGT), L405R (Figure 3.11). As this does not affect a restriction enzyme site it cannot be confirmed directly by restriction enzyme digestion and would require an amplification refractory mutation system (ARMS) to be designed.

Normal 5'CTTAAA   CC TTG AA   T   CCCCC

C.Lo.   G
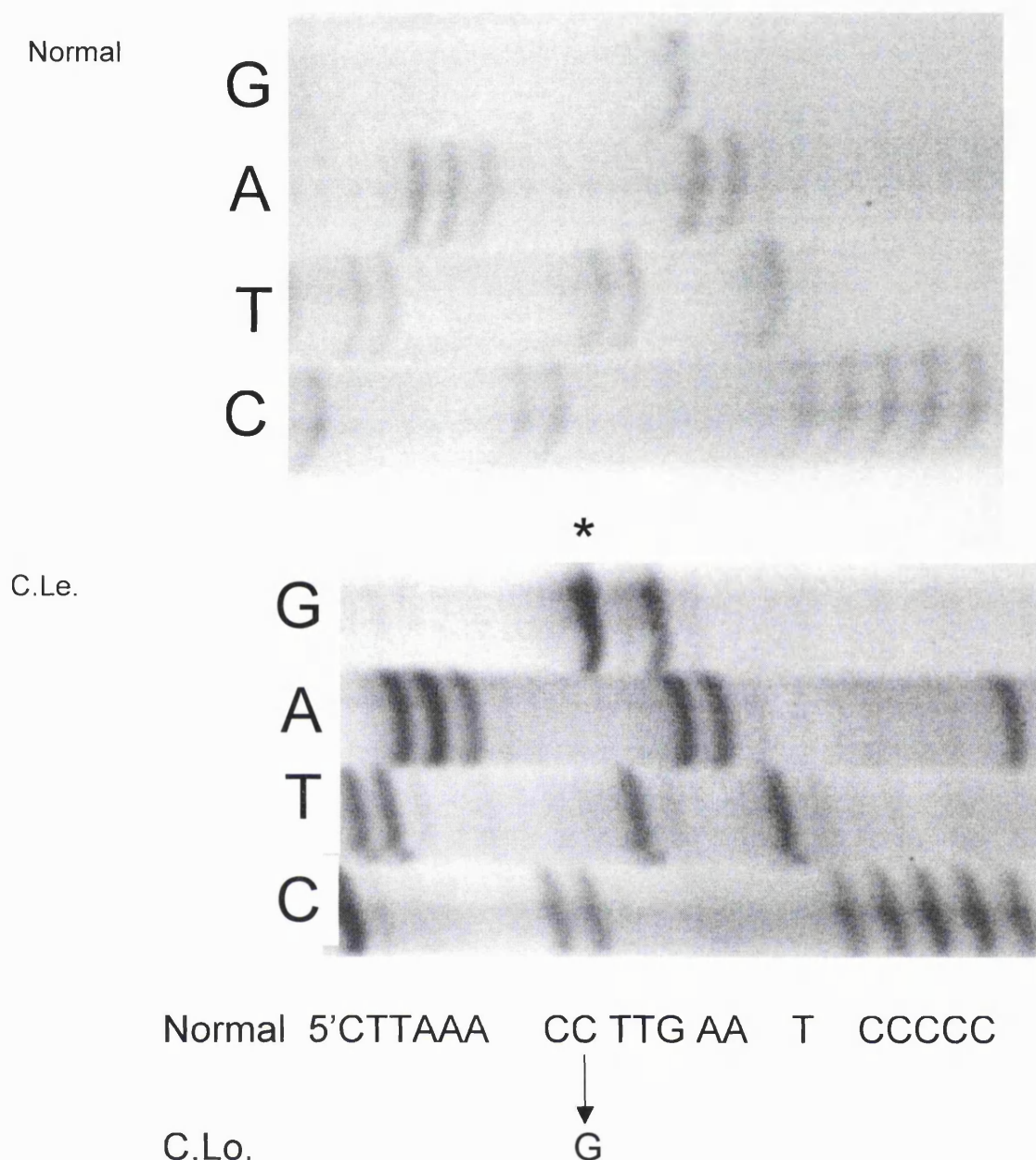
Figure 3.11 Sequencing of exon 7 from patient C.Lo. and control.

A homozygous point mutation, C→ G, was identified in patient C.Lo. (marked *) compared to the control sequence.

The mutation did not occur in a CpG 'hotspot' as is often the case with missense mutations (Cooper and Youssoufion, 1988). Leucine is a smaller, neutral, hydrophobic amino acid usually found in the protein core whereas arginine is a large, basic amino acid which would usually be found on the outside of a protein. The substitution changes a very hydrophobic residue to a very hydrophilic residue but since the surrounding residues are hydrophilic, this might not cause a large structural change (Kyte and Doolittle, 1982). However,

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it also changes a neutral side chain to a basic one in an area where residues are mainly neutral with some acidic residues (Fisher and Aronson, 1989, Skelly, personal communication). This could affect bonding and the conformation of the polypeptide.

The fact that this patient has a milder form of the disease and that the mutated amino acid is not in a conserved region (Fisher and Aronson, 1989) suggests that this region is not very important in the function or folding of the polypeptide.

3.4.2.3. Identification of mutations when no conformational changes were seen on SSCP analysis

In patients C.Le., O.B. and H.A. the SSCP patterns were not informative and each exon was subsequently sequenced. SSCP generally detects between 80 and 95% of mutations since some mutations do not change the conformation of the single- or double- stranded molecule. SSCP sensitivity is thought to be influenced by the size of the DNA molecule and the position of the mutation rather than the type of mutation.

In this study and a previous study carried out at ICH, analysis of the FUCA1 gene in typical fucosidosis patients resulted in the identification of unique conformational changes in 12 out of 14 patients i.e. 86% success rate. This rate is similar to that seen in other mutation detection studies for lysosomal storage diseases performed at ICH using the same methodology, 83% for analysis of iduronate sulphate sulphatase gene in 18 Hunter patients (Goldenfum et al., 1996), 81% for analysis of the α- galactosidase A gene in 21 Fabry patients (Davies et al., 1994), 88% for the analysis of the α- N-acetylglucosaminidase gene in 14 out of 16 Sanfilippo B patients (Beesley et al., 1998). The efficiency of the technique is high but it has limitations. Generally, there are few false positives with this technique but a negative result is not indicative of a normal sequence.

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Patient O.B.

Patient O.B. was classified as an atypical fucosidosis patient. The level of α- L- fucosidase in her plasma, and the fact she does not appear to have a mutation in the FUCA1 gene on SSCP analysis, suggest that she probably has a pseudodeficiency of α- L- fucosidase coupled with a disorder which accounts for her clinical symptoms. If leukocytes had been available an assay of α- L- fucosidase could have been carried out to confirm that this patient had the pseudodeficiency.

Patient C.Le.

Patient C.Le. was diagnosed as a classic fucosidosis patient clinically and enzymatically yet no conformational changes were detected on SSCP analysis under various conditions. Previous studies also failed to find any conformational changes at 4°C or room temperature. Sequence analysis of exon 1 for patient C.Le. revealed a heterozygous G → T transversion at nucleotide 52 of codon 18, which results in the change of a glycine codon (GGA) to a stop codon (TGA), G18X (Figure 3.12).

Sequence analysis of the paternal grandparents (G.Le. and W.Le.) and maternal grandmother (D.M.) showed that the paternal grandfather (W.Le.) was heterozygous for this G18X mutation whereas both grandmothers (G.Le. and D.M.) were normal. This is supported by enzyme activity measurements. The specific α- L- fucosidase activity in leukocytes of G.Le. was within the normal range whereas activity of W.Le. was 48nmol/h/mg protein, consistent with his being a heterozygote. As this mutation alters a Dde I restriction site, a digest can be used to analyse any further samples from this family to detect the G18X mutation.
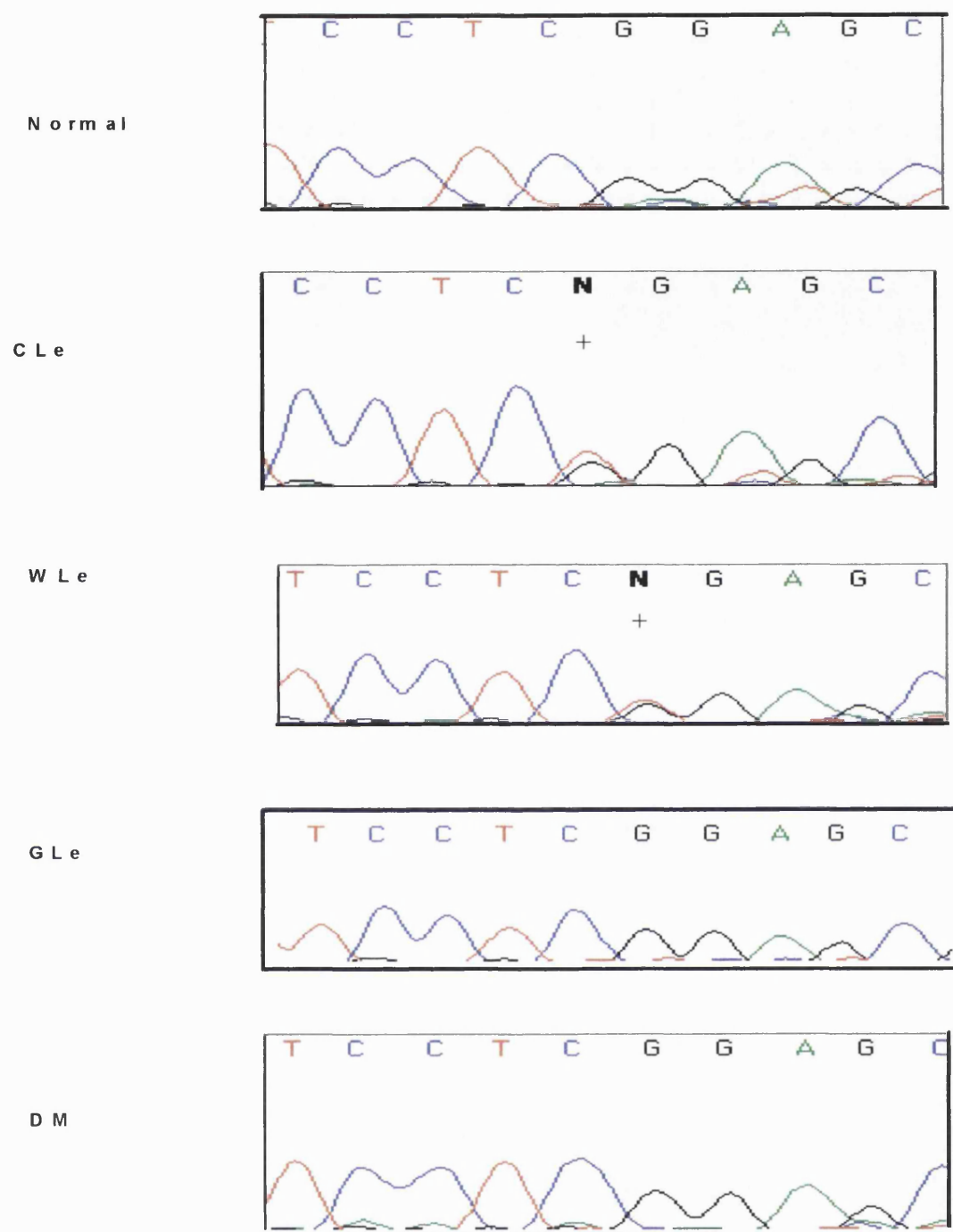
Figure 3.12 Sequencing of exon 1 from normal control, patient C.Le. and three of his grandparents

A heterozygous point mutation G → T was identified in patient C.Le. (marked+) W.L.e.

(paternal grandfather) was a heterozygote for this mutation (marked +) but both G.Le. (paternal grandmother) and D.M. (maternal grandmother) were normal.

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G18X was the only sequence alteration identified in exon 1 of this patient, who must be a compound heterozygote. Therefore, all eight exons were sequenced, in both forward and reverse directions, to try to identify the other mutation in any of the exonic or flanking regions but no sequence changes were detected. This suggests that the other mutation is within the regulatory region of the gene, forms a splice site within an intron or is a large deletion of one or more exons. In the case of a large heterozygous deletion, PCR would amplify up the normal copy of the sequence so that it could not be detected by sequencing. Western blotting results demonstrated a reduction of normal length α- L- fucosidase in this patient suggesting that there was not a large deletion or introduction of new amino acids into the protein which would result in a mutant polypeptide of different length.

G18X, introduces a stop codon close to the end of the signal peptide. This would not produce a truncated protein since the signal peptide is removed during post- translational processing. The mutation could cause exon skipping, production of a read- through polypeptide or a severe reduction of mRNA.

Patient H.A.

Patient H.A. was a classic fucosidosis patient clinically and biochemically but no conformational changes were detected on SSCP analysis. Sequence analysis of each of the eight exons of H.A. revealed a homozygous mutation in exon 1. The mutation was a T → G transversion at nucleotide 106 in codon 36, which changes the amino acid tryptophan (TGG) to a glycine (GGG), W36G (Figure 3.13). This mutation alters restriction sites for Eae I, Gdi II and Sau 96I and could therefore be identified in family members by restriction enzyme digestion.

CCCC AGACT GGCCGAGCCT

Normal

CCCCAGAC GGGCCGAGCCT
*

HA
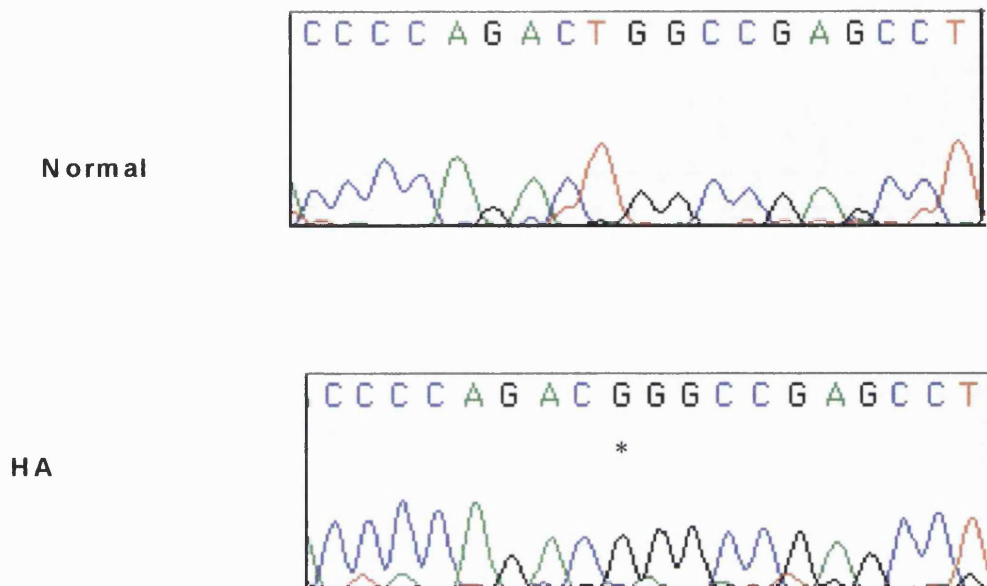
Figure 3.13 Sequence analysis of exon 1 in patient H.A. and a normal control.

A homozygous mutation, T → G, was identified in patient H.A. (marked*) compared to the control sequence.

This mutation did not occur in a CpG island. The replacement of a tryptophan residue with glycine changes the amino acid side chain at position 36 from a large neutral one to a small neutral one. Glycine is known to influence protein folding due to its involvement in dihedral angles. Tryptophan is hydrophobic whereas glycine is hydrophilic (Kyte and Doolittle, 1982). Comparison of this amino acid residue in α- L- fucosidase polypeptide from human, rat, Dictyostelium discoideum (Fisher and Aronson, 1989) and dog (Skelly, personal communication) reveals that the sequence around the mutated tryptophan is fairly conserved in all four cases. The mutated tryptophan itself is conserved in all the species. This implies that this region may be important in the catalytic mechanism or maintenance of the active conformation of the enzyme and that mutation of the tryptophan could inactivate the enzyme.

3.4.2.4. Identification of putative disease- causing mutations by sequencing alone

Patients Z.T. and M.C. were diagnosed after the acquisition of automated sequencing therefore each exon was sequenced by this method.

Patient Z.T.

All exons of new patient Z.T. were analysed by sequencing and a homozygous deletion of nucleotides 157-8 (G and T) in codon 153 of exon 2 was detected (Figure 3.14). Deletion of these bases results in a frameshift (V153fs) that changes the amino acid sequence at the point of the mutation from KNVGPHRDLV in the normal to KNGASSGFG in patient Z.T. causing the formation of a premature stop codon nine amino acids downstream from the site of deletion. This could have similar effects to those discussed previously in patients with premature stop codons. Small deletions are usually caused by repetitions in the surrounding sequence. The deleted GT in V153fs is not part of a repetitive sequence but is followed by a run of Gs.
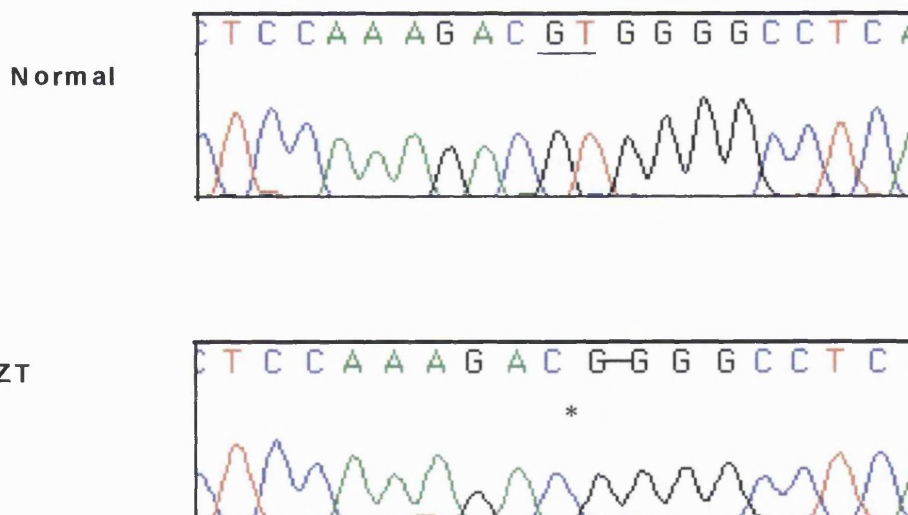
Figure 3.14 Sequence analysis of exon 2 for a control and patient Z.T.
This revealed a homozygous deletion of two bases (marked *) compared with the normal (underlined).

Patient M.C.

Automated sequencing of amplified exon 1 and its flanking regions identified a sequence alteration in patient M.C., a homozygous point mutation of C → G at nucleotide 14 in codon 5 (Figure 3.15). This transversion replaces the normal amino acid proline (CCG) with arginine (CGG) and is therefore described as P5R. Sequence analysis of the parents of this atypical patient, E.C. and G.C., revealed that they were both heterozygous for this sequence change (Figure 3.15).
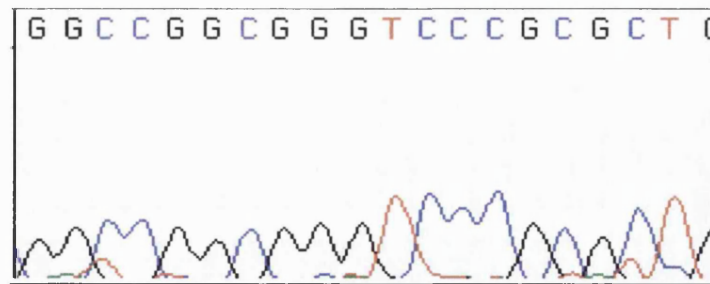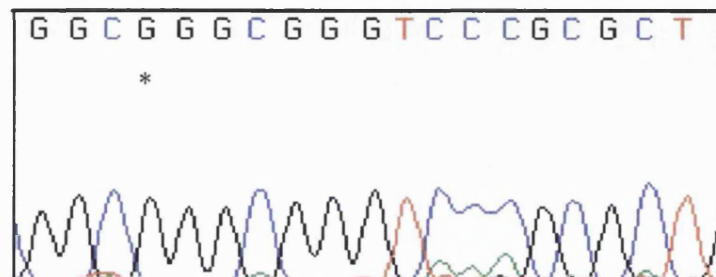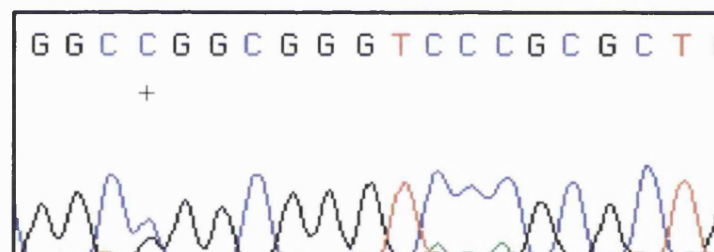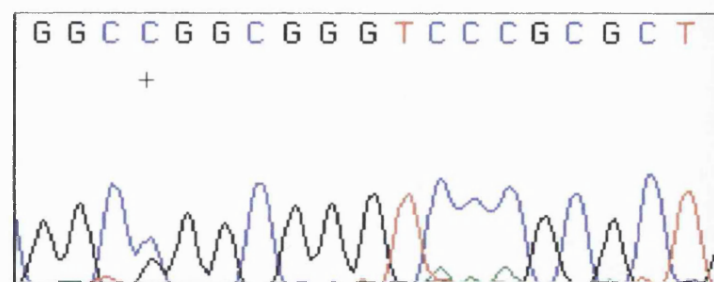
Patient M.C.

Figure 3.15 Sequence analysis of exon 1 in patient M.C. (marked*), and her parents E.C.
and G.C. (marked +).

Patient M.C. is atypical since she is assymptomatic and her α- L-fucosidase activity indicates she might be a heterozygous carrier for the disease or she may have the pseudodeficiency. Her DNA was screened using sequencing to check for a heterozygous mutation. The homozygous sequence change, P5R, was identified suggesting that this is a polymorphism. P5R was not identified in any other atypical patients but it was detected in 2 out of 20 controls as a heterozygous change by restriction enzyme digestion, adding further evidence that it is a polymorphism. P5R was previously identified in another patient A.M. (Cragg et al., 1997). A.M. was unusual in that two possible homozygous disease- causing mutations were identified, P5R in exon 1 and Y330fs in exon 6.

P5R is within the putative 22 amino acid signal peptide (Occhiodoro et al., 1989) which has several features in common with other signal peptide sequences. It is rich in uncharged amino acids with a hydrophobic core consisting largely of hydrophobic amino acids with no acidic amino acid residue present. The structure of the signal peptide is thought to be important for its recognition and insertion into translocation channels of the ER (Section 1.2.1). The substitution of a proline by an arginine, replaces a neutral and hydrophobic imino acid with a basic residue which might interact ionically with other amino acid residues. Proline residues disrupt ordered secondary structures of polypeptides due to their structure and they are usually found in bends rather than in α- helices or β- sheets. Arginine residues also occur in bends but are widely found in α- helices and β- sheets as well, therefore, there may not be a large disruption of protein structure with this change. Both residues are hydrophilic and lie within the hydrophilic region of the signal peptide. The substitution also changes a neutral amino acid to a basic residue, which may affect bonding and therefore the conformation of the protein. However, there are other basic amino acids in this region; two arginine residues in fact surround the proline at position 5. The residue is only conserved across human and dog and is not in a highly conserved region. The level of conservation of a mutated amino acid appears to be a key indicator when predicting the effects of missense mutations. At least three other non-pathogenic polymorphisms have been identified in the signal peptide sequence of another lysosomal hydrolase, α- L- iduronidase (Scott et al., 1995). Since the plasma α- L- fucosidase levels

of M.C. were lower than normal it may be that this polymorphism has some effect on the enzyme activity. This missense mutation is not in a CpG island (Cooper and Youssoufian, 1988).

3.5. Discussion

A summary of the mutations found in this study is given in Table 3.5. A summary of the biochemical and genetic characterisation of fucosidosis patients carried out in this study and previous studies is given in appendix 3.1.

Patient	Exon	Sequence change	Mutation	Amino acid change
M.C.	1	CCG TO CGG	P5R polymorphism	Pro to Arg
C.Le.	1	GGA to TGA*	G18X*	Gly to stop*
H.A.	1	TGG to GGG	W36G	Trp to Gly
Z.T.	2	2bp deletion	V153fs or nt457 (del2)	Val to Gly
D.P.	4	TGG to TGA	W228X	Trp to stop
S.D.	4	10bp deletion	I221fs or nt666 (del 10)	Ile to Met
C.Lo.	7	CTT to CGT	L405R	Leu to Arg

Table 3.5 Sequence changes identified in the FUCA1 gene in this study
*indicates a heterozygous mutation

The six mutations and a polymorphism identified in this study were novel. Five out of six of the mutations identified were homozygous suggesting that these patients result from consanguineous marriages or populations in which the concentration of the mutant allele is high. C.Le. is a compound heterozygote but only one mutation was identified despite sequencing the entire coding and flanking regions. C.Le. is Caucasian and his parents were non-consanguineous.

A previous study identified P5R as a putative disease-causing mutation in a classic fucosidosis patient (Cragg et al., 1994). This study suggests that it is in fact a polymorphism but it is still possible that it modulates α- L- fucosidase activity to cause the low α- L- fucosidase activity of M.C. and her parents.

Ideally, further studies should be carried out to express the mutations and determine their effects on the enzyme activity. Cotton and Scriver (1998) outlined the criteria needed to prove that a mutation causes a disease. In this study the type of mutation, level of conservation of the sequence, family information and putative effects of the sequence change were considered. However, at least 100 normal chromosomes should be analysed for each mutation to check whether they are polymorphisms.

Genotype/ phenotype correlation in fucosidosis

The severity of the disease appears to be independent of the α- L- fucosidase activity because the α- L- fucosidase activity is decreased to less than 1% of the control mean in all cases of fucosidosis. The natural substrates of α- L- fucosidase vary between different cell types and tissues. Hence, the residual enzyme activity measured using the artificial substrate, 4-methylumbelliferyl- α- L- fucopyranoside, may not reflect the physiological activity of the enzyme in different cell types. A similar lack of correlation between the residual activity measured using a synthetic substrate and the severity of the disease is found in many other lysosomal storage diseases.

Different amounts of cross- reacting α- L- fucosidase protein were detected in the patients but they did not correlate with the severity of the disease. The sizes of the major bands of α- L- fucosidase detected by Western blotting in this study were similar to those reported previously for the normal enzyme. Although the bands were less intense or absent in extracts of patient cells than in normal controls the levels of α- L- fucosidase protein detected in fucosidosis patients in this study were relatively high. In a previous study of 23 patients the amount of CRIM determined by radioimmunoassay in fibroblast and lymphoblastoid cells had been found to be below 6% of the normal mean (Willems et al., 1991). Other studies have also found a substantial reduction in α- L- fucosidase protein in patient material (Thorpe and Robinson, 1978; Alhadeff and Andrews- Smith, 1980; Andrews- Smith and Alhadeff, 1982; Johnson and Dawson, 1985). However a few patients with substantial intracellular α- L- fucosidase protein have been reported (Alhadeff et al., 1978; Yang et al., 1992, 1993). The differences between studies could be due to the

specificities of antibodies used, which might detect different epitopes, or to different detection methods used in Western blotting, which might have different sensitivities. The study could also have contained an unrepresentative proportion of patients with high levels of CRIM. It should be noted that on Western blotting one would expect false negatives if a disease- causing mutation alters an epitope so that the antibody used cannot recognise it.

Other Western blotting studies have identified glycosylated α- L- fucosidase as having 2 forms of higher molecular weights, 50 and 53kDa (Johnson and Dawson, 1985), 51 and 56kDa (Johnson et al., 1992), 58 and 60 (DiCioccio and Brown, 1988). Since calibration using molecular weight markers varies, especially between different laboratories, the difference in molecular weight compared to other studies is not significant.

The present observation of two iso-forms of liver α- L- fucosidase is consistent with previous studies (Johnson et al., 1992). The presence of a third band in fibroblasts suggests tissue specific differences in the expression of α- L- fucosidase iso-forms. Iso-forms of α- L- fucosidase are thought to differ in their glycan chains as well as in their polypeptide size, possibly due to normal allelic variation, alternative splicing, proteolytic processing and/ or post translational processing modifications other than those due to glycosylation (Johnson et al., 1992; Shoarinejad et al., 1994). Differences in glycan processing could explain differences in the molecular mass of iso-forms, as a high mannose glycan has a mass of 1500Da and a biantennary complex chain has a mass of 2250Da.

In lymphoblastoid cells three major bands were identified with Mr 76, 62 and 50kDa. The 50kDa band was less intense in the patients suggesting that it represented α- L- fucosidase. DiCioccio and Brown (1988) identified the presence of two intracellular forms of α- L- fucosidase with molecular mass of 58 and 60 kDa on Western blotting of lymphoblastoid cell extracts. They also reported faint bands of 105 and 75kDa which were considered to be contaminants. The finding of higher molecular weight bands in the present study raises the possibility that there might be higher molecular weight α- L- fucosidase in lymphoblastoid cells. This would seem to be the case since in patient C.Le. there is an increase in the highest molecular weight band and a

decrease in the two smaller bands suggesting that an alternative form or abnormal intermediate is accumulating due to the mutations in this patient. Higher molecular weight forms of α- L- fucosidase have also been identified in other tissues. Using gel filtration Troost et al. (1976) estimated that the molecular weight of a α- L- fucosidase iso-form was approximately 75-85kDa in leukocytes. Therefore there could be tissue- specific, alternatively spliced precursor forms of α- L- fucosidase as suggested by Johnson et al. (1992) who reported two iso-forms of 51 and 56 kDa in human liver.

The residual α- L-fucosidase activity and the amount of cross reacting α- L- fucosidase protein in patients depend predominantly on the specific mutation in the FUCA1 gene, although other genetic factors may affect the catalytic process and the substrate load presented to cells.

Missense mutations

Substitutions are likely to be deleterious if they alter a specific residue which is critical to function (e.g. a residue involved in catalysis or glycosylation) or alter the folding of the protein so that it is unable to function and possibly be secreted or degraded. Therefore, a detailed knowledge of enzyme structure and expression studies are important in predicting the effects of this class of mutations. The three- dimensional structure of α- L- fucosidase is not known so the best indication of the importance of amino acids is the level of conservation between species. The fact that residue P5 of α- L- fucosidase is only conserved between human and dog and is not in a conserved region supports the hypothesis that this is probably a polymorphism which may moderate a second mutation. The mutation in C.Lo., L405R, is not in a conserved region and the patient has a mild phenotype. The mutations in patients G.S. and H.A. are both conserved residues in conserved regions. G.S. has a severe form of the disease but the phenotype of H.A. is not known. The results of this study therefore demonstrate that the level of conservation of the mutated residue may be a good indication of the clinical phenotype.

Mutations causing production of a premature stop codon

A growing amount of evidence suggests that the predominant effect of a premature termination codon is either a severe reduction in the levels of mRNA

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prior to its accumulation in the cytoplasm (Baserga and Benz, 1992) or the skipping of the exon in which the premature termination codon is located (Dietz et al., 1993). If the exon skipping does not cause a frameshift and the deleted amino acids are not essential for function, this may give a less active, shorter polypeptide and a milder phenotype. If the reading frame were not conserved, there would be a frameshift and probably formation of a truncated protein, which may be non-functional due to loss of a critical group of amino acids. One effect of a premature stop codon can be the production of a truncated polypeptide but this is rarely demonstrated in vivo (Lehrman et al., 1987). A truncated, mutant polypeptide may fail to exit from the ER and be degraded (Lau and Neufeld, 1989; Lippincott et al., 1988). Another possibility is that an amino acyl-tRNA reads the stop codon and inserts an amino acid thereby suppressing the stop signal. This would result in synthesis of a mutant read-through polypeptide with a molecular size indistinguishable from the normal protein as proposed by Yang et al. (1992).

Premature termination codons resulting from point mutation

Two nonsense mutations were identified in this study, G18X in patient C.Le and W228X in patient D.P. It is difficult to determine the effects of the W228X mutation identified since protein or mRNA analysis was not possible. A similar homozygous mutation, W183X, has been reported previously (Cragg et al., 1997). In this case, the patient had negligible cross reacting material suggesting that the mutation caused production of an unstable mRNA or polypeptide.

Patient C.Le. had negligible α- L- fucosidase activity in lymphoblastoid cells but Western blotting demonstrated the presence of high amounts of normal length α- L- fucosidase protein (64% of control) in these cells. Two larger bands were also detected with molecular masses 62 and 76kDa. The 62kDa band was less intense than in controls (76%) but the 76kDa band was consistently more intense than in controls (177%). Only one mutation was found in C.Le. making it difficult to correlate phenotype with genotype. The result from Western blotting suggests two possible effects for this mutation, the production of a read- through polypeptide or of unstable mRNA. If a read- through polypeptide were produced the substitution would probably cause

disease since G18 is in a highly conserved region within the signal peptide but the residue itself is only conserved in human and dog. This substitution would be at the end of the signal peptide and might interfere in the cleavage of the signal peptide and proteolytic processing resulting in decreased rate of production of processed polypeptide or an inactive precursor, consistent with the increased amount of a presumed precursor seen on Western blotting. A severe reduction in mRNA, and therefore catalytically active enzyme produced, is possible if the second mutation in the patient causes production of a catalytically inactive protein which is seen on Western blotting.

Nonsense mutations have been identified in both mild and severe patients. In most cases there is negligible cross- reacting material suggesting that a read- through polypeptide, is not being made. Therefore, an unstable truncated or full- length mRNA or protein is probably being produced. Since the position of the nonsense mutation does not correlate with the severity of disease, it is unlikely that stable truncated proteins are produced. Further studies on mRNA from these patients would be necessary to establish the effect of these mutations on splicing and translation of the α- L- fucosidase gene.

Premature termination codons resulting from small deletions and insertions

Small insertions and deletions are common in non- coding DNA but rare in coding DNA (Strachan and Read., 1996). Often the effect of these mutations is to cause a frameshift and the formation of a premature termination codon. Two such mutations were identified in this study, V153fs and I221fs in patients Z.T. and S.D. respectively, due to small deletions. The mechanism of such small gene deletions is influenced by the local DNA sequence environment (Krawezak and Cooper, 1991). The effect of these mutations is predicted to be the same as that of nonsense mutations, as they all result in the production of a premature stop codon in frame. This will either result in a full- length read- through protein, truncated protein, exon skipping, or reduced levels of mRNA. The effect of the mutations identified in this study on the mRNA and therefore on protein would require further studies and are difficult to predict.

Western blotting was carried out on material from patients M.B. and A.M. in this study. Their mutations had been identified previously. Patient M.B. has a severe form of the disease and his mutation is a 10bp deletion causing a frameshift and production of a premature termination codon 3' to the mutation. There is a relatively high amount of normal length CRIM therefore the mutation probably produces an inactive read- through protein.

Patient A.M. has a moderate- severe phenotype and is homozygous for both Y330fs and the polymorphism P5R. Y330fs is an insertion of one T which causes a frameshift and the production of a premature termination codon 3' of the mutation. Since there are low levels of full length α- L- fucosidase protein in the patient it would seem that Y330fs is producing reduced levels of mRNA.

The large variety of mechanisms by which a premature termination codon could effect production of α- L- fucosidase makes correlation of phenotype and genotype difficult. Further studies on the structure of α- L-fucosidase, function of amino acids and the effects of the individual mutations on mRNA and polypeptide could help to establish genotype/ phenotype correlations.

Splice site mutations

The mutation of S.B. was identified previously and is a homozygous G to A transition at the first position of the donor splice site of intron 5 (Cragg et al., 1997). In this study almost normal levels of cross- reacting α- L- fucosidase protein were observed. However, previous studies show that it is very difficult to predict the effect of a splice site mutation, especially without studying the mRNA (Kuivaniermi et al., 1990).

Distribution of mutations in the FUCA1 gene

A total of 26 different mutations and two polymorphisms have now been identified in the FUCA1 gene. Of these, two were splice site mutations, five were missense mutations, nine formed premature termination codons and ten caused frameshifts. The mutations causing frameshifts included eight deletions, one duplication and one insertion.

The frequency of mutation within an exon will generally depend on the functional importance of the amino acids encoded, the number of repeated

sequences and the occurrence of methylated CpG islands. The mutations are fairly evenly spread amongst the exons except for exon 8 (Figure 3.16). The sequence of exon 8 is less conserved than other exons so sequence changes might not be pathogenic. Exons 1 and 5 also have lower frequencies of mutation compared to other exons and their sequences are also less conserved. However, exon 7 has a high number of mutations but a low level of sequence conservation suggesting that other factors are influencing the frequency of mutation in this exon. Exons 1, 5 and 8 have the lowest frequencies of mutation. As these exons were more difficult to amplify and sequence some mutations might not have been detected.
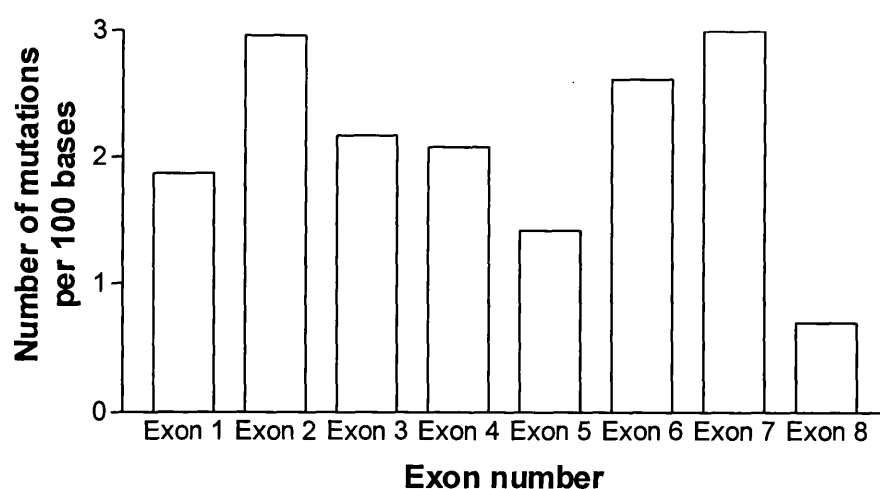
Figure 3.16 Distribution of mutations in the FUCA 1 gene. The X axis represents the number of mutations per 100 bases identified so far in each exon.

Choice of patient cell lines for gene transfer studies

The choice of fibroblasts for the gene transfer studies was limited to cells from patients M.B., G.S. and A.M. It was decided to use the fibroblasts from patient A.M. because the cells had a low concentration of cross- reacting material. It was hoped that this would obviate the possible problem of protein-protein interactions between the residual inactive protein and that produced by the introduced gene on transfection. The residual α- L- fucosidase activity in this cell line was low, increasing the chance of detecting enzyme activity due to introduction of α- L- fucosidase cDNA into the cells. A.M. had the normal haplotype Q281 which could offer the opportunity to distinguish between protein

expressed from endogenous and introduced DNA, if the introduced DNA had the Q/R281 polymorphism, by isoelectric focusing.

Since fucosidosis cell lines do not grow very well, some optimization and other control experiments were repeated on fibroblasts from patient G.S., who had negligible α- L- fucosidase activity, a relatively low level of cross- reacting material and a clearly defined missense mutation (N329Y).

Development of integrin- mediated gene delivery system for fibroblasts in culture

4.1. Introduction.

The use of retroviral, adenoviral and adeno- associated virus vectors for gene therapy for lysosomal storage diseases has been investigated extensively in model systems (Section 1.6.5). At the start of this study both adeno- associated viral vectors and non-viral systems were examined as possible gene therapy strategies. Difficulties with producing the adeno- associated viral vector and results from parallel studies in our group led to a decision to concentrate on the non-viral gene delivery system. The aim of this approach was either to introduce the gene directly into the target cells or to produce a graft of genetically modified cells which could secrete the missing enzyme, which could then be taken up from the circulation by the target cells.

Detailed investigations into the conditions for optimal delivery of genes into fibroblasts in culture using a non-viral system are described in this Chapter. The technique exploits non- covalent interactions amongst protein, DNA and lipid molecules to form a liposome- integrin targeting peptide- DNA (LID) complex which is capable of delivering the cargo DNA from the exterior of a cell to its nucleus. This is achieved by first "docking" with an integrin cluster at the cell surface, followed by internalisation through an endocytotic route with avoidance of direct delivery to lysosomes and delivery of the DNA to the nucleus. The peptide used is a bi-functional molecule with a DNA- binding domain consisting of a stretch of 16 L- lysine residues and a cell surface integrin- binding domain which can include the arginine- glycine- aspartic acid (RGD) integrin ligand motif in a cyclic 12-25 amino acid residue sequence. The liposome component brings about the escape of the DNA- peptide complex from the endosomes, thus averting its degradation by the lysosomes. Once the plasmid DNA is delivered to the nucleus it is expressed episomally.

The efficiency of this system of gene delivery depends on formation of a stable complex and optimal binding to integrin on the cell surface. In view of the complexity of the system, experiments were carried out to optimise the composition of the complex and the internalisation and expression of reporter

genes in fibroblasts. The different peptides and liposomes used to form complexes are listed in Table 2.8.

4.2. Preliminary experiments using unmodified vector

Initial experiments were carried out using a formulation of the transfection complex based on the original protocol described by Hart et al. (1998) for the introduction of luciferase cDNA into COS7, A375M and ECV304 cell lines. Each complex was calculated to contain about 5000 peptide molecules per plasmid (Hart et al., 1997).

β- Galactosidase, from the pAB11 plasmid, was used as the reporter gene in the preliminary experiments but subsequently luciferase was used for the experiments. Most experiments were carried out on control normal fibroblasts, but some were performed on both control and patient fibroblasts to determine if the results were comparable. All transfections examining one set of transfection conditions were carried out in triplicate and each experiment was repeated at least twice. In all of the experiments an untransfected control was included in which cells were incubated with OptiMEM alone.

The transfection complex was prepared by mixing the three components in the following ratio: 1μg plasmid carrying lac Z cDNA: 4μg peptide 6: 0.75μg Lipofectin in OptiMEM serum- free medium. Normal fibroblasts were incubated for 6 hours in medium containing the transfection complex and then transferred to normal medium and incubated for a further 48 hours. The cells were then harvested and stained for β- galactosidase activity histochemically. About 1% of cells were transfected as indicated by lac Z expression (Figure 4.1).

Figure 4.1 Transfection of normal human fibroblasts by vector containing lac Z gene.

Vector: Lipofectin- peptide 6- lac Z plasmid (weight ratio 0.75:4:1)

Transfection time: 4h   Expression time: 48h

This experiment was then repeated using LID complexes containing luciferase cDNA (Figure 4.2) to give a quantitative measurement of gene expression with the unmodified system.
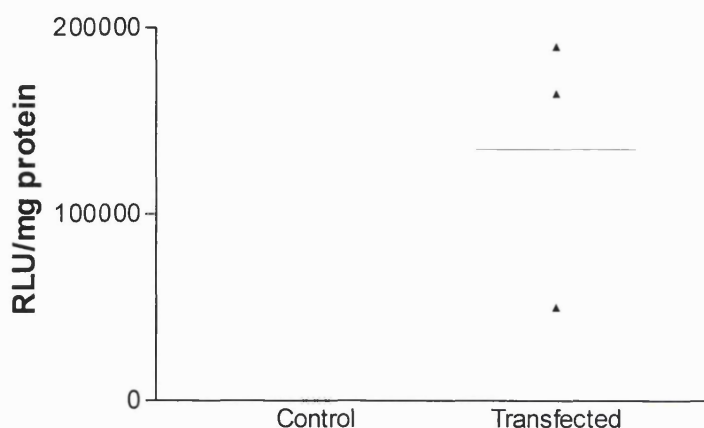
Figure 4.2 Transfection of normal human fibroblasts by vector containing luciferase gene
Vector: Lipofectin- peptide 6- lac Z plasmid (weight ratio 0.75:4:1)
Transfection time: 4h   Expression time: 48h
Luciferase measurements were performed in triplicate and standardised to total protein concentration of each sample and expressed as relative light units (RLU) / mg of protein. The line represents the mean of three observations. The control is untransfected cells.

This was the first attempt to transfect fibroblasts using this transfection system. Gene transfer was observed but it was inefficient compared to that seen in other cell lines. Transfection of COS7, A375M or ECV304 cells with similar complexes resulted in levels of expression which were 1-2 orders of magnitude greater than that observed in this experiment. Therefore the composition of the complex and conditions were varied in order to improve the levels of reporter enzyme activity.

4.3. Optimization of the transfection protocol

4.3.1. Coating cell culture plates with extracellular matrix proteins

Tissue culture plates were coated with the extracellular matrix proteins, fibronectin or gelatin, prior to seeding with the fibroblasts to be transfected, to determine if this would improve cell binding and growth of the patient fibroblasts and increase the reporter enzyme activityafter transfection of both normal and patient cells (Figures 4.3 and 4.4).
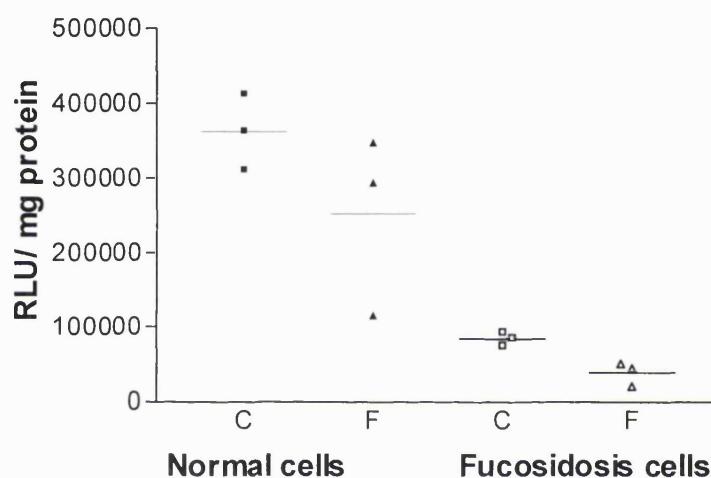
Figure 4.3 Transfection of normal and fucosidosis patient fibroblasts in uncoated plates and plates coated with fibronectin.

C= control, uncoated plates   F= fibronectin- coated plates

Vector: Lipofectin- peptide 1- luciferase plasmid (weight ratio 0.75:4:1)

Transfection time: 6h   Expression time: 68h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. C is the control of untransfected cells.

Figure 4.4 Transfection of normal and fucosidosis patient fibroblasts in uncoated plates and plates coated with gelatin.

C= control, uncoated plates   G= gelatin- coated plates

Vector: Lipofectin- peptide 1 - luciferase plasmid (weight ratio 0.75:4:1)

Transfection time: 6h   Expression time: 68h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. C is the control of untransfected cells.

Transfection gave less luciferase activity for both normal and fucosidosis fibroblasts when cell culture plates were coated with fibronectin or gelatin. The growth of fucosidosis fibroblasts appeared, by light microscopy, to be improved when grown on extracellular matrix protein substrates. The transfection of fucosidosis fibroblasts, on uncoated plates, produced about four times less activity than for normal fibroblasts.

4.3.2. Composition of transfection complex

4.3.2.1. Optimal concentration of plasmid DNA

The effect of changing the concentration of plasmid DNA in the complex over a 3- fold range was investigated (see Figure 4.5).
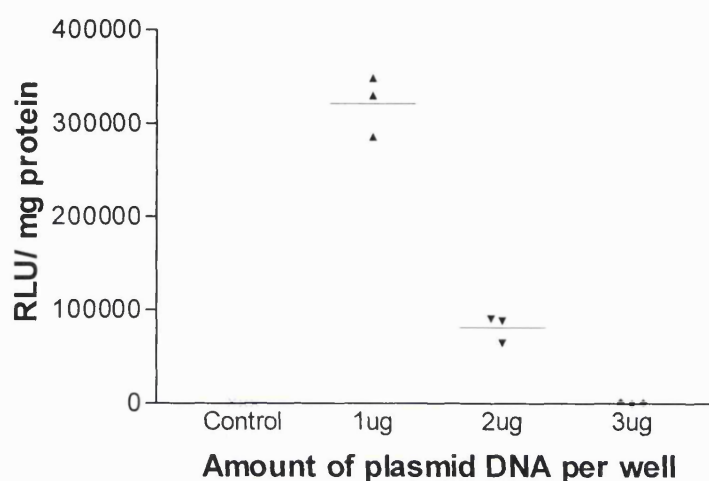
Figure 4.5 Transfection of normal fibroblasts with transfection complexes containing different amounts of plasmid (μg).
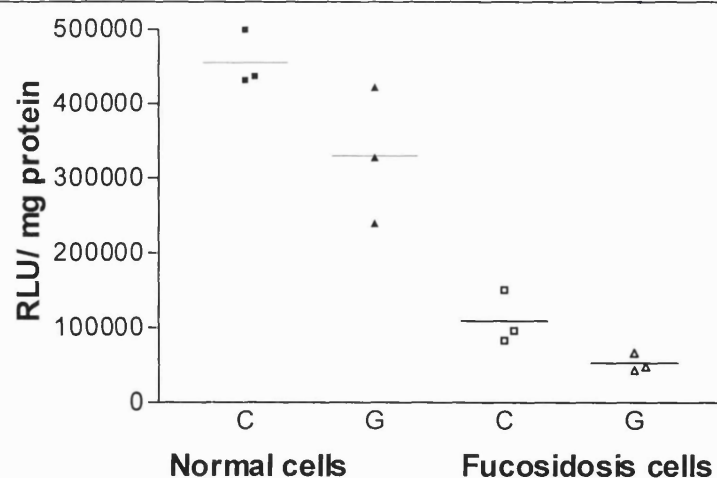
Vector: Lipofectin- peptide 1- luciferase plasmid (weight ratio 0.75:4:1-3)

Transfection time: 6h   Expression time: 48h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein. The line represents the mean of three observations. The control is untransfected cells.

Optimal transfection was observed with 1μg plasmid DNA. Increasing the amount of DNA decreased the luciferase activity and also increased the amount of cell death as observed by examination with a light microscope. This would suggest that the cells are sensitive to the uptake or presence in the medium of larger amounts of LID complex.

4.3.2.2. Different integrin-targeting peptides

Details of the structures of the different peptides used in this study are given in Table 2.7. The original protocol had been optimised using ECV304 cells and peptide 6 in the complex. Peptide 6, [K_{16}]GACRRETAWACG, is a specific $\alpha_5\beta_1$ integrin ligand (Koivunen et al., 1995). Since different cells express different classes of cell surface integrins, a number of hybrid transporter peptides with different sequences surrounding the arginine-glycine-

aspartic acid (RGD) or integrin-targeting domain were tried. Peptide 1 ([K$_{16}$]GACRGDMFGCA) (Hart et al., 1994, 1995) and peptide 5 ([[K$_{16}$]GACDCRGDCFCA) (Kouvinen et al., 1995; Hart et al., 1997) contain RGD sequences which bind to α_5 and α_v integrins. The integrin-binding domain of peptide 5 has a particularly high affinity for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Kouvinen et al., 1995). Peptide 6 is specific for $\alpha_5\beta_1$ integrins (Kouvinen et al., 1995). Peptide 7 is specific for α_4 integrins. Fibroblasts have been reported to express $\alpha_v\beta_5$ (Memmo and McKeown- Longo, 1998) and $\alpha_5\beta_1$ integrins (Dalton et al., 1995).

Transfection complexes containing the integrin-targeting peptides 1,5,6 or 7 were prepared with pAB11 plasmid DNA and Lipofectin with a constant charge ratio. The charge ratios of LID complexes may affect their transfection efficiency and were deduced from the molecular weight and charge parameters in Table 2.8. Liposome- DNA complexes for control lipofection (i.e. without peptide) consisted of Lipofectin and DNA in a transfection- optimised weight ratio of 5:1. The complexes were used to transfect normal human fibroblasts and the level of expression of β- galactosidase activity was determined histochemically (Table 4.1).

Peptide	Cells expressing β-galactosidase
None	0%
1	10 %
5	8%
6	5%
7	1%
Lipofection	1%

Table 4.1 Transfection of normal fibroblasts with transfection complexes containing peptide 1,5, 6 or 7 or by lipofection.

Vector: Lipofectin- different peptides- luciferase plasmid (weight ratio 0.75: 4: 1).

Transfection time: 4h   Expression time: 48h

The results demonstrated that the Lipofection was ineffective with only 1% of cells expressing β-galactosidase. Peptide 1 was found to be the most efficient in delivery of lac Z reporter gene to normal fibroblasts with a transfection efficiency of about 10% of the cells. Other peptides showed a lower transfection efficiency in the following order: 5> 6> 7.

To check that the integrin binding ligand was mediating transfection an experiment comparing peptides 1,11 and K16 (Figure 4.6) was carried out. K_{16} contains 16 lysines but lacks the integrin- binding domain of the other peptides and it has no known affinity for integrins. Peptide 11 is identical to peptide 1 except it contains an RGE sequence, rather than RGD, and does not bind integrins. Complexes were made with weight ratios which produced complexes of the same charge ratio.

Figure 4.6 Transfection of normal fibroblasts with transfection complexes containing peptide 1, 11 or K₁₆.

Vector: Lipofectin- different peptides- luciferase plasmid (weight ratio 0.75: 4: 1).

Incubation time: 1h   Transfection time: 6h   Expression time: 68h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein. The line represents the mean of three observations. The control is untransfected cells.

Transfection complexes containing the peptides lacking the integrin binding domain, peptide 11 and $K_{16,}$ did not give luciferase expression. This is strong evidence that transfection with this system is mediated by ligand interaction with integrin.

4.3.2.3. Peptide: DNA ratio

The effect of changing the ratio of peptide: DNA was studied (Figure 4.7).

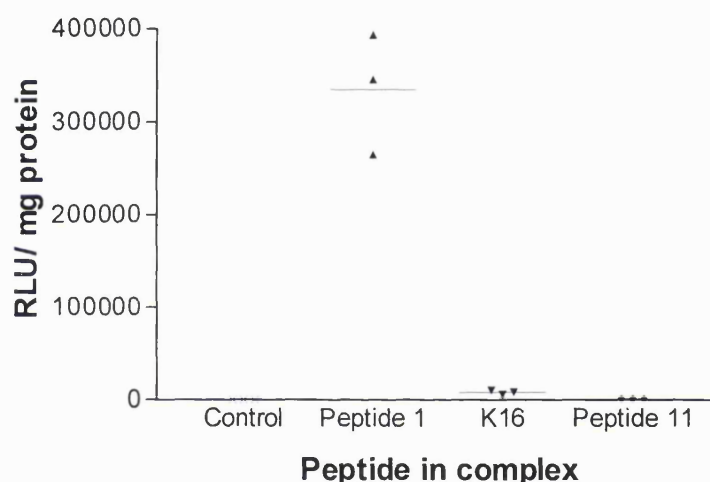**Figure 4.7 Transfection of normal fibroblasts with transfection complexes containing peptide: DNA plasmid ratios of 4:1, 5:1 or 6:1.**

Vector: Lipofectin- peptide 1- luciferase plasmid (weight ratio 0.75: 4-6: 1).

Transfection time: 6h   Expression time: 42h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein. The line represents the mean of three observations. The control is untransfected cells.

The highest level of gene expression was achieved with a ratio of 4μg peptide to 1μg plasmid DNA. Increasing the ratio to 5:1 or 6:1 reduced transfection efficiency. This is probably due to an excess amount of free peptide which would compete with the transfection complex to bind to the available cell surface integrins. Reducing the ratio below 4:1 could be beneficial.

4.3.2.4. Different liposomes and liposome concentrations

Receptor- mediated gene transfer involves the internalisation of complexes into endosomes. The complex must then leave the endosome in order to deliver DNA to the nucleus. A number of agents have been reported to aid the escape of complexes from endosomes. It has been reported that the incorporation of the cationic liposome 3β[N-(N',N'-dimethylaminoethane) carbamoyl]cholesterol (DC-chol)/ dioleoyl phosphatidylethanolamine (DOPE) into antibody-poly- L- lysine conjugates increases transfection efficiency (Trubetskoy et al., 1992). This is presumed to be due to lipid-mediated

destabilisation of the endosomal membrane. The cationic liposome, Lipofectin, enhances transferrin- mediated gene delivery (Cheng, 1996) and also integrin-mediated gene delivery (Hart et al., 1998). In this study various liposome formulations were incorporated into the transfection complex to determine which is the most successful in enhancing gene expression. The structures of the liposomes used are given in Table 2.8.

Lipofectin

Changing the concentration of lipofectin in LID complexes was investigated (Figure 4.8).
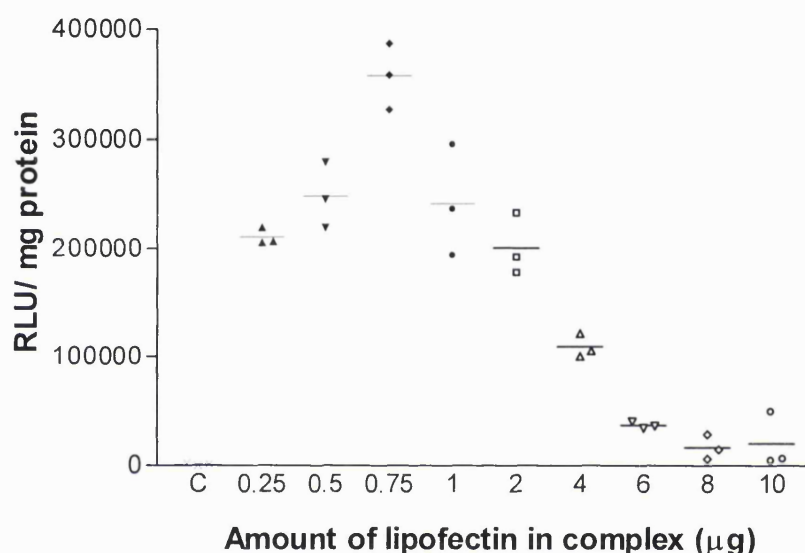
Figure 4.8 Transfection of normal fibroblasts with transfection complexes containing between 0.25 and 10μg of lipofectin.

Vector: Lipofectin- peptide 1- luciferase plasmid (weight ratio 0-10: 4: 1).

Transfection time: 6h   Expression time: 48h
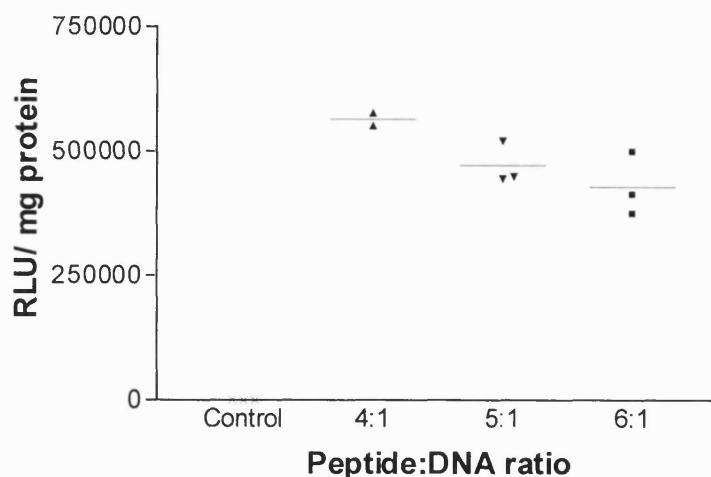
Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. The control is untransfected cells.

0.75μg Lipofectin per μg DNA gave the highest luciferase activity. Increasing Lipofectin above this amount decreased the expression of luciferase in a dose-dependent manner.

Lipofectamine

Lipofectamine is a 3:1 (w/w) formulation containing the polycationic lipid 2,2-dioleyloxy-N [2 (sperminecarboxyamido) ethyl]-N, N-dimethyl-1-propanium trifluoroacetate (DOSPA) and the neutral lipid dioleyl phosphatidylethanolamine (DOPE). Lipofectamine has been shown to give higher transfection efficiencies than Lipofectin in many cell types (Gao and Huang, 1995). This is probably due to the presence of the highly positively charged spermine headgroup in DOSPA. This enables Lipofectamine to condense DNA more efficiently (Sorgi et al., 1997) and therefore produce smaller complexes (Yang and Huang, 1998).

The effect of altering the concentration of Lipofectamine in LID complexes was examined and compared to the optimal concentration of Lipofectin (Figure.4.9).

Figure 4.9 Transfection of normal fibroblasts with transfection complexes containing between 1 and 25μg of Lipofectamine.

Vector: Lipofectamine- peptide 1 - luciferase plasmid (weight ratio 0.75: 4: 1).

Transfection time: 6h   Expression time: 48h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. The control (C) was untransfected cells.

LF are cells transfected with LID complexes containing Lipofectin in the ratio 0.75: 4: 1

It was observed that transfection complexes containing Lipofectamine transfected fibroblasts less efficiently than those containing Lipofectin, and that the luciferase expression decreased with increasing Lipofectamine.

Tfx liposomes

Tfx liposomes are formulations prepared by the Mirus Corporation. Information on their composition was not available. The use of Tfx 1, 2 or 3 in LID complexes was studied and compared to the optimal concentration of Lipofectin (Figure 4.10).

Figure 4.10 Transfection of normal fibroblasts with transfection complexes containing liposomes Tfx 1, 2 or 3.

Vector: Tfx liposome- peptide 1 - luciferase plasmid (weight ratio 2: 4: 1).

Transfection time: 6h   Expression time: 48h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.
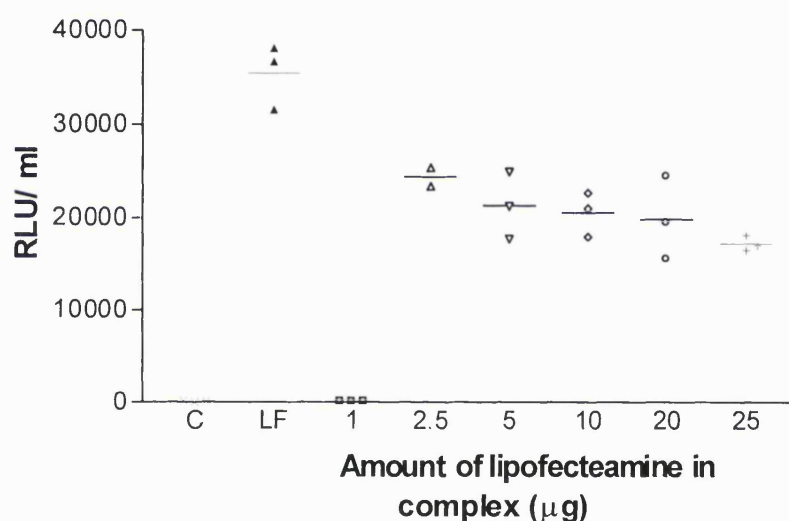
The line represents the mean of three observations. The control (C) is untransfected cells.

LF are cells transfected with LID complexes containing Lipofectin

There was very little luciferase expression using the Tfx liposomes in LID complexes compared to Lipofectin.

4.3.2.5. Addition of calcium to the transfection complex

Addition of 2mM calcium during and after transfection increases the efficiency of histone H1- mediated gene transfer since calcium is involved in membrane processes such as endocytosis and exocytosis (Haberland, 1998). Therefore the effect of addition of 2mM calcium chloride to the complex was investigated (Figure 4.11).

Figure 4.11 Effect of calcium on transfection of normal fibroblasts.

Vector: Lipofectin- peptide 1 - luciferase plasmid ± 2mM calcium (optimal weight ratio).

Transfection time: 6h   Expression time: 38h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. The control is untransfected cells.

The addition of calcium decreased transfection efficiency.

4.3.3. Preparation of complex

4.3.3.1. Order of mixing

Changing the order in which the components of non- viral transfection complexes are mixed has been demonstrated to effect the efficiency of transfection (Cheng, 1996). Transfection complexes were prepared with peptide 1 (I), pGL2 plasmid DNA (D) and Lipofectin (L) mixed in all the possible orders (Figure 4.12).
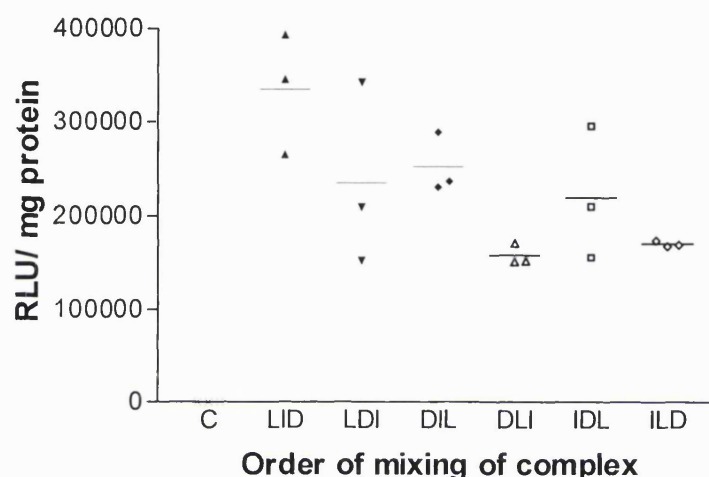
Figure 4.12 Transfection of normal fibroblasts with transfection complexes formed by mixing the components in different ways.

L= Lipofectin, I= Integrin targeting peptide, D= DNA therefore ILD indicates that I was added to the container first then L then D, with mixing after each stage.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

Transfection time: 6h   Expression time: 68h

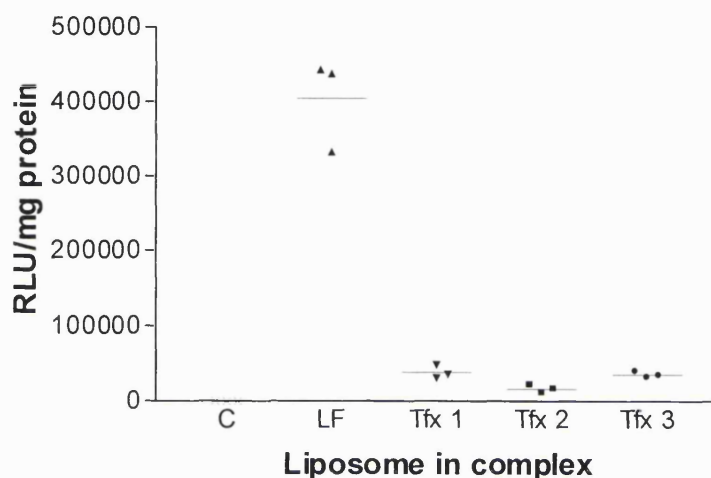Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein. The line represents the mean of three observations. The control is untransfected cells.

The order of mixing the transfection complex components altered the expression levels greatly. Addition of peptide to Lipofectin followed by the DNA gave the highest expression level. Since LI is different from IL there may be some interaction between the components and the plastic container.

4.3.3.2. Incubation of Lipofectin and peptide before addition of plasmid DNA

The effect of mixing Lipofectin and peptide and pre- incubating at room temperature before adding the DNA and incubating the complex was examined (Figure 4.13).

Figure 4.13 Transfection of normal fibroblasts with transfection complexes which had been prepared by first mixing Lipofectin with peptide and adding the plasmid DNA either immediately or after 30 min.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

Transfection time: 6h   Expression time: 68h

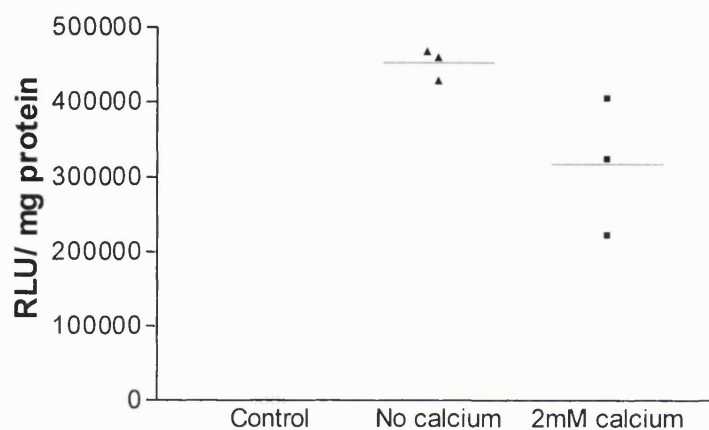Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. The control is untransfected cells.

Pre- incubation of Lipofectin and peptide before the addition of the DNA decreased the luciferase expression. This pre- incubation may increase the interaction of the complex components with the container.

4.3.3.3. Transfection complex formation time

The effect of altering the time for complex formation was analysed (Figure.4.14).

Figure 4.14 Transfection of normal fibroblasts with transfection complexes which had been incubated for different times between mixing of the transfection complex and transfection.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

Transfection time: 6h   Expression time: 42h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein. The line represents the mean of three observations. The control is untransfected cells.

Incubation of the complex for 30 minutes before it was added to the cells was found to give the highest luciferase activity of the time points which were assessed.

4.3.3.4. Optimal temperature for formation of transfection complex.

Changing the temperature of complex formation was investigated (Figure 4.15).

Figure 4.15 Effect of temperature of formation of complexes on transfection.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

Transfection time: 6h   Expression time: 39h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. The control is untransfected cells.

Room temperature was subsequently used since it was convenient and gave a high activity.

4.3.4. Factors affecting the transfection process

4.3.4.1. Presence of calcium

Having demonstrated that calcium does not have a beneficial effect when added to the transfection complex its addition to cells during transfection was studied (Figure 4.16).

Figure 4.16 Transfection of normal fibroblasts in the presence and absence of 2mM calcium.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

Transfection time: 6h   Expression time: 38h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.
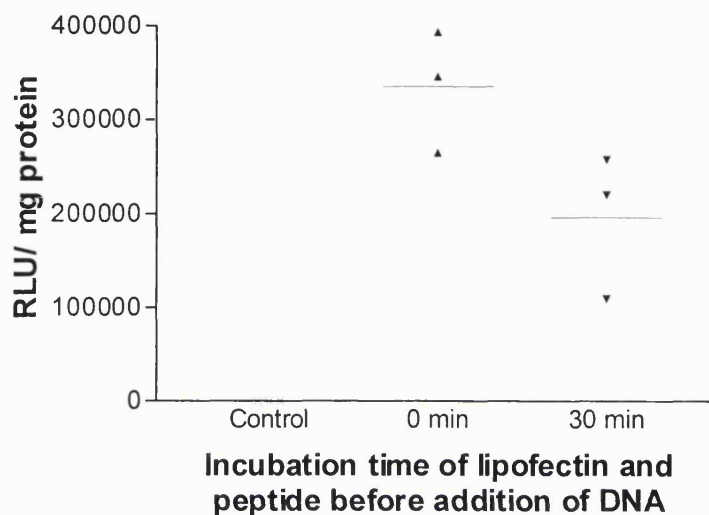
The line represents the mean of three observations. The control is untransfected cells.

Addition of calcium during transfection did not increase the efficiency of transfection and possibly had a detrimental effect.

4.3.4.2. Washing cells before transfection

Washing human fibroblast cells five times with phosphate- buffered saline (PBS) before transfection with LID complex was examined (Figure 4.17).

Figure 4.17 The effect of washing normal fibroblasts with PBS before transfection.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

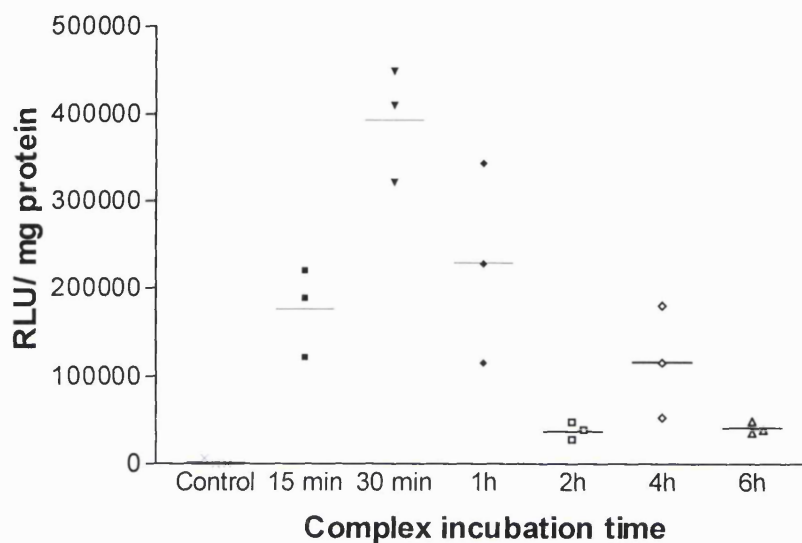Transfection time: 6h   Expression time: 42h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. The control is untransfected cells.

Washing cells extensively before transfection decreased the luciferase expression since it probably removed the ions associated with integrins which are essential to their function.

4.3.4.3. Addition of fetal calf serum

Various amounts of fetal calf serum (FCS) were added to normal and fucosidosis human fibroblasts just before transfection with LID complexes to simulate physiological conditions (Figure 4.18).

Figure 4.18 The effect of addition of FCS to normal and fucosidosis fibroblasts just before transfection.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

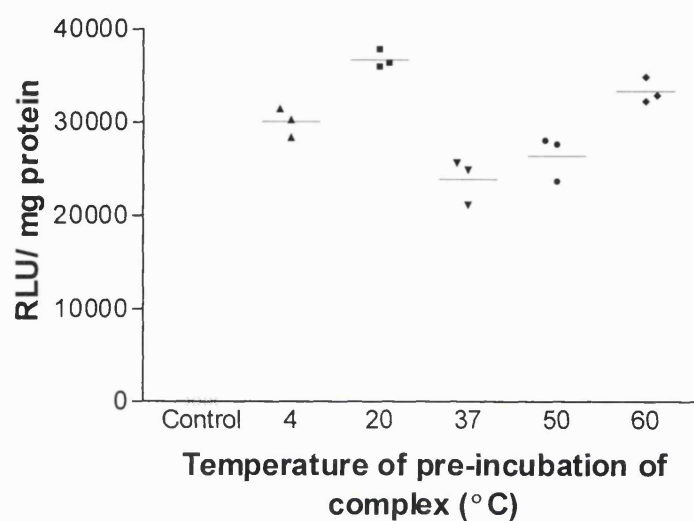Transfection time: 6h   Expression time: 46h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein. The line represents the mean of three observations. The control is untransfected cells.

The presence of very low concentrations of FCS inhibited transfection in a dose-dependent response. A concentration of 50% completely prevented transfection or expression.

4.3.4.4. Centrifugation

Transfection using the synthetic cationic lipid Transfectam (Promega) and the cationic, organic molecule polyethylenimine have been shown to be enhanced after mild centrifugation of the complex onto the cell line (Boussif et al., 1996). The effect of centrifugation of transfection complexes onto the cells at 2000 x g, 4°C for 20 min was investigated (Figure 4.19).

Figure 4.19 The effect of centrifugation of normal fibroblasts at the start of transfection.
Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).
Transfection time: 20 min centrifugation (2000 x g, 4°C) followed by 6h in incubator
Expression time: 42h
Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.
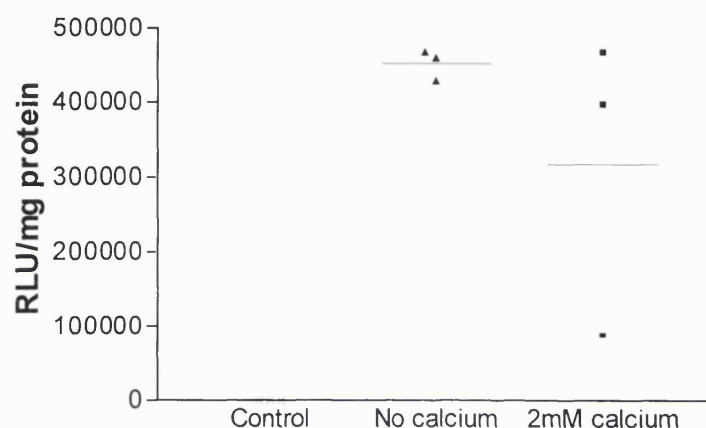The line represents the mean of three observations. The control is untransfected cells.

Centrifugation of the complex after it was added to the cells increased the efficiency of transfection markedly. A further experiment was carried out to establish the optimal centrifugation time and temperature (Figure 4.20).
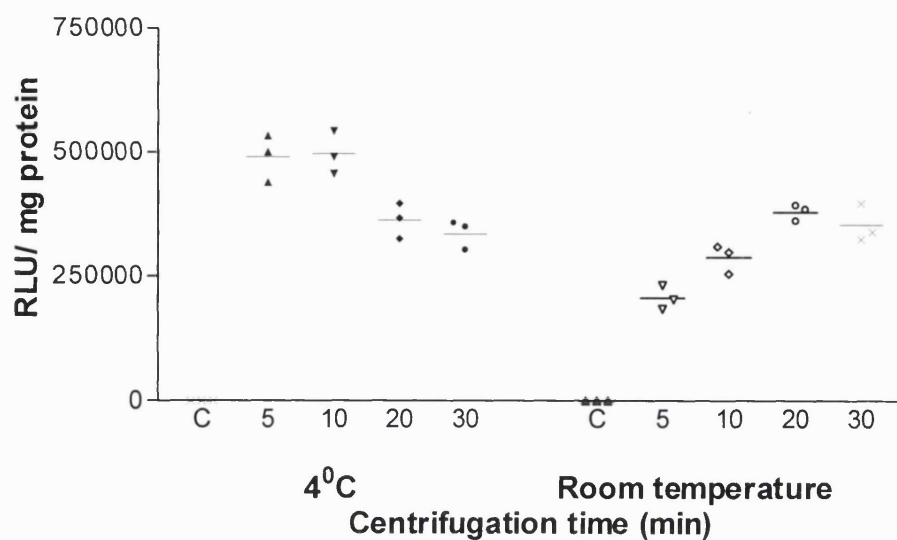
Figure 4.20 The effect of centrifugation of normal fibroblasts at the start of transfection at 4°C or room temperature for various times.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

Transfection time: 5- 30 min centrifugation (2000 x g, 4°C or room temperature) followed by 6h in incubator.   Expression time: 38h
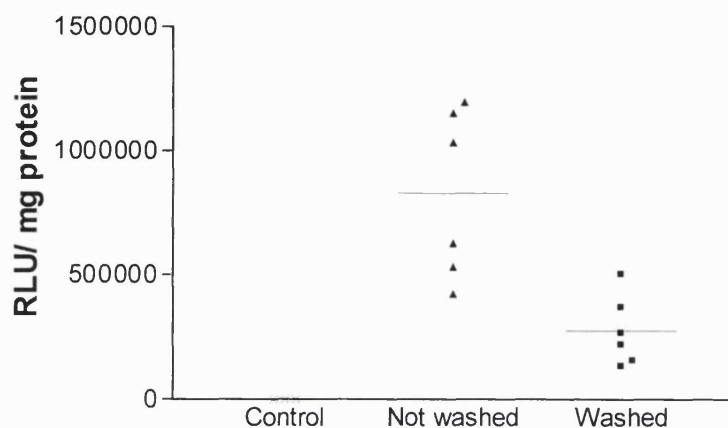
Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. The control (C) is untransfected cells.

Luciferase expression was the highest when the complex was centrifuged onto the cells at 4°C for 5 or 10 minutes. At 20 minutes the temperature did not matter but at 5 minutes it had an effect.

4.3.4.5. Suspension of cells

Cells in suspension may have more integrins available to bind the complexes since they are not bound to extracellular substrate. Transfecting normal fibroblasts when in suspension after trypsinization was studied (Figure 4.21).

Figure 4.21 Normal fibroblasts were transfected while either adhering to well plates or whilst in suspension.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

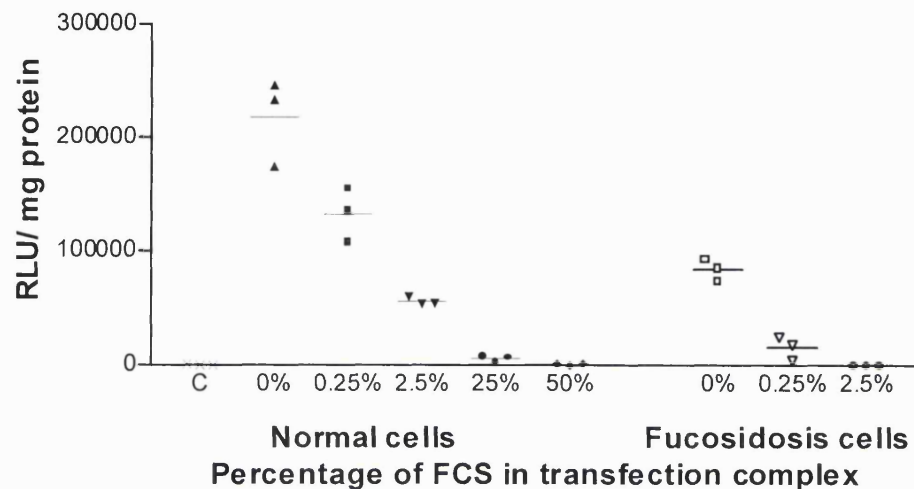Transfection time: 6h   Expression time: 38h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. The control is untransfected cells.

Transfection of cells in suspension did not give any luciferase expression.

4.3.4.6. Transfection time

The transfection time, during which cells are exposed to the transfection complex, was altered to see if it affected the luciferase expression after transfection (Figure 4.22).

Figure 4.22 Normal fibroblasts were transfected for different amounts of time.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

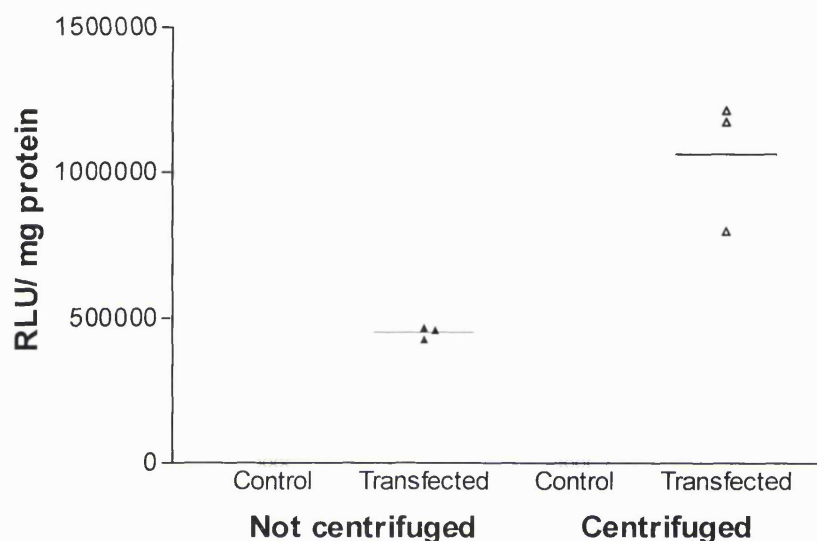Transfection time: 2-24h   Expression time: 18h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. The control is untransfected cells.

Transfection for six hours gave the highest levels of luciferase expression. Activity increased with transfection time up to 6 hours since with time more of the RGD peptide will bind to the integrin receptor and be endocytosed. There is a limited number of integrins on the cell surface but they are recycled. An incubation of 18h may increase the toxicity of the protocol.

4.3.5. Expression time

Once the cells have been exposed to the complex and given time to take up the gene they are then incubated with medium to allow expression of the transferred gene.

4.3.5.1. Determination of the optimal expression time for lac Z reporter gene

The affect of altering the expression time after transfection with LID complexes containing the LAC Z reporter gene was investigated (Table 4.2).

Expression time	Cells expressing β-galactosidase
24h	15%
48h	12%
72h	5%

Table 4.2 Investigation of expression time for transfection of normal human fibroblasts by vector containing lac Z gene

Vector: Lipofectin- peptide 6- lac Z plasmid (weight ratio 0.75:4:1)

Transfection time: 6h   Expression time: 24-72h

The highest level of expression for β-galactosidase was at 24 hours.

4.3.5.2. Determination of the optimal expression time for luciferase reporter gene

The expression time after transfection with LID complexes containing a luciferase reporter gene was examined (Figure 4.23).
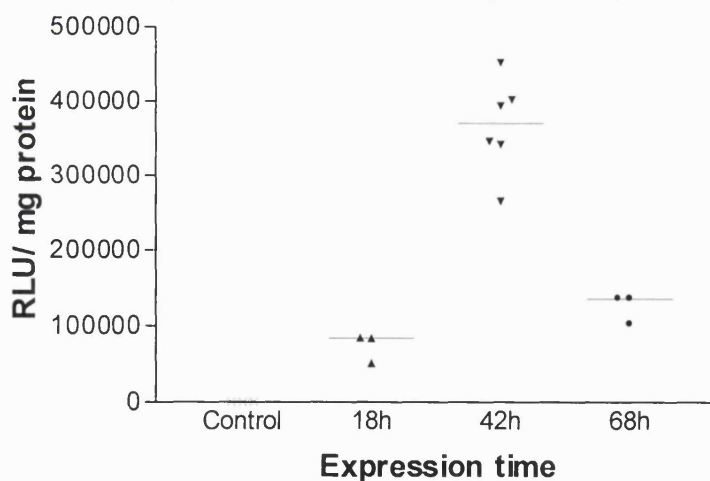
Figure 4.23 Investigation of expression time for transfection of normal human fibroblasts by vector containing luciferase gene.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

Transfection time: 6h   Expression time: 18-68h

Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. The control is untransfected cells.

The highest level of expression of luciferase, 367 000 RLU/mg protein, was reached at 42 hours.

4.3.6. Addition of calcium during expression

The effect of adding 2mM calcium chloride to the growth medium in the expression period might affect membrane processes and was therefore investigated (Figure 4.24).
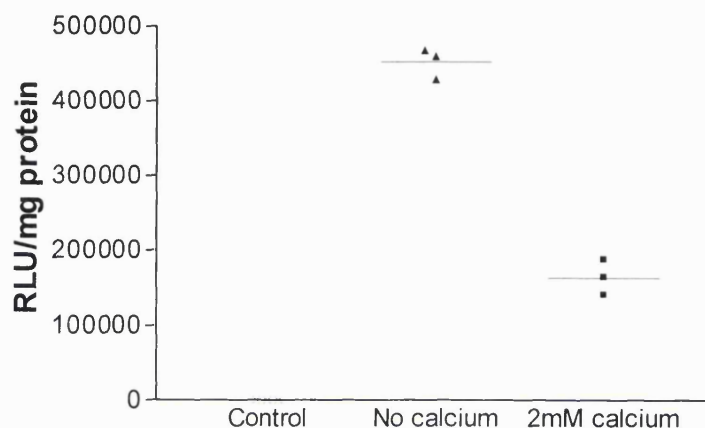
Figure 4.24 The effect of addition of 2mM calcium chloride to the growth medium in the expression period.

Vector: Lipofectin- peptide 1 - luciferase plasmid (optimal weight ratio).

Transfection time: 6h   Expression time: 38h

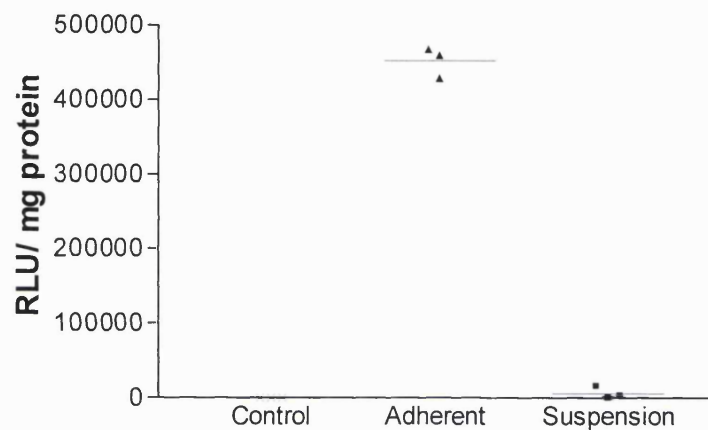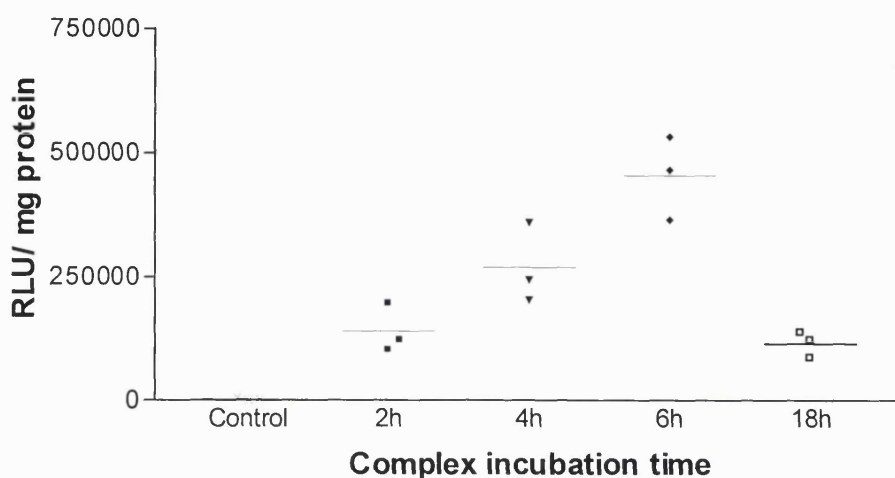Luciferase measurements were performed in triplicate and expressed as RLU / mg of protein.

The line represents the mean of three observations. The control is untransfected cells.

Addition of calcium chloride decreased expression of luciferase approximately 3-fold.

4.4. Discussion

Coating cell culture plates with extracellular matrix proteins

The use of fibronectin- or gelatin- coated plates improved cell growth but decreased the transfection efficiency of cells grown on them. Fibroblasts have been reported to express $\alpha_v\beta_5$ (Memmo and McKeown- Longo, 1998) and $\alpha_5\beta_1$ integrins (Dalton et al., 1995). Since the RGD sequence in fibronectin also binds to these integrins (Hynes, 1992) the decrease in transfection could be due to direct competition between fibronectin and the LID complexes for the cell surface integrins. However, there is also decreased transfection when plates are coated with gelatin, which does not bind directly to these integrins and would therefore not compete directly. Generally, cell adhesion to extracellular matrix proteins such as fibronectin or gelatin stimulates increased expression or activation of cell surface integrins (Hynes, 1992). The attachment of cells to extracellular matrix proteins on the flask surface may cause the integrins to concentrate in focal adhesions on the area of the cell surface which is attached. Localisation of integrins in the area of cell surface attached to the flask would decrease the number available to bind LID complexes on the unattached surface.

Composition of transfection complex

The optimal composition of complexes containing peptide 1 or 6, DNA and Lipofectin for the transfection of fibroblasts is the same as that reported previously for COS7, A375M and ECV304 cells (Hart et al., 1995, 1998). 0.75μg Lipofectin, 4μg peptide 1 and 1μg plasmid DNA per well produced the best luciferase expression. It is possible that these proportions of Lipofectin, peptide and DNA allow optimal complex formation and that this is more important than the type of cell to be transfected. A precise ratio of DNA: liposome: peptide is therefore important for optimal transfection. Unbound Lipofectin has been reported to have a cytotoxic effect. Brief centrifugation of complex and removal of the supernatant, containing the unbound components which may be toxic to cells, has been recommended by Hofland et al. (1996).

Transfection was carried out with different peptides containing an integrin ligand. Peptide 1 gave the highest luciferase expression after transfection of

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fibroblasts, followed by peptide 5, then 6 and finally 7. Peptides 1 and 5 both bind to α_v integrins, suggesting that these are expressed on the surface of fibroblasts. Transfection with complexes containing peptide 7 did not lead to expression. This peptide binds to α_4 integrins. Therefore, the lack of transfection suggests that α_4 integrins are not expressed or not active on the surface of fibroblasts. The nature of the peptide sequence surrounding the integrin- binding motif appears to be important when transfecting different cell types which probably express different integrins. Although it is possible to determine which integrins are expressed on a cell type using FACs analysis, it is the number of activated integrins on the cell surface which will determine the level of LID- mediated transfection.

The use of different liposomes in LID complexes showed that Lipofectin gave the most efficient reporter gene expression. Lipofectamine is generally a more effective transfection agent than Lipofectin (Gao and Huang, 1995). However, when it is used in LID transfection complexes the positively charged spermine headgroup of its DOSPA component may interfere with complex formation. Conversely, the positive charge may lead to stronger ionic interactions within the complex so that they fail to dissociate and therefore inhibit nuclear expression.

Calcium affects the formation of the transfection complex and makes transfection less efficient. This may be due to the positively charged calcium ions interfering in the electrostatic interactions involved in LID complex formation. The calcium chloride may also interact directly with DNA, disrupting the phosphate groups.

Preparation of transfection complex

The protocol developed for the preparation of transfection complex to transfect A375M, COS7 and ECV304 cells was also found to be optimal for transfection of fibroblasts. This suggests that the preparation of transfection complex will not need to be optimised for different cell lines. The complex should be mixed in the order Lipofectin- peptide- DNA. The addition of the two cationic components (Lipofectin and peptide) first probably allows them equal access to plasmid DNA giving better complex formation for transfections. Previous studies on transferrin- mediated gene transfer demonstrated that the

order of mixing of transferrin, Lipofectin and DNA affected the efficiency of gene product expression (Cheng, 1996). The optimal complex formation period is 30 min at room temperature. Conversely, Lipofectin- DNA complexes transfect cells more efficiently when incubated for 40 min at 45°C before transfection compared to thirty minutes at 22°C (Yang and Huang, 1998).

Transfection conditions

Various alterations to the transfection conditions were examined. The presence of calcium during transfection did not increase the luciferase expression. Washing cells prior to transfection decreased expression of the delivered gene. This might be because washing with PBS removes divalent cations which are essential for integrin function. Even the presence of small amounts of FCS decreased the efficiency of transfection. This inhibition is probably due to serum proteins binding to the cationic complex and raises the possibility that the complexes may be ineffective if used intravenously. However, studies on other transfection systems have shown cross-species differences to inhibition with serum (Yuda et al., 1996; Liu et al., 1995). Therefore FCS inhibition does not give an accurate forecast of what would happen if these complexes were injected into humans and further studies might include examining the effect of human serum on transfection.

Centrifugation of the LID complexes on to cells increased the efficiency of gene delivery and expression. This is probably because the complex is brought into closer contact with the cells at the start of the transfection period by the centrifugation. LID complexes were not able to transfect cells in suspension. The binding of transfection complex to fibroblasts in suspension may prevent them from settling down, adhering and growing. Another possibility is that during trypsinization the integrins are cleaved or internalised and new integrins do not move to the cell surface when the cells are left in suspension.

The optimal transfection time was found to be about 6 hours. An evaluation of DMRIE/DOPE liposomes complexed with fluorescently labelled DNA showed that the process of DNA entry into cells is relatively slow, with most of the complex internalised by 6 hours (Zabner et al., 1995). This suggests that the mechanisms of internalisation of liposome complexes and LID complexes could be similar.

Expression period

It has been demonstrated that commonly used methods of transient expression induce a cellular stress response. Maximal stress- mediated enhancement of the CMV promotor does not occur until cells have recovered for 24 hours following transfection (Andrews et al., 1997). Therefore, expression of introduced genes increases over the first 24 hours after transfection.

Expression with the LID system is only transient since the DNA is not integrated but is expressed episomally and will therefore be diluted out on cell division. The DNA can also be degraded by nucleases and the promotor silenced. It is hoped that with the development of human artificial chromosomes there will be plasmid replication, nuclear retention and consequently prolonged expression.

Other modifications of the complex may increase the transfection efficiency for fibroblasts and other cells. Most non-viral gene therapy protocols are being developed by trial and error because the kinetics of complex formation and the mechanisms of cellular uptake and intracellular trafficking of the complexes remain poorly understood. Little is known about the parameters that influence complex formation and the characteristics of the complex that are relevant to transfection efficiencies. Once these mechanisms are better understood, protocols could be improved using a more rational approach.

Fibroblasts have given poorer transfection luciferase expression for LID transfection than other cells. Cell type- dependent variability has been reported in other non-viral systems such as lipofection (Zabner et al., 1995; Matsui et al., 1997). These cell type differences may be at a number of stages of the process: complex binding, complex uptake, intracellular transport, nuclear entry and exclusion of DNA from the nucleus. Different cell types might differ in their abilities to take up particles of different size, to exclude endogenous DNA from the nucleus and therefore in their ease of transfection (Gao and Huang, 1995; Zabner et al., 1995; Coonrod et al., 1997).

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4.5. Conclusions

The optimal system used LID complexes containing Lipofectin, peptide 1: plasmid in a ratio of 0.75: 4:1.(with 1μg plasmid per well).This was mixed in the order Lipofectin then peptide then DNA and incubated at room temperature for 30min . The LID complex was then added to the cells which were centrifuged at 2000 x g for 10min at 4°C and placed in an incubator for 6h. The two alterations to the protocol for the transfection of fibroblasts which gave the largest improvements in transfection efficiency were changing the peptide used in the complex to peptide 1 and centrifugation of the complex onto the cells at the start of transfection. The additional step of centrifuging the transfection complex onto the cells at the start of transfection was found to double gene expression. These experiments demonstrate that LID complexes are able to transfect fibroblasts. The luciferase activity due to transfection was improved to levels similar to those demonstrated in other cell lines (A375M, COS7 and ECV304). These conditions were then used to transfect patient and normal fibroblasts with LID complexes containing lysosomal enzyme cDNA.

This study demonstrates that many aspects of this system, such as the component ratios, are optimal for all cell types. However, the peptide within the transfection complex and the transfection time can vary for different target cell types and optimal expression times can vary with different gene cargoes. An important question raised is why are fucosidosis patient fibroblasts transfected less efficiently than control fibroblasts. Further research on this could provide an insight into how transfection of cells from patients with lysosomal storage disease might be improved.

LID- mediated delivery of human α- L- fucosidase and α- galactosidase A cDNAs into fibroblasts from fucosidosis and Fabry patients

5.1. Introduction

Previous work with the LID non- viral gene therapy system described the use of vectors carrying reporter genes to transfect A375 cells (melanoma cells), COS7 cells (monkey kidney epithelial cells) and ECV304 cells (spontaneously transformed human umbilical vein endothelial cells) (Hart et al., 1998). In this chapter the use of this gene transfer system to transfect fibroblasts from patients with lysosomal storage diseases with the normal cDNA encoding the enzyme that is defective, is described. This is the first application of this system to transfect cells from human patients and evaluate its use for gene therapy of human lysosomal storage diseases.

LID complexes containing α- L- fucosidase cDNA or α- galactosidase A cDNA were used to transfect fibroblasts from fucosidosis and Fabry disease patients, respectively. Similar experiments were also carried out in normal cells to investigate the expression of the transgene in the presence of normal levels of the enzyme. The possible effect of lysosomal storage on transfection was assessed by transfecting fibroblasts from Fabry patients with LID complexes containing α- L- fucosidase cDNA. The effect of transfection on native endogenous lysosomal enzymes was also investigated.

5.2. Transfection of human fibroblasts with LID complexes containing α- L- fucosidase cDNA

Having established valid analytical procedures and assessed their reproducibility, experiments were carried out using the optimal transfection conditions from Chapter 4 (apart from the initial experiment on the choice of α- L- fucosidase plasmids which was carried out under sub- optimal conditions). In the experiments that follow the enzyme activities are expressed as units of activity (nmol/h) since this allowed the intracellular activity and the activity secreted into the medium to be compared. However, in each experiment the specific enzymic activity was also determined to assess whether any of the changes observed were due to changes in protein concentration (data not shown). The same trends were seen for total and specific activities confirming that changes in total activities reported were not due to differences in concentration between assays.

5.2.1. Transfection of fucosidosis fibroblasts with LID complexes containing α- L- fucosidase cDNA

Plasmid

Fucosidosis fibroblasts (A.M.) were transfected with LID complexes containing different α- L- fucosidase cDNA expression plasmids (pLF, pLNCF and pLFSN, Figure 2.1 p85) and the α- L- fucosidase activity of the transfected fibroblasts was determined (Figure 5.1).
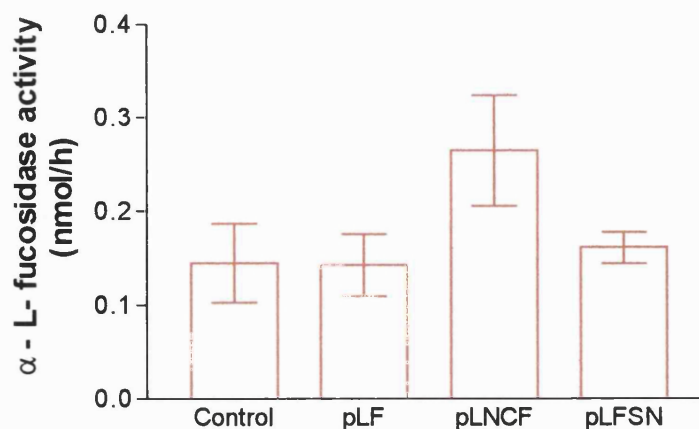
Figure 5.1 Expression of α- L- fucosidase in fucosidosis fibroblasts (A.M.) transfected with LID complexes containing the plasmids pLF, pLFSN or pLNCF.

The error bars represent ± 1 standard deviation of the mean activity in 4 wells assayed in triplicate. The control is untransfected fucosidosis fibroblasts.

pLNCF, where α- L- fucosidase was expressed from a CMV promotor rather than a viral LTR, was found to produce the highest α- L- fucosidase activity. Transfection of fucosidosis fibroblasts with LID complexes containing pLF and pLFSN did not produce significant differences in α- L- fucosidase activity. pLNCF was therefore used in all subsequent experiments.

Intra- and extracellular activity of transgene in transfected cells

Fucosidosis fibroblasts were transfected with LID- containing α- L- fucosidase cDNA in pLNCF and the accumulated α- L- fucosidase activity in the intracellular and extracellular fractions was determined 2, 4 and 6 days after transfection on different wells of cells (Figure 5.2).
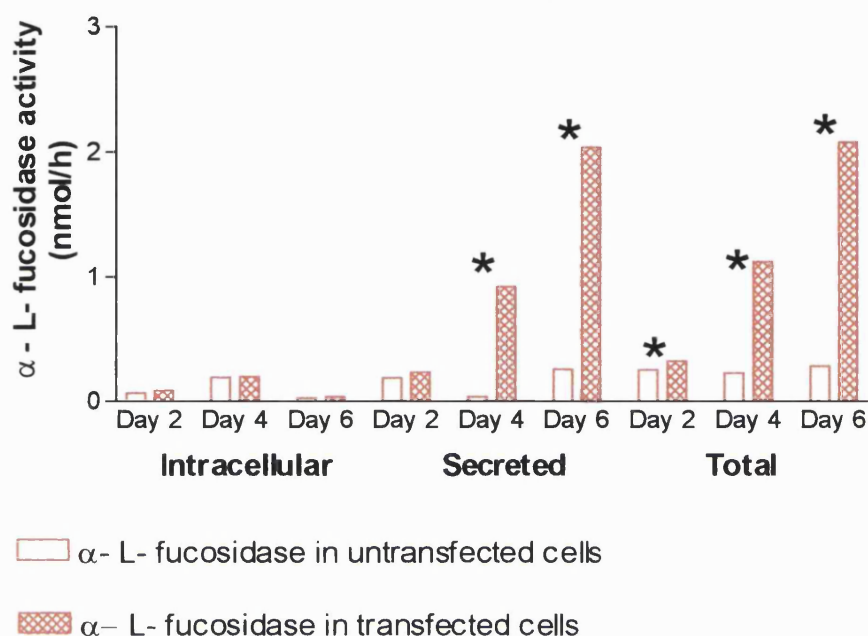
Figure 5.2 Transfection of fucosidosis fibroblasts (A.M.) with LID complexes containing α- L- fucosidase cDNA in pLNCF.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

This experiment demonstrated that LID complexes containing α- L- fucosidase cDNA successfully transfected fucosidosis fibroblasts but that the resultant enzyme was mainly secreted. The secreted α- L- fucosidase activity showed a significant increase 4 days after the transfection of fibroblasts with LID complexes containing the pLNCF construct. This increase was reflected in the total activity in the culture, the combined intracellular and extracellular activity, which was significantly greater in the transfected cultures at all time points. The intracellular α- L- fucosidase activity showed an increase as a result of transfection which was, however, not significant. The small increase in

intracellular activity after transfection was consistent with preliminary experiments in this study. The total activity in the cultures at day 6 was 7 times higher compared with the untransfected cultures. However, it represented about 10% of the level in normal cultures. This increase in activity would probably have corrected the fucosidosis cells metabolically if it had been intracellular. However the enzyme was mainly secreted and any correction would depend on whether the enzyme could be taken up by cells.

Preliminary experiments using immunofluorescent staining for α- L-fucosidase protein *in situ* and FACS analysis showed no differences in intracellular α- L- fucosidase protein expression associated with cells after transfection (data not shown).

Intra- and extracellular activities of native endogenous lysosomal enzymes in transfected cells

In the previous experiment it was concluded that after transfection the transgene is expressed but its product is mainly secreted. This could be a general feature of transfection with LID complexes or it could be due to a problem in the production, intracellular transport or processing of the transgene products. Therefore, in the next stage of this study, the effect of transfection on the distribution of endogenous lysosomal enzymes in fucosidosis fibroblasts was studied. Fucosidosis fibroblasts were transfected with LID- containing α- L-fucosidase cDNA and the intra- and extracellular activities of α- galactosidase and β- hexosaminidase activities were determined 2, 4, and 6 days after transfection (Figure 5.3 and 5.4).
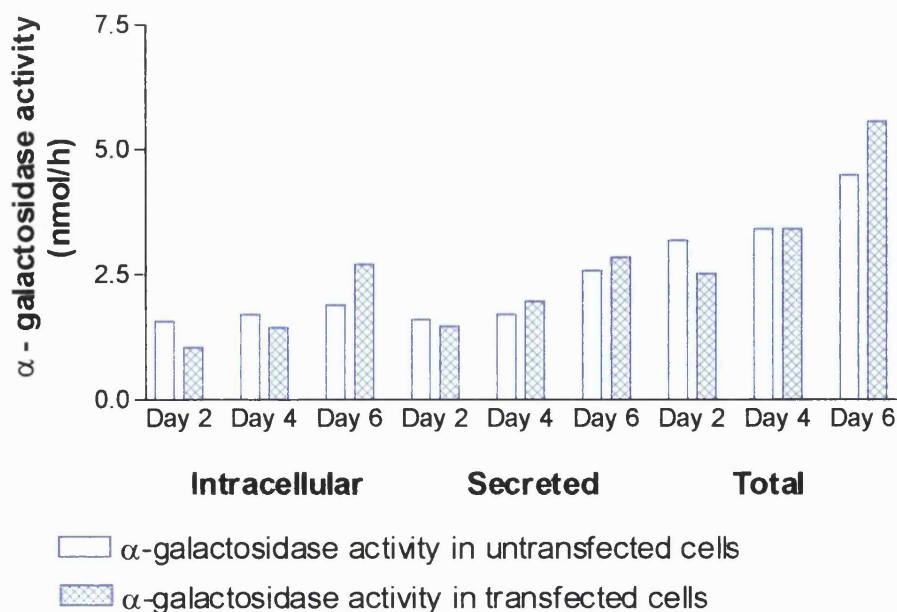
Figure 5.3 Transfection of fucosidosis fibroblasts with LID complexes containing α- L-fucosidase cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.
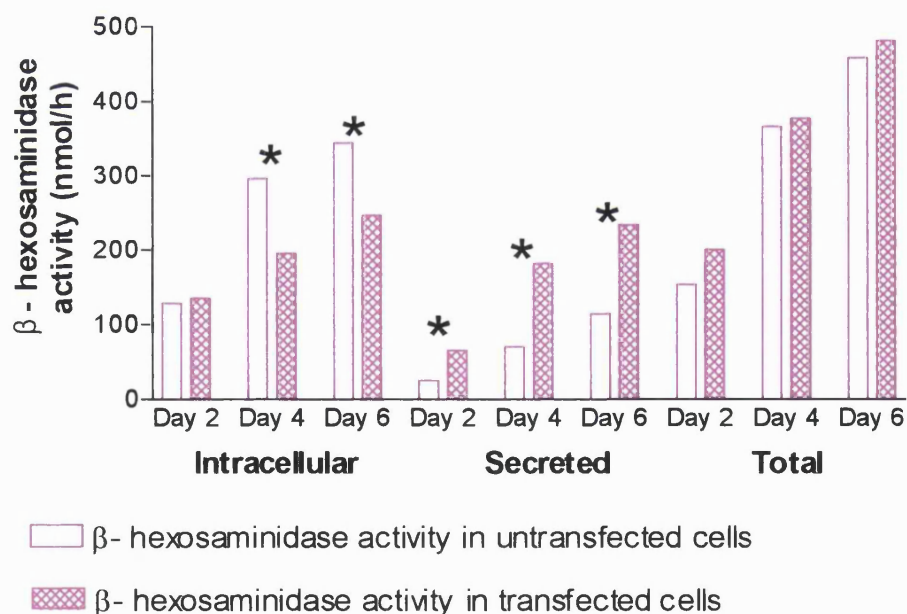
Figure 5.4 Transfection of fucosidosis fibroblasts with LID complexes containing α- L-fucosidase cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

No significant change was found in the levels of intracellular and secreted α- galactosidase enzyme activity as a result of transfection with LID complexes containing α- L- fucosidase cDNA. On the other hand, transfection of the fibroblasts produced a significant decrease in the intracellular activity of the native β- hexosaminidase which was concomitant with a significant increase in the extracellular levels of the enzyme. The percentage of the total enzyme which was secreted increased after transfection. This suggests that transfection may have a non- specific effect on native endogenous lysosomal enzymes.

5.2.2. Transfection of normal fibroblasts with LID complexes containing α- L- fucosidase cDNA

Intra- and extracellular activity of transgene in transfected cells

Normal fibroblasts were transfected with LID complexes containing α- L- fucosidase cDNA. The intracellular and secreted α- L- fucosidase activities were measured after 2, 4 and 6 days (Figure 5.5).

Figure 5.5 Transfection of normal fibroblasts with LID complex containing α- L- fucosidase cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

Transfection of normal fibroblasts produced a decrease in the intracellular enzyme activity, which became significant 6 days after transfection. At day 6 the intracellular activity was less than half the level of normal cultures. There was a significant increase in the secreted amount of α- L- fucosidase in the transfected cells reaching 11.5 fold by day 6. The overall total activity in the culture was increased significantly as a result of transfection. The observed increase in total activity reached about 150% of the normal activity by day 6. The transgenic activity increased by 15 units in normal cultures compared to 2 units in fucosidosis cultures suggesting that transfection of normal cultures was more efficient. Transfection with α- L- fucosidase cDNA has clearly altered the distribution or synthesis of the endogenous α- L- fucosidase.

After transfection of normal fibroblasts the decrease in intracellular activity over 6 days could be a non- specific effect of transfection or a specific effect on the lysosomal system. As the intracellular activity in untransfected normal cells increases over this period, this suggests that there is a loss of endogenous activity, which is greater than the amount of acquired transgenic activity. It further suggests that the secreted fraction was not being recaptured by the normal cells. However, transfection of fucosidosis cells does not lead to this decrease in intracellular enzyme and there is a small increase in intracellular activity. As the endogenous α- L- fucosidase activity in fucosidosis fibroblasts is very low it is possible to measure the acquired transgenic activity. It is concluded that in transfected fucosidosis fibroblasts some transgenic activity is either directly targeted to the lysosome or recaptured from the medium.

Intra- and extracellular activities of native endogenous lysosomal enzymes in transfected cells

LID complexes containing α- L- fucosidase cDNA were used to transfect normal fibroblast cells and after 2,4, and 6 days the intracellular and extracellular α- galactosidase and β- hexosaminidase activities were measured (Figure 5.6 and 5.7).
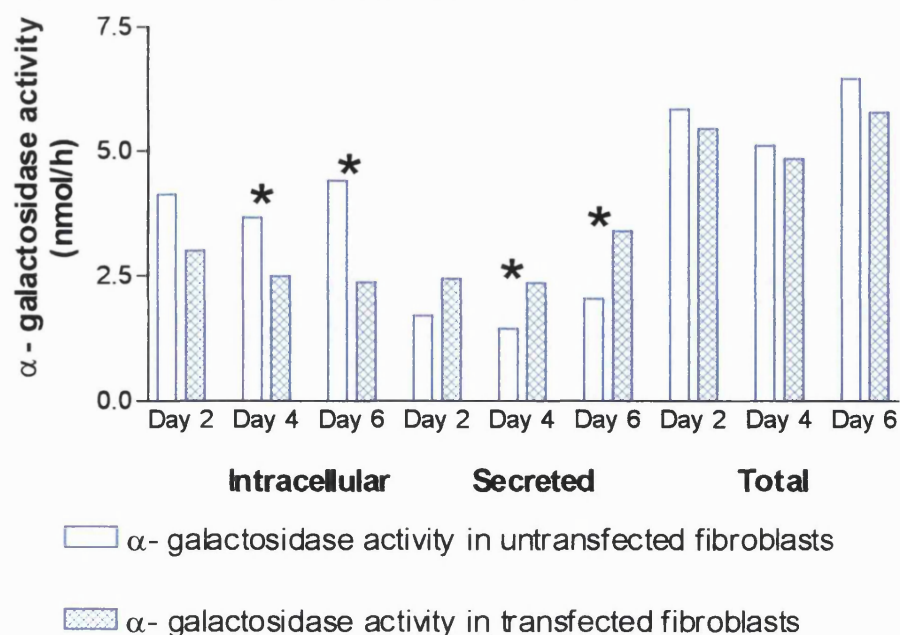
Figure 5.6 Transfection of normal fibroblasts with LID complex containing α- L-fucosidase cDNA.

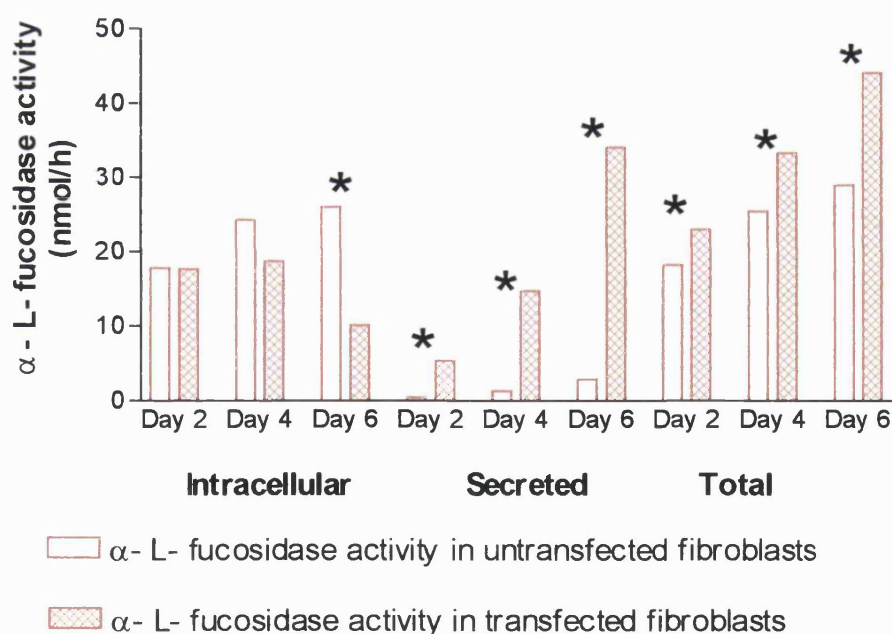Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.
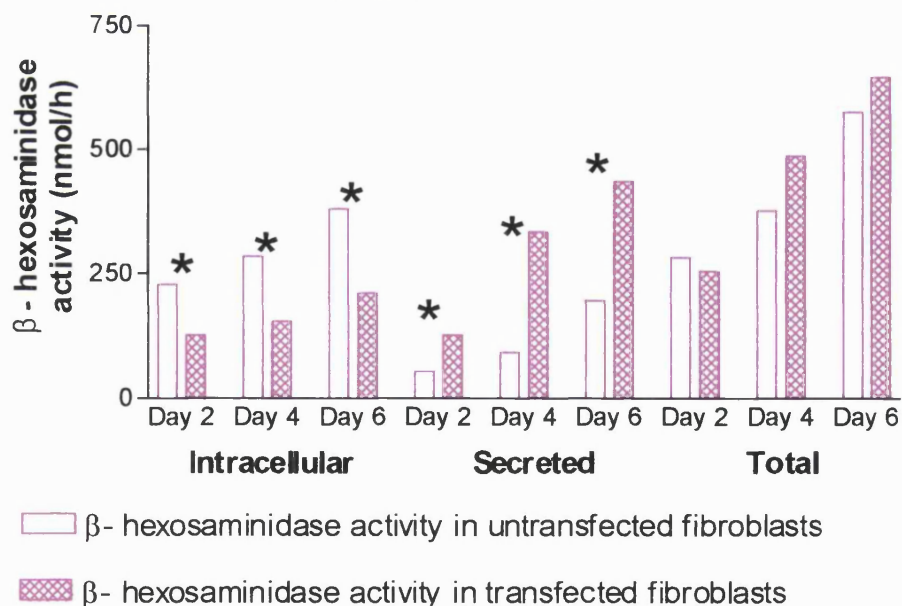
Figure 5.7 Transfection of normal fibroblasts with LID complex containing α- L-fucosidase cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

There were no significant changes in the total α- galactosidase or β-hexosaminidase in cultures of normal cells after transfection with α- L-fucosidase cDNA. However, there were significant increases in the secreted α-galactosidase (days 4 and 6) and β- hexosaminidase (days 2, 4 and 6) and concomitant significant decreases in the intracellular activity of both enzymes at the same time points.

5.2.3. Transfection of Fabry fibroblasts with LID complexes containing α- L- fucosidase cDNA

Intra- and extracellular activity of transgene in transfected cells

Fabry disease fibroblasts have normal levels of α- L- fucosidase but have a deficiency of α- galactosidase A. They contain storage products specific to Fabry disease. Fabry disease fibroblasts were transfected with LID complexes containing α- L- fucosidase cDNA and the α- L- fucosidase activity of the intracellular and secreted fractions were determined 2,4 and 6 days after transfection (Figure 5.8).

Figure 5.8 Transfection of Fabry fibroblasts with LID complexes containing α- L-fucosidase cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

After transfection there was a significant increase in total α- L-fucosidase enzyme activity compared to untransfected cells. The increase in total activity of the enzyme was due to an increase in the secreted form of the enzyme rather than that of the intracellular form. Transfection increased production of α- L- fucosidase enzyme but the additional enzyme molecules were not delivered to the lysosome but were instead secreted out of the cell. However, the intracellular activity decreased following transfection and on days 4 and 6 the decrease was significant. In this respect the Fabry fibroblasts behaved similarly to normal fibroblasts. This suggests that the transfection of cells that had normal levels of α- L- fucosidase caused a loss of intracellular endogenous enzyme which was not compensated by the acquired intracellular transgene activity. It is interesting to note that a small increase in intracellular transgenic activity is only observed when cells deficient in the enzyme are transfected (Figure 5.2). Therefore, there may be some targeting of transgene activity to the lysosomes, directly or indirectly via secretion and uptake in storage disease fibroblasts.

Transfection of Fabry fibroblasts, like fucosidosis fibroblasts, produced less enzyme activity from the transgene compared with that of normal fibroblasts. This suggests that the pathological state of the lysosomal storage disease fibroblasts effects their response to the transfection.

Intra- and extracellular activities of native endogenous lysosomal enzymes in transfected cells

Fabry fibroblasts were transfected with LID- containing α- L- fucosidase cDNA. The intracellular and extracellular activities of the endogenous α-galactosidase and β- hexosaminidase were measured 2,4, and 6 days after transfection (Figure 5.9 and 5.10).

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Figure 5.9 Transfection of Fabry fibroblasts with LID complexes containing α- L-fucosidase cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.
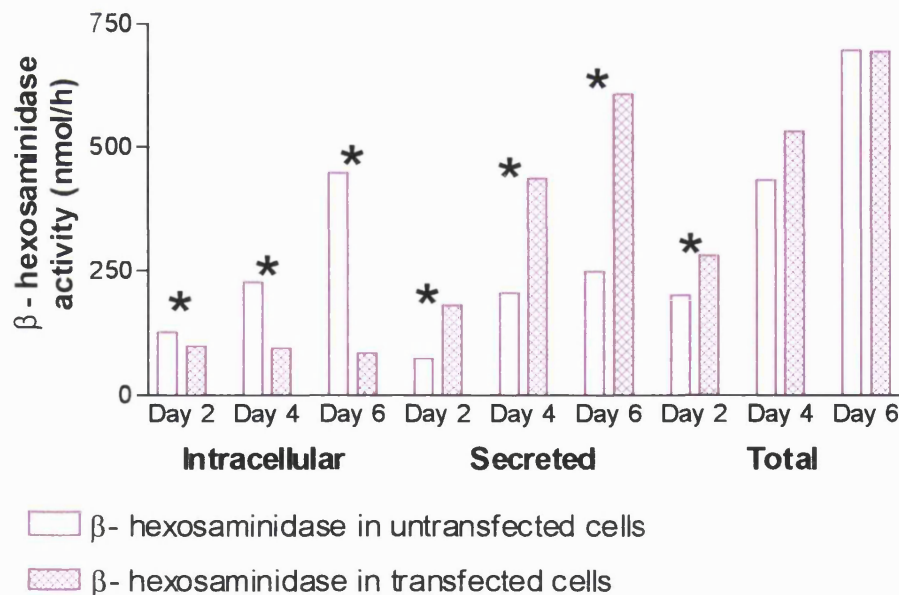
Figure 5.10 Transfection of Fabry fibroblasts with LID complexes containing α- L-fucosidase cDNA.

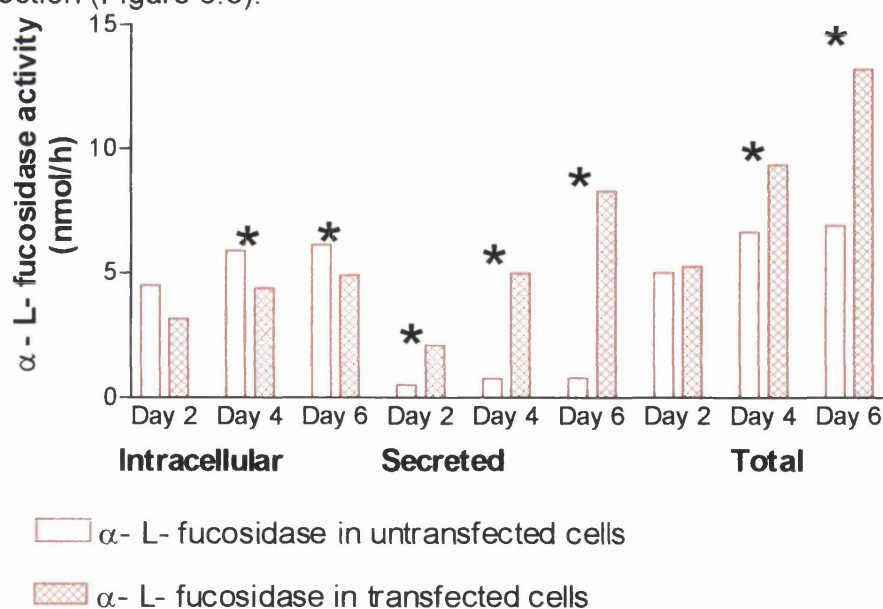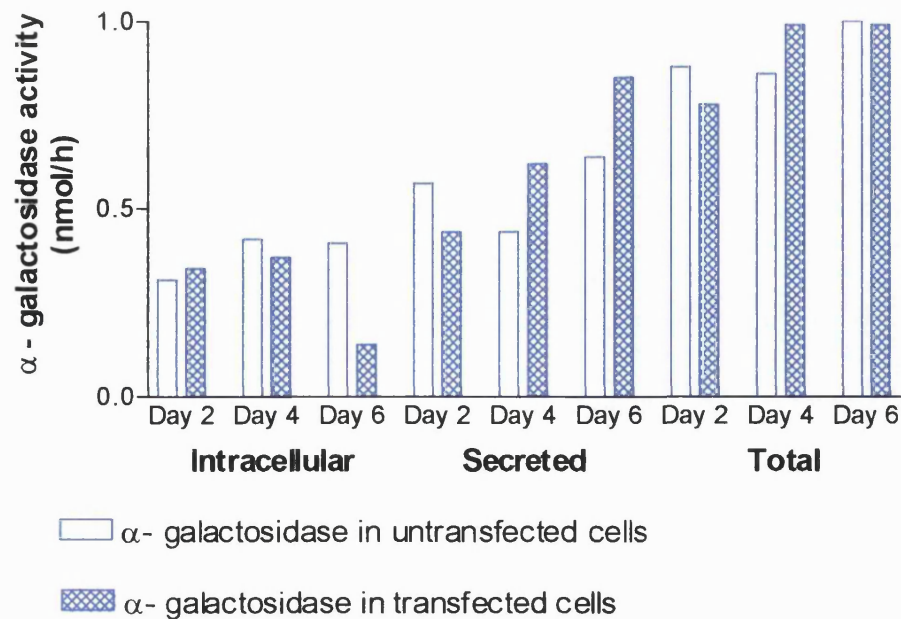Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

Since Fabry fibroblasts are deficient in α- galactosidase A activity they had a very low activity of this enzyme and showed no significant changes as a result of transfection with α- L- fucosidase cDNA. On the other hand, the intracellular β- hexosaminidase activity decreased after transfection and the extracellular activity increased. There was no difference between the effect of transfection with LID complexes containing α- L- fucosidase cDNA on the normal endogenous lysosomal enzymes of Fabry, normal and fucosidosis fibroblasts.

5.2.4. Effect of Lipofectin, Integrin- targeting peptide and DNA on endogenous α- L- fucosidase, α- galactosidase and β- hexosaminidase in normal fibroblasts

Normal fibroblasts were transfected with Lipofectin alone, plasmid containing α- L- fucosidase cDNA alone, integrin- targeting peptide alone, Lipofectin and DNA, Lipofectin and integrin- targeting peptide, integrin- targeting peptide and DNA and LID. The α- L- fucosidase, α- galactosidase and β- hexosaminidase activities of the intracellular and secreted fractions were determined 6 days after transfection.

Small decreases in intracellular α- L- fucosidase α- galactosidase and β- hexosaminidase activity and increases in their extracellular activity were observed in all mock transfections where α- L- fucosidase cDNA was present (data not shown). However, in the absence of cDNA the treatment of fibroblasts with other components of the LID complex had no effect on the intra- and extracellular lysosomal enzyme activities. Hence, these studies indicated a possible effect of the introduction of foreign DNA on the physiology of the cell.

5.2.5. Characterisation of secreted transgenic α- L- fucosidase

After transfection with LID complexes containing cDNA for a lysosomal enzyme the newly synthesised exogenous lysosomal enzyme appears to be secreted from cells. To determine if the secreted enzyme had passed through the normal secretory route via the endoplasmic reticulum and Golgi apparatus, its glycosylation was assessed by chromatography on concanavalin A-Sepharose. Fucosidosis patient fibroblasts were transfected with LID

complexes containing α- L- fucosidase cDNA and placed in OptiMEM serum-free medium. At the same time a flask of normal cells was transferred to OptiMEM medium. After 6 days the medium was removed from both flasks and concentrated by centrifugal ultrafiltration using Centriplus concentrators. The concentrated samples were applied to concanavalin A- Sepharose. All of the α- L- fucosidase activity in the culture medium of both normal fibroblasts and transfected fucosidosis fibroblasts bound to concanavalin A- Sepharose. This demonstrated that the secreted α- L- fucosidase was glycosylated.

5.3. Transfection of cells with α- galactosidase A cDNA

Having evaluated the LID system for α- L- fucosidase and fucosidosis the use of the system for α- galactosidase A and Fabry disease was investigated in the same way. The α- galactosidase A cDNA and Fabry disease fibroblasts used in this study had been fully characterised previously.

5.3.1. Transfection of Fabry fibroblasts with LID complexes containing α- galactosidase A cDNA

Intra- and extracellular activity of transgene in transfected cells

Fabry fibroblasts were transfected with LID complexes containing α-galactosidase A cDNA and the intracellular and extracellular α- galactosidase activity was determined 2, 4 and 6 days after transfection (Figure 5.11).
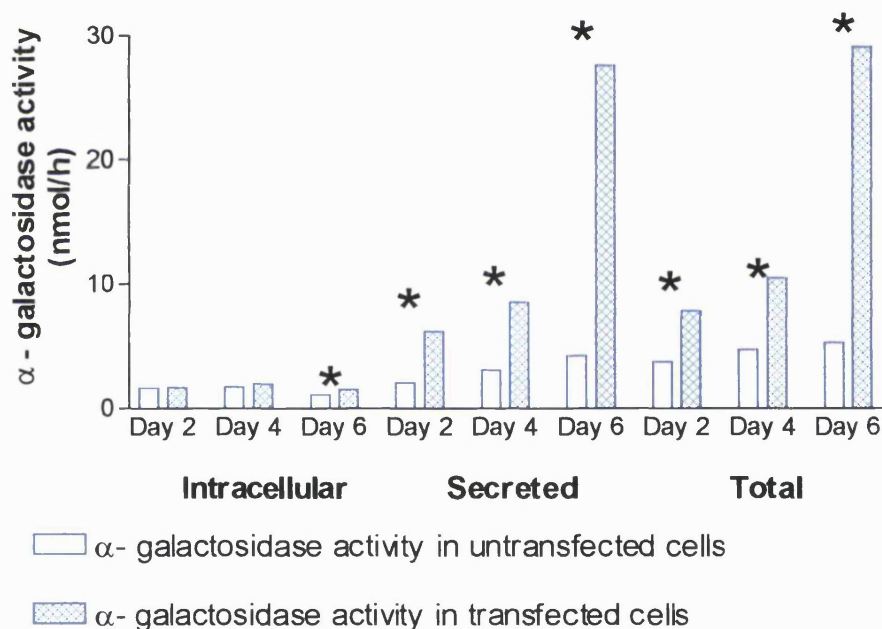
Figure 5.11 Transfection of Fabry fibroblasts with LID complexes containing α-galactosidase A cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

The intracellular α- galactosidase activity increased after transfection and was significantly greater than the activity in untransfected cells at day 6 The extracellular α- galactosidase activity increased after transfection. The total α- galactosidase activity at day 6 was about 5 times more than the levels in untransfected Fabry fibroblasts. In comparison, transfection of fucosidosis fibroblasts with LID- containing α- L- fucosidase gave a total α- L- fucosidase activity 7 times that in untransfected fucosidosis fibroblasts at day 6.

Transfection of Fabry fibroblasts with LID complexes containing α-galactosidase A demonstrated the same trend observed when fucosidosis fibroblasts were transfected with LID complexes containing α- L- fucosidase. Thus when lysosomal storage disease fibroblasts are transfected with cDNA for the appropriate lysosomal enzyme the production of the enzyme increases to levels that would be expected to correct the fibroblasts. However, this increase is mainly in the secreted activity, suggesting that the transgene product is not targeted correctly to the lysosomes. This could be of therapeutic value if the secreted enzyme is taken up by the mannose- 6- phosphate receptor on the plasma membrane of deficient cells and targeted to their lysosomes.

Intra- and extracellular activities of native endogenous lysosomal enzymes in transfected cells

Fabry fibroblasts were transfected with LID complexes containing α-galactosidase A cDNA and the intra- and extracellular activities of α- L-fucosidase and β- hexosaminidase were determined 2, 4 and 6 days after transfection (Figure 5.12 and 5.13).

Figure 5.12 Transfection of Fabry fibroblasts with LID complex containing α-galactosidase A cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.
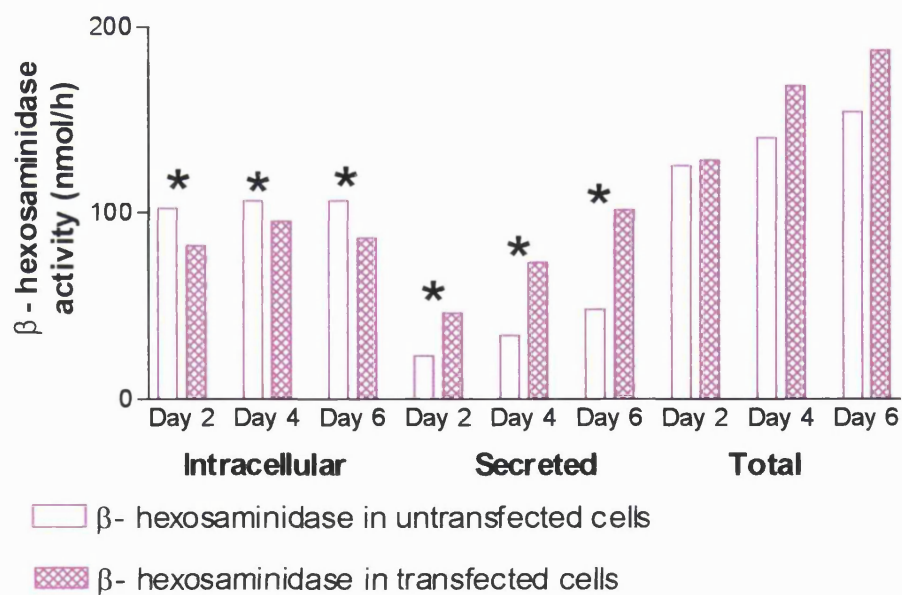
Figure 5.13 Transfection of Fabry fibroblasts with LID complexes containing α-galactosidase A cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

There was no significant change in the total α- L- fucosidase activity after transfection but there was a significant increase in extracellular α- L- fucosidase and a concomitant significant decrease of intracellular α- L- fucosidase. The percentage of total α- L- fucosidase which was secreted increased after transfection. Similar significant changes in the distribution of β- hexosaminidase were also observed. The same change in distribution of endogenous non- deficient lysosomal enzymes was observed when fucosidosis fibroblasts were transfected with LID complexes containing α- L- fucosidase cDNA (Figures 5.3 and 5.4).

5.3.2. Transfection of normal fibroblasts with LID complexes containing α- galactosidase A cDNA

Intra- and extracellular activity of transgene in transfected cells

Normal fibroblasts were transfected with LID complexes containing α- galactosidase A cDNA and the intracellular and extracellular α- galactosidase A activity was determined 2, 4 and 6 days after transfection (Figure 5.14).

Figure 5.14 Transfection of normal fibroblasts with LID complex containing α- galactosidase A cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

Transfection caused significant increases in the total α- galactosidase activity at each time point compared to control. This increase was due to an increase in the extracellular fraction of the α- galactosidase. There were small decreases in the intracellular levels, which were only significant for day 4. At day 6 the total α- galactosidase activity in transfected cells had increased by 50% of the untransfected value. The amount of α- galactosidase activity produced after transfection of Fabry fibroblasts and normal fibroblasts with LID-containing α- galactosidase A cDNA was similar suggesting a comparable transfection efficiency.

Intra- and extracellular activities of native endogenous lysosomal enzymes in transfected cells

Normal fibroblasts were transfected with LID complexes containing α-galactosidase A cDNA. The intracellular and secreted α- L- fucosidase and β-hexosaminidase activities were determined 2, 4 and 6 days after transfection (Figure 5.15 and 5.16).

Figure 5.15 Transfection of normal fibroblasts with LID complex containing α-galactosidase A cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.
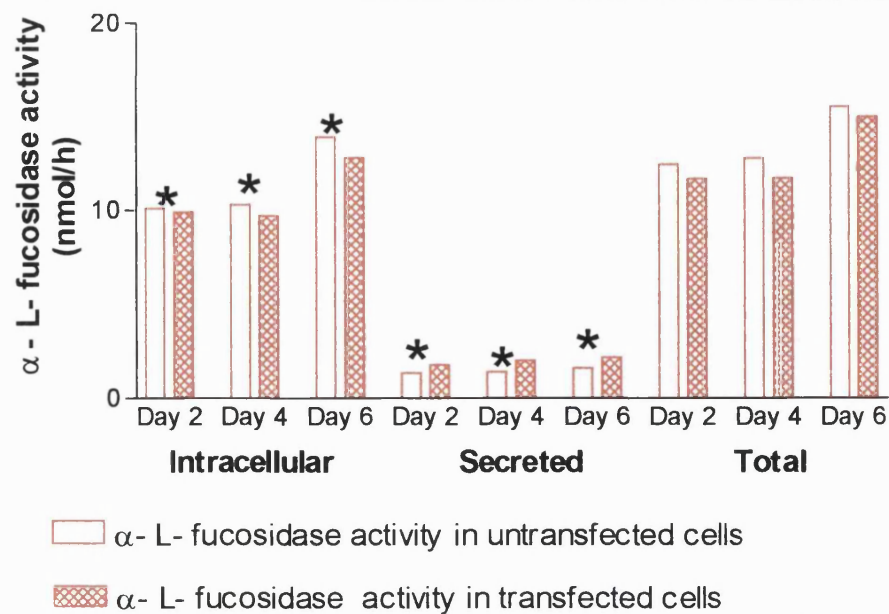
β- hexosaminidase in untransfected cells

β- hexosaminidase in transfected cells
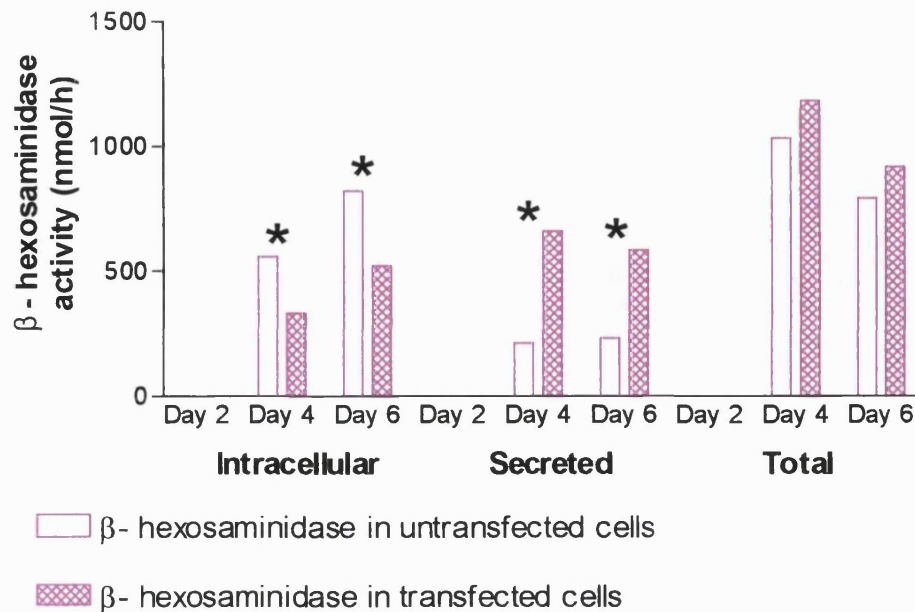
Figure 5.16 Transfection of normal fibroblasts with LID complex containing α-galactosidase A cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

Data not available for day 2

There was a significant increase in secreted α- L- fucosidase and a significant decrease of intracellular α- L- fucosidase after the transfection of normal cells by α- galactosidase A cDNA. The percentage of total α- L-fucosidase which was secreted increased after transfection. A small but significant increase of secreted β- hexosaminidase was also observed with a small significant decrease of intracellular β- hexosaminidase. There were no significant differences in the total β- hexosaminidase activity. The percentage of total β- hexosaminidase activity secreted increased after transfection of normal fibroblasts with LID complexes containing α- galactosidase A cDNA.

5.3.3. Transfection of transformed human umbilical vein endothelial cells (ECV304) with LID complexes containing α- galactosidase A cDNA

Endothelial cells are the main cell type affected in Fabry disease and therefore the main target for therapy. Transfer of α- galactosidase A cDNA into ECV304 cells, a transformed endothelial cell line, was therefore investigated. LID complexes containing peptide 6, which has been reported to be the best integrin- targeting peptide to transfect this cell line, were used (Hart et al., 1997).

Intra- and extracellular activity of transgene in transfected cells

ECV304 cells were transfected with LID complexes containing α- galactosidase A cDNA and the intracellular and extracellular α- galactosidase A activity was determined 2, 4 and 6 days after transfection (Figure 5.17).
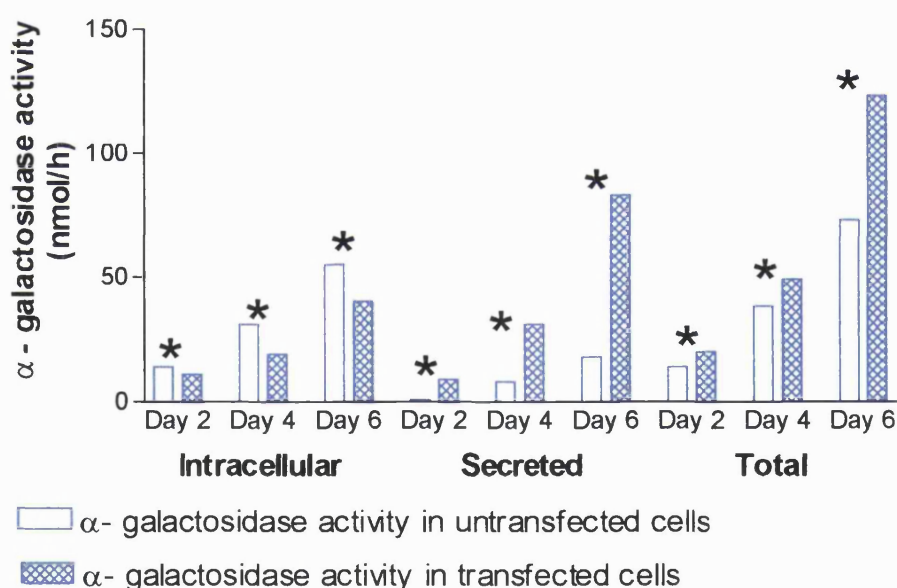
Figure 5.17 Transfection of ECV304 cells with LID complex containing α- galactosidase A cDNA.

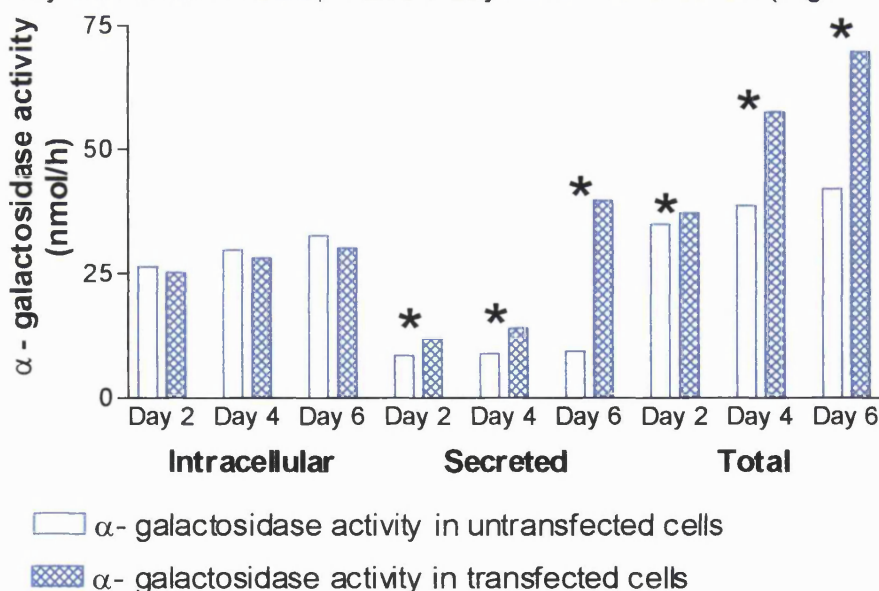Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

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There was a significant increase in total α- galactosidase activity after transfection due to an increase in the secretion of α- galactosidase. At day 6 the total α- galactosidase in transfected ECV304 cells was 65% above that of untransfected ECV304 cells. There were small but significant decreases in the intracellular α- galactosidase levels due to transfection. This is the same pattern as seen for transfection of normal fibroblasts (Section 5.3.2).

Intra- and extracellular activities of native endogenous lysosomal enzymes in transfected cells

ECV304 cells were transfected with LID complexes containing α- galactosidase A cDNA. 2, 4 and 6 days after transfection the intracellular and extracellular α- L- fucosidase and β- hexosaminidase activities were determined (Figure 5.18 and 5.19).

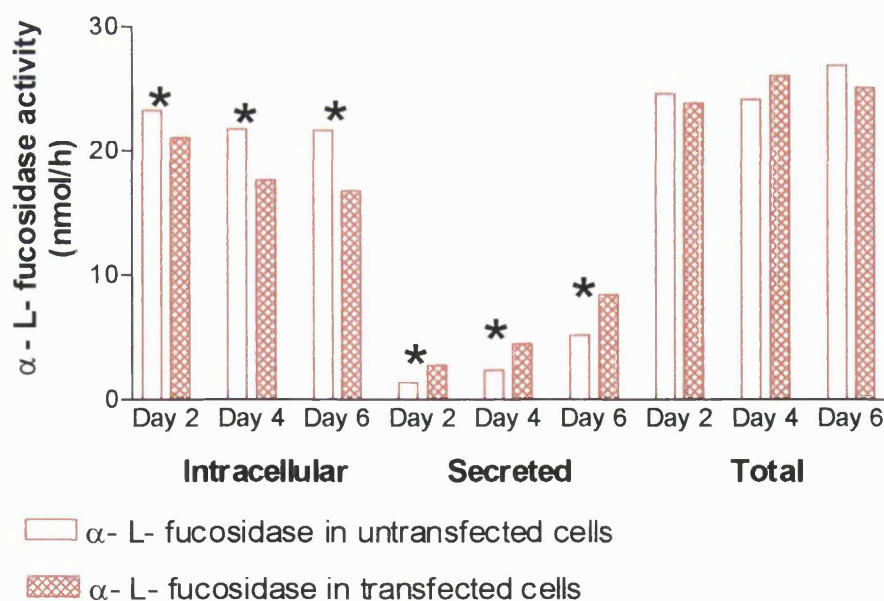**Figure 5.18 Transfection of ECV304 cells with LID complex containing α- galactosidase A cDNA.**

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

Small increases of secreted α- L- fucosidase were seen after transfection of ECV304 cells with LID complexes containing α- galactosidase A cDNA. These differences were significant 4 and 6 days after the transfection.

There were significant small decreases of intracellular α- L- fucosidase. These differences were equivalent so that no significant differences were observed between the total α- L- fucosidase activity of transfected and untransfected cells. The percentage of total α- L- fucosidase which was secreted increased after transfection.
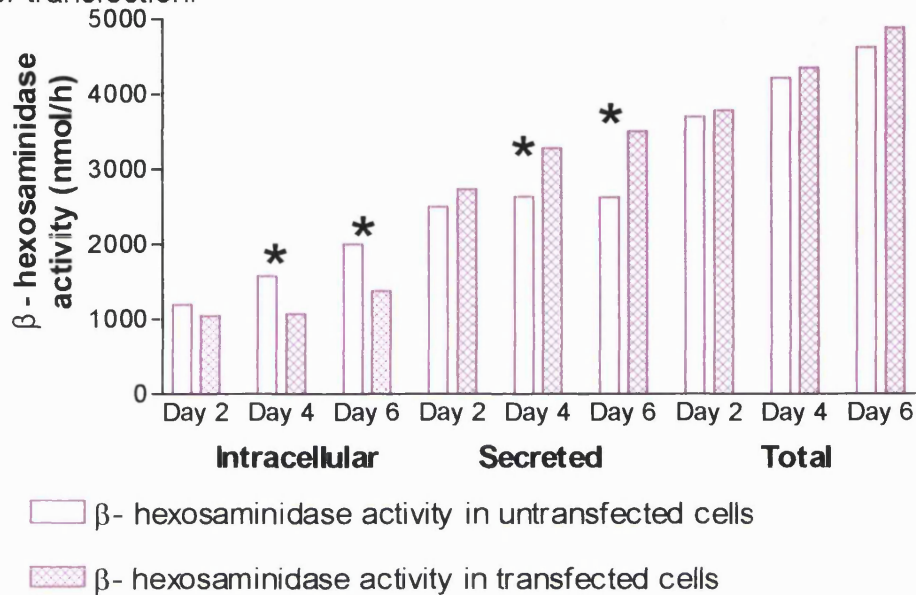
Figure 5.19 Transfection of ECV304 cells with LID complex containing α- galactosidase A cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

A small significant decrease in intracellular β- hexosaminidase after transfection was also observed with a small significant increase in secreted β- hexosaminidase. There were no significant differences in the total β- hexosaminidase activity. The percentage of total β- hexosaminidase activity secreted increased after transfection of ECV304 cells with LID complexes containing α- galactosidase A cDNA.

Transfection of ECV304 cells demonstrated similar results to transfection of normal fibroblasts. This is probably because they both have a full complement of lysosomal enzymes before transfection. Transfection caused a very small decrease of intracellular lysosomal enzymes, a small increase in

secretion of native lysosomal enzymes and a large increase in secretion of the transgene product.

5.4. Transfection of lysosomal storage disease fibroblasts with LID complexes containing the cDNA which encodes a non- lysosomal enzyme

Transfection of cells with LID complexes containing lysosomal enzyme cDNA causes a small decrease in the intracellular, endogenous lysosomal enzymes and a small increase in their secretion. This may be due to abnormal lysosomal enzyme processing or to the disruption of lysosomes. Hence the effect of transfection of cells with LID complexes containing cDNA for the non-mammalian enzyme luciferase was investigated.

Fabry fibroblasts were transfected with LID complexes containing luciferase cDNA. The intracellular and extracellular α- L- fucosidase, α-galactosidase and β- hexosaminidase activities were determined 2, 4 and 6 days after transfection (Figure 5.20, 5.21 and 5.22).

☐ α- galactosidase activity in untransfected cells

▨ α- galactosidase activity in untransfected cells
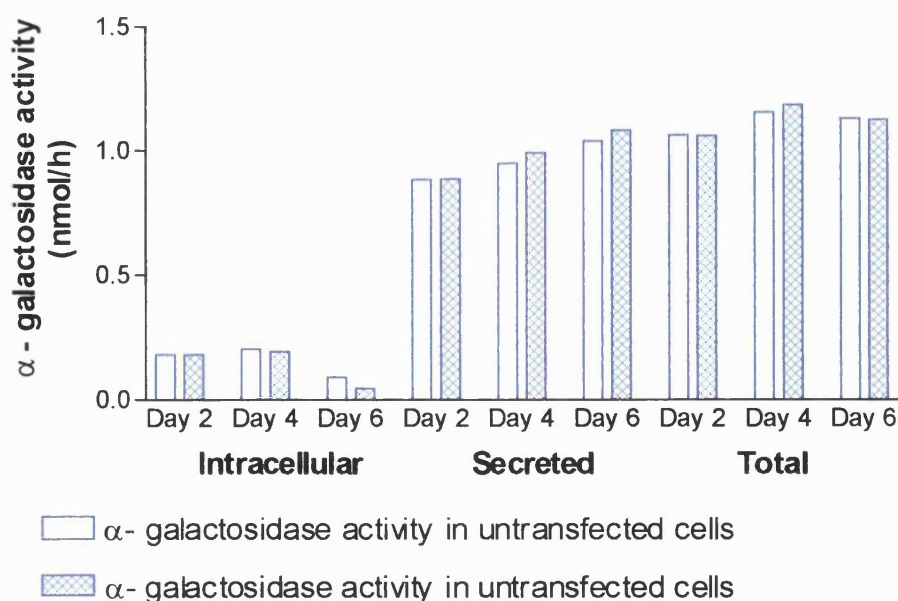
Figure 5.20 Transfection of Fabry fibroblasts with LID complex containing luciferase cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.
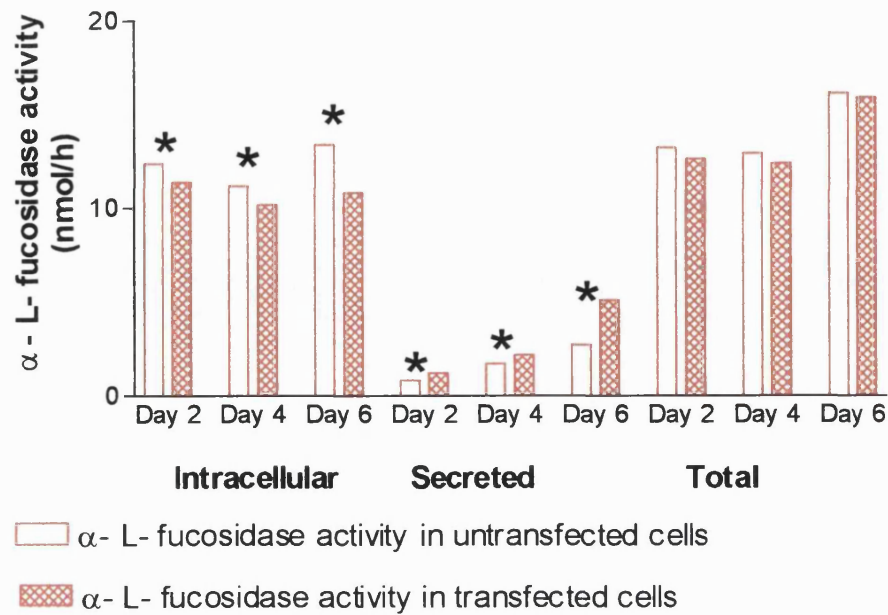
Figure 5.21 Transfection of Fabry fibroblasts with LID complex containing luciferase cDNA.

Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.
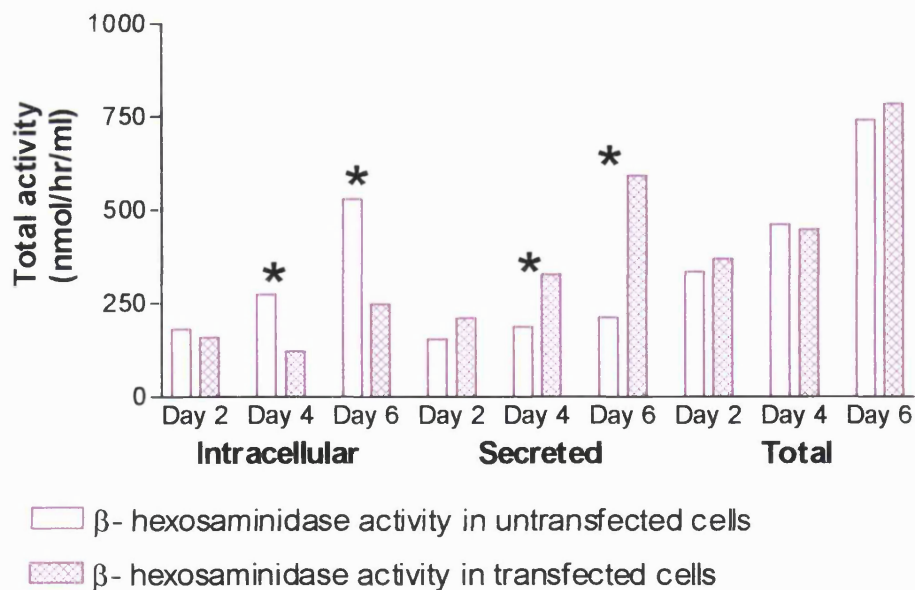
Figure 5.22 Transfection of Fabry fibroblasts with LID complex containing luciferase cDNA: distribution of β- hexosaminidase.
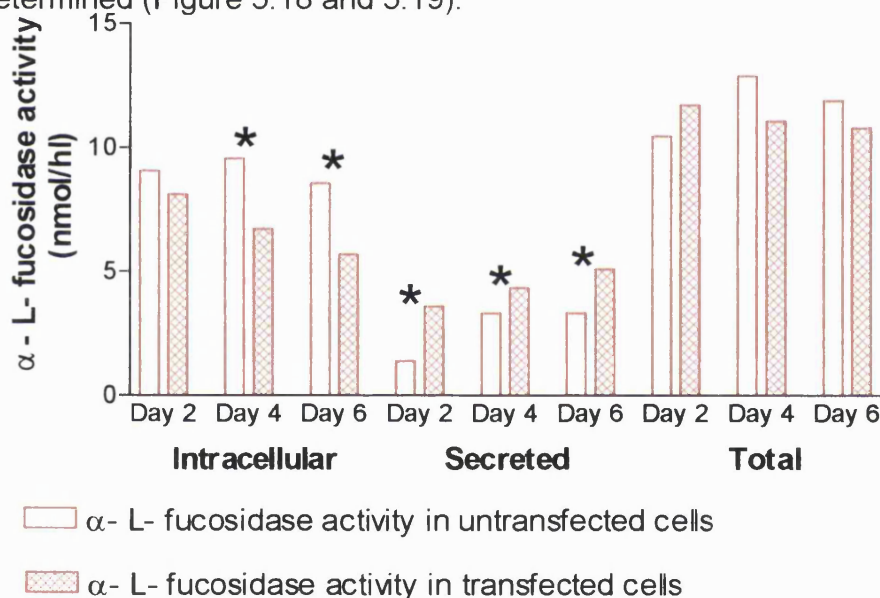
Each bar represents the mean of the activities for three wells of cells which were assayed in triplicate.

* denotes that the enzyme activities of the transfected and untransfected cells were significantly different at a 99% level using a student t- test.

Fabry patient fibroblasts have very low levels of α- galactosidase. There were no significant differences in the intracellular, secreted or total α-galactosidase activity after transfection of Fabry patient fibroblasts with luciferase cDNA. In contrast, small but significant increases of secreted α- L-fucosidase were seen after transfection of Fabry patient fibroblasts with luciferase cDNA. There were corresponding significant, small decreases in the intracellular α- L- fucosidase. A small decrease in intracellular β-hexosaminidase was also observed after transfection, with a concomitant small increase in secreted β- hexosaminidase. There were no significant differences in the total β- hexosaminidase activity. The percentage of total β-hexosaminidase activity secreted increased after transfection of Fabry patient cells with luciferase cDNA.

These results suggest that the changes observed in non- deficient lysosomal enzymes after transfection are the result of transfection and do not depend on the type of enzyme being introduced or its cellular destination. Transfection seems to cause a very small decrease of endogenous intracellular lysosomal enzymic activity and a small increase in secretion. The large amounts of transgene secreted were much greater than these non- specific changes. These results suggest that there is a problem of targeting all lysosomal enzymes synthesised after transfection or that there is disruption of the lysosomal system.

5.5. Discussion

The levels of enzymic activity in cultures varied considerably between experiments because of differences in the rates of growth of different cell lines. Therefore comparisons have been made on the basis of changes in the distribution of enzymic activity for comparable cultures rather than changes in absolute specific activities. This variation between experiments is due to the different cell lines used and differences in protein levels between experiments. To minimise differences between experiments, all experiments on the same cell line transfected with the same LID complex were carried out at the same time. The α- galactosidase activity measured in these experiments was due to both the A and B forms (approximately 95% is in the A form). Therefore, when α-

galactosidase A was deficient α- galactosidase B would contribute to a measurement of α- galactosidase activity.

Transfection of fibroblasts deficient in an enzyme with the LID- containing cDNA for that enzyme produced overall increases in the target enzyme. This increase was 7- fold for α- L- fucosidase in fucosidosis fibroblasts and 5- fold for α- galactosidase in Fabry fibroblasts. These increases would be enough to correct the cells metabolically if the active enzyme had been delivered to lysosomes. However, the increased activity was mainly in the secreted fractions and would only correct cells it was taken up by cell surface mannose- 6- phosphate receptors and targeted to the lysosomes. There were very small increases in intracellular activity which, considering transfection generally depleted the intracellular lysosomal enzymes, suggests that small amounts of transgene products may be targeted to the lysosomes. Transfection did not affect the total synthesis of the non- deficient lysosomal enzymes but did cause a slight increase in the fraction that was secreted.

LID complexes containing α- L- fucosidase and α- galactosidase A cDNA were also used to transfect normal cells (both fibroblasts and ECV304 cells). The increases in the total activity of the transgene were greater in normal cells than in the lysosomal storage disease cells. This suggests that the lysosomal storage disease cells may be transfected less efficiently. Transfection of normal fibroblasts with LID- containing α- L- fucosidase or α- galactosidase A cDNA produced increases of 50% in the α- L- fucosidase or α- galactosidase activities in the cultures after 6 days. ECV304 cells were also efficiently transfected with LID- containing α- galactosidase A cDNA demonstrating that endothelial cells could be successfully targeted with this system for Fabry disease. Transfection of normal fibroblasts and ECV304 cells did not affect the total synthesis of endogenous lysosomal enzymes but caused a slight increase in their secretion. This was not compensated for by the intracellular activity of the transgene, as was seen in deficient cells. The fact that this intracellular increase only occurred in deficient fibroblasts suggested that this phenomenon depended on the original intracellular levels of the enzyme within the cells or that the phenomenon also occurs in normal cells but is not detected. Since transfection of normal cells with LID- containing luciferase cDNA effected endogenous

lysosomal enzymes in the same way it is concluded that transfection and not the specific cDNA used causes the increased secretion. The intracellular decreases were balanced by increased secretion. It may be that transfection made the cells more sensitive to serum withdrawal and that this increased the secretion of lysosomal enzymes. Secretion of lysosomal enzymes could also be part of a stress response to transfection. Secondary lysosomes may have released their contents into the medium or all newly synthesised enzymes may have been secreted.

The secreted overexpressed enzyme bound concanavalin- A Sepharose suggesting that it had been glycosylated in the endoplasmic reticulum. Preliminary experiments were unable to demonstrate uptake of the secreted enzyme into lysosomal storage disease fibroblasts. Re- uptake by other deficient cells would need to be established if this is to be a viable method for gene therapy of lysosomal storage diseases.

5.6. Conclusions

Transfection of fibroblasts from patients with lysosomal storage disease with LID complexes containing cDNA for the appropriate lysosomal enzyme produces large amounts of the enzyme which are secreted from the cells. Although this enzyme would appear to be glycosylated in the ER it is not known if it has the mannose- 6- phosphate recognition marker required for correct targeting to the lysosomes. The LID transfection system had small effects on endogenous lysosomal enzymes. The main questions raised are why the transgene products are secreted and whether they can be recaptured by other deficient cells and correct the disease phenotype.

Discussion

Biochemical and genetic characterisation of material from fucosidosis patients

The biochemical and genetic characterisation of material from fucosidosis patients allowed the identification of suitable cell lines for *in vitro* studies using integrin- mediated gene delivery. It also provided important information for the diagnosis, detection of carriers and prenatal diagnosis in the affected families. Definitive diagnosis is based on enzyme analysis in white blood cells but enzyme analysis can be ambiguous for carrier detection because of the overlap in reference ranges of carriers and normals. The identification of disease-causing mutations is therefore important for carrier detection and confirmation of diagnosis. The strategy for identification of disease- causing mutations used in this study was only partially successful since SSCP analysis was not informative for 3 out of 6 mutant alleles and none of the patients had the previously reported recurrent mutations. Since recurrent mutations are probably limited to specific ethnic groups their detection by restriction enzyme digestion should only be carried out where the ethnic origin of the patient is appropriate. With the advent of automated sequencing and in light of the fact that a sequence change may not necessarily be disease- causing it is prudent to sequence each exon and the intron boundaries for each patient and using SSCP to screen for mutations is therefore irrelevant.

There was no correlation between the residual enzyme activity in fucosidosis patients and their clinical profile. Future studies with natural substrates might establish a correlation. However, it is likely that other factors as well as the specific mutations affect the phenotype such as environmental variables, polymorphisms and the genetic background of the patient. This was demonstrated in two siblings with the same mutation but different phenotypes (Willems et al., 1988b).

Sequencing identified six novel putative disease- causing mutations bringing the total number of known mutations in FUCA1 to 27. Five out of six patients were homozygous and the sixth was a compound heterozygote in whom only one putative disease- causing mutation was found. Each of the patients had

unique mutations with no common mutations or hot spots found. In patient M.C.
a polymorphism was identified which was previously thought to be a disease-
causing mutation. This polymorphism may modulate α- L- fucosidase activity.

Evaluation of the use of integrin- targeting non- viral vectors for treatment of lysosomal storage disorders

This study was the first attempt to use integrin- targeting LID complexes
to introduce a normal gene into cells containing 2 mutant alleles. It was
anticipated that this would produce over- expression of the enzyme encoded by
the exogenous gene, thereby correcting the enzyme defect.

The protocol that had been developed for transfection of COS7, A375M
and ECV304 cells with LID complexes (Hart et al., 1998) was optimised for the
transfection of fibroblasts. Subsequent transfection of fibroblasts from patients
with a lysosomal storage disease with LID complexes containing the cDNA for
the appropriate enzyme demonstrated an increased expression of the enzyme.
However, the majority of the newly synthesised enzyme was secreted and there
were only extremely small increases in intracellular enzyme activity.
Transfection also affected the distribution of endogenous lysosomal enzymes
between the intracellular and extracellular compartments.

Efficiency of transfection

Initial experiments demonstrated that about 1% of normal fibroblasts
were transfected, as indicated by lac Z expression. This was inefficient
compared to the transfection of COS7, A375M and ECV304 cells using the
same protocol, where greater than 50% of the cells were transfected (Hart et
al., 1998). Extensive optimisation studies improved reporter gene expression to
levels comparable to those achieved in the other cell lines. LID transfection is
more efficient then lipofection (Hart et al., 1998) and compares favourably with
other non- viral gene delivery systems. However, the efficiency of transfection
observed with non- viral gene delivery is generally well below that of viral
systems. Non- viral gene delivery complexes can be inhibited by serum
components binding to the complex and activating immune clearance of the
complexes when administered systemically (Gregoriadis, 1991). Preliminary
studies suggest that this may also be the case with LID complexes, limiting their

use to transfection of depot organs, such as the lung or fibroblast neo-organs. Another potential limitation of this system is that the introduced DNA remains episomal and therefore only provides transient expression of the introduced gene. Therefore high efficiency of transfection and repeated administration would be required. Repeated administration would be difficult using neo- organs therefore the use of a depot organ would be necessary. It is not known if repeated administration would have immunological consequences *in vivo*. With the development of human artificial chromosomes it may become possible to introduce the gene to be expressed in a human artificial chromosome within LID complexes so that plasmid replication and nuclear retention are possible. This would prolong expression from a single administration of complex. The expression from LID complexes for fibroblast implants could be increased by utilising the selectable markers in the introduced plasmids to select transfected cells.

This study also demonstrated that normal fibroblasts were transfected more efficiently than patient fibroblasts. Lysosomal storage probably disrupts the general metabolism of cells, including the interlinked lysosomal and endosomal systems, and this may affect transfection. Storage may also effect the expression of glycoproteins, such as the integrins. FACS analysis using antibodies to integrins would show whether there were different concentrations of integrins being expressed on normal patient cells. Ideally antibodies should be used which only bind to integrins in their active form since it is the number of active integrins that determines the level of transfection. Two-dimensional electrophoresis coupled with immuno- staining might also reveal differences in the protein expression between fibroblasts from patients with lysosomal storage diseases and normal fibroblasts.

Effect of transfection on the distribution of the endogenous and exogenous lysosomal enzymes

Irrespective of what DNA was included in the LID complex, there was a small intracellular decrease of endogenous lysosomal enzyme activity and a concomitant increase in secreted lysosomal enzyme activity. This was found in all cell types and with DNA encoding both lysosomal and non- lysosomal enzymes. Preliminary experiments suggested that this effect was due to the

DNA component of the complex rather than the Lipofectin or integrin- targeting peptide.

The increased lysosomal enzyme activity in the extracellular compartment after transfection probably includes both enzyme expressed from the transgene and also endogenous lysosomal enzyme activities. The recombinant transgene product is newly synthesised enzyme which is probably secreted before it reaches the lysosome. The endogenous lysosomal enzyme activity that is secreted may originate from the misrouting of newly synthesised enzymes or from the release of mature intralysosomal enzyme. Therefore, transfection appears to cause a general defect in the processing and transport of newly synthesised lysosomal enzymes, including the enzyme expressed from the exogenous cDNA. There are several possible explanations for this misrouting of newly synthesised lysosomal enzymes.

Secretion of over- expressed recombinant lysosomal enzymes is common but this is always accompanied by a large increase in the intracellular activity of the enzyme. Recombinant enzyme, which has been secreted due to over- expression, has been found to have heterogenous glycosylation with a majority bearing high mannose glycans suggesting lack of processing in the Golgi apparatus (Matsuura et al., 1998). It was proposed that this might be due to the relative inefficiency of the glycosylation processing machinery, particularly the transferases for galactose and sialic acid in the Golgi network. Over-expression of lysosomal proteases in cancer has been demonstrated to produce precursors with complex type oligosaccharides rather than the normal high- mannose type resulting in increased secretion (Braulke 1996). In this study, the secreted enzyme was found to bind to the lectin, concanavalin A, demonstrating that it was glycosylated. Further studies are required to determine the exact nature of the glycans on the secreted enzyme. Western blotting of secreted enzyme from untransfected and transfected cells after treatment with various endoglycosidases would determine if there were any differences in glycosylation or protein processing. However, it is unlikely that the moderate increases of enzyme activity observed in this study would cause over-saturation of the processing machinery to the extent that none of the enzyme is correctly glycosylated and targeted.

A study by Akli et al. (1996) examined adenoviral- mediated gene transfer of hexosaminidase α chain into Tay- Sachs disease fibroblasts. Adenovirus exploits integrins as a means of entering the cell. Although the enzyme deficiency was corrected and enzyme activity was secreted as judged by an assay with an artificial substrate, only 40-84% of normal intracellular enzyme activity and no secreted enzyme activity were detected with a natural substrate. Western blotting of normal cells detected the 54kDa hexosaminidase α- chain but in infected cells there were also two larger precursors (Akli et al., 1996). It was proposed that these precursors are active with the artificial substrate explaining the discrepancy in results. The recombinant enzyme was qualitatively different from the endogenous enzyme. Therefore, it is possible that recombinant enzyme which was over- expressed did not mature to active hexosaminidase A but was secreted as a precursor or other intermediate. Similar discrepancies were found in adenoviral transfer to correct glycogen storage in fibroblasts from patients with glycogen storage disease type 2 (Nicolino et al., 1998). This raises the possibility that the secreted enzyme in this study is not the mature form and this would need to be investigated further.

It has also been reported that over- expression of recombinant lysosomal enzymes in cells causes aggregation of the newly synthesised enzyme when it reaches the more acidic environment of the trans- Golgi network (Ioannou et al., 1992). Most of the aggregated enzyme is secreted since the mannose- 6- phosphate is not accessible to the receptors in the Golgi. It is possible that in this study the rate of synthesis of the enzyme caused aggregation and therefore secretion. However, the absence of a large increase in intracellular activity argues against this (Matsuura et al., 1998).

Another explanation for the secretion of enzyme after LID transfection is that the secreted enzyme has not acquired the mannose- 6- phosphate lysosomal marker. This question could be answered by determining if the secreted enzyme binds to a mannose- 6- phosphate receptor. Experiments were carried out to see if the secreted enzyme could be taken up by enzyme-deficient cells in the presence and absence of added mannose- 6- phosphate, which would indirectly demonstrate the presence of mannose- 6- phosphate. However, it was not possible to demonstrate any uptake even for enzyme secreted by normal cells. The inability to demonstrate uptake was due to the

low levels of activity used. Concentration of larger amounts of secreted activity is necessary. The Q/R281 polymorphism could be used to determine if all the secreted enzyme was transgenic in origin due to the different DNA sequence and the production of different electrophoretic isoforms. The development of a DNA plasmid expressing a green fluorescent protein/ α- L- fucosidase fusion protein would provide a visual marker of the processing of the introduced gene allowing investigation of the processing of the recombinant protein.

The secretion of the recombinant enzyme in the present study could be due to aberrant glycosylation, incomplete maturation, aggregation or failure to acquire the mannose- 6- phosphate recognition marker. However, the level of synthesis of recombinant enzyme in this study seems unlikely to saturate any of these processing steps and these possibilities would not explain the effects of transfection on the endogenous lysosomal enzymes, which are not being over-expressed.

It is possible that the secretion of newly synthesised lysosomal enzymes is a stress response. Commonly used methods of transient transfection have been demonstrated to induce cellular stress responses (Coonrod et al., 1997) which can depend on the transfection time in serum- free medium (Andrews et al 1997). The use of serum-free medium during and after LID transfection may have caused a stress- response in the fibroblasts. The stress response may cause secretion of lysosomal enzymes, particularly where over- expression is occurring. However, in preliminary experiments cells were placed in normal growth medium immediately after transfection and the level of intracellular activity was not different from when serum- free medium was used.

Another possible reason for the misrouting of newly synthesised lysosomal enzymes is that integrin- mediated signalling is being activated and affecting lysosomal enzyme transport. Adenoviral internalisation is also mediated by integrins (Croyle et al., 1998) and it is blocked by the phosphatidylinositol- 3 kinase (PI3K) inhibitor, wortmannin. PI3K activity has been implicated in protein trafficking and phagocytotic and endocytotic events (Ireton et al., 1996; Li et al., 1998). It is thought that different pathways exist for different ligands in receptor- mediated endocytosis but they probably feed into a common signalling pathway for endocytosis. PI3K activation is also thought to be involved in the export of mannose- 6- phosphate receptors (MPR) from the

trans- Golgi network to clathrin coated vesicles necessary for the insulin- like growth factor (IGF)- induced redistribution of MPR300 to the cell surface (Korner and Braulke, 1996, Gaffet et al., 1997). The MPR300 also acts as an IGFII receptor. This receptor redistribution increases the uptake of lysosomal enzymes but the sorting of newly synthesised enzymes in fibroblasts is not impaired (Braulke, 1996). Integrin- mediated transfer probably does not cause a defect in lysosomal enzyme sorting via a signalling pathway since it seems to be the DNA in the complex that mediates this non- specific transfection effect. However the complexity of these systems highlights the need for further study into the mechanisms of gene- transfer systems.

Another aspect of LID- transfection which could possibly have deleterious consequences in fibroblasts from patients with lysosomal storage diseases is the chemical effect of transfection agents such as Lipofectin. It has been proposed that Lipofectin aids endosomal escape of complexes (Farhood et al., 1995; Xu and Szoka, 1996; Hart et al., 1998). In this process the liposomes mix with lipids in the vesicle membrane. Therefore, cationic lipids will be introduced into the endosomal membrane and consequently to other membranes in the cell. This could lead to damaging ion pair formation between the cationic lipids and cellular anionic lipids, such as cardiolipin in the mitochondrial membrane (Felgner et al., 1994; Felgner et al., 1997). Changing the composition of membranes may also affect receptors. Purified integrins placed in liposomes have different ligand specificities depending on the lipids in the membrane (Conforti et al., 1990).

The secretion of activity after LID- transfection could be of therapeutic value if the activity was taken up by other cells via the mannose- 6- phosphate receptors on the plasma membrane. However, preliminary experiments suggest that this is not the case. Large amounts of secreted enzyme remain in the medium with no significant increase of intracellular enzyme activity over 6 days suggesting that the secreted activity is not being extensively recaptured by cells. However, it is interesting to note that there may be some degree of targeting or recapture of recombinant enzymes in deficient cells, but not in normal cells. In cells that are deficient for the enzyme being transfected there is a small increase in intracellular enzymic activity whereas in cells with normal levels of the transfected enzyme there is a small decrease in intracellular

enzymic activity. The decrease in normal cells is of the same magnitude as the decreases seen in other endogenous lysosomal enzymes as a non- specific consequence of transfection. If addition of mannose- 6- phosphate to the media blocked the increase in intracellular enzyme in these experiments it would demonstrate that enzyme was being taken up from the medium.

Evaluation of gene therapy methods for lysosomal storage diseases

In this study the total recombinant enzyme activity was up to 70% of normal and the transfected cells secreted up to 11 times more enzyme than normal fibroblasts. In most other studies in which fibroblasts from patients with lysosomal storage diseases were transfected only the intracellular enzyme activity was measured and transgene expression up to 300% of normal levels has been recorded (Fink et al., 1990; Rommerskirch et al., 1991; Peters et al., 1991; Ohashi et al., 1993). A study using adenoviral vectors to infect Tay-Sachs fibroblasts demonstrated 100% of normal enzyme activity intracellularly and 25 times normal levels of secreted enzyme (Akli et al., 1996). Therefore, LID transfection has demonstrated surprising efficiency for a non- viral vector system but does not give the best long- term production of secreted recombinant enzyme compared to viral gene transfer. In some cases viral vectors also have the advantage of long term expression. To provide a good alternative to viral gene therapy for these diseases long term expression after LID- transfection would be necessary. The two possibilities for this would be through the development of human artificial chromosomes or the repeated administration of LID- complexes to the lung. Other potential limitations in the use of LID transfection for treatment of lysosomal storage diseases include the possibility of immunogenicity of both the transgene product and if the complex is administered *in vivo* of the complex components. The proposed mechanism of action of LID complexes suggests that they may rupture lysosomes which could have detrimental effects. There are also the practical aspects of treatment of patients such as large-scale production, stability, toxicity and ease of administration.

In a study using retroviral vectors to transfect fucosidosis fibroblasts expression was up to 30-50 times higher than levels in normal cells, although the normal levels were lower than would be expected (Occhiodoro et al., 1992). Consequently, retroviral gene transfer of α- L- fucosidase into hematopoietic

stem cells was carried out in 6 dogs with fucosidosis (Ferrara et al., 1997). The results were disappointing with very poor engraftment and failure of engraftment in all the dogs analysed. Plasma α- L- fucosidase activities remained within the affected range. The apparent failure of this study is due to difficulties of gene therapy in bone marrow stem cells. These problems are because stem cells are quiescent and retroviral transduction requires cells to be dividing. Therefore, although the retroviral vectors are more efficient than LID complexes in respect of enzyme activity produced in transfected patient fibroblasts, the non- viral vectors and other types of viral vectors may have an advantage in gene transfer to quiescent cells.

Two separate studies on retroviral gene transfer for Fabry disease have been carried out. A retroviral bicistronic vector was developed which expressed both α- galactosidase A and a multiple drug resistance gene (MDR) (Sugimoto et al., 1995). The vincristine- resistant retrovirally transduced clones gave high α- galactosidase A activity. In the second study a retroviral vector was used to transfer the α- galactosidase A gene into patient fibroblasts and immortalised B cell lines from patients with Fabry disease (Medin et al., 1996). The cells were corrected for the metabolic defect and also secreted α- galactosidase A which was shown to be taken up by other uncorrected cells in a mannose- 6- phosphate receptor- dependent manner. Adenovirus- mediated gene transfer into Fabry knockout mice via intravenous injection resulted in elevation of α- galactosidase A to normal levels in all tissues, including liver, lung, kidney, heart, spleen and muscle (Ziegler et al 1999). Concomitant with the increase in enzyme activity there was a significant decrease in the globotriaosylceramide storage in all tissues. The α- galactosidase A activity in plasma was taken up by patient fibroblasts in culture in a mannose- 6- phosphate- dependent manner. However, enzyme activity declined rapidly so that by 12 weeks there was only 10% of the activity seen at day 3. Re-administration of the adenoviral vector was facilitated by transient immunosupression using a monoclonal antibody against CD40 ligand. Therefore, this treatment is very promising for the treatment of Fabry disease where target tissues are mainly amenable to systemic administration of vectors. The adenoviral vector system produces more enzyme activity for a longer period than LID- complex transfection. Since LID complexes would also face problems with the immune system when

injected intravenously it is unlikely that LID complex transfection in its current form would provide a more effective treatment for Fabry disease than adenoviral vectors.

Prospects for treatment of lysosomal storage diseases

There are ongoing clinical trials for the gene therapy of Hurler disease, Hunter disease and Gaucher disease. Only restricted data is available from these trials at present. Two clinical trials are being carried out on retroviral gene therapy for Hurler disease. Fisher has registered a trial in France but there are no reports on its progress. In the U.K. three patients have undergone transplantation with genetically modified autologous bone marrow (Fairbairn et al., 1997, 1999). There were detectable levels of α- L- iduronidase in peripheral blood and bone marrow when transplantation was carried out following myeloablative conditioning. However, after 74 days there was no detectable enzyme activity. Two clinical trials have been registered for Hunter disease. Zachello proposed a trial using adenovirus- mediated gene therapy of patients in Italy but it is not known if this trial has started. Whitley has been carrying out a trial using retroviral vectors in the U.S.A. Three clinical trials of retroviral gene therapy for Gaucher disease are being carried out in the U.S.A. by Barranger, Karlsson and Schuening. Retroviral gene transfer into haematopoietic stem cells of patients within clinical trial is producing evidence of transduction, competitive engraftment of genetically corrected haematopoietic stem cells, expression of the glucocerebrosidase transgene and the suggestion of a clinical response (Barranger, 1999). The difference in the efficacy of retroviral gene therapy for bone marrow stem cells may be due to immunogenicity, disease- specific differences or to differences in the transduction protocols.

In fucosidosis bone marrow transplantation has provided some clinical improvement for dogs and then patients (Taylor et al., 1986a, 1986b, 1987a, 1988, 1989a, 1989b, 1989c, 1992; Vellodi et al., 1995) This led to experiments on bone marrow gene therapy in fucosidosis dogs which produced disappointing results (Ferrera et al., 1997). However, similar protocols have shown promise for Gaucher disease (Barranger, 1999). Therefore, modification of transduction protocols may produce an effective form of therapy. On the other hand, the development of other vector systems to transduce bone marrow

stem cells may overcome the inherent problems of trying to transduce a quiescent population of cells with retroviral vectors.

Clinical trials are also being carried out on patients with lysosomal storage diseases using enzyme replacement therapy and substrate deprivation. A phase II clinical trial for glycogen storage disease type II involves 4 infants less than 1 year old and 3 patients with late onset disease (Van der Ploeg et al., 1999). This trial is in its early stages and although results have not been reported they are promising enough for the trial to continue. Enzyme replacement therapy for Fabry disease using enzyme produced in Chinese hamster ovary cells is also underway (Desnick et al., 1999). 15 patients with classic Fabry disease were enrolled and intravenous enzyme administration decreased the accumulated globotriaosylceramide storage in plasma, liver, heart and kidneys. It appears that enzyme replacement therapy will provide benefit to patients with non- neuropathic lysosomal storage diseases. However, it is unlikely to provide great improvement to symptoms of the central nervous system or joints since these sites are generally inaccessible to enzyme circulating in the vascular system.

Substrate deprivation for glycosphingolipidoses is another promising treatment. N- butyldeoxynojirimycin (NB- DNJ), an inhibitor of glycosphingolipid biosynthesis, is currently undergoing clinical evaluation in type I Gaucher patients in Europe and Israel and in Fabry patients in the U.S.A. (Platt et al., 1999). The outcome of these trials is not yet known although preclinical work in mice was promising with an increase in life expectancy. However, this approach is only likely to produce improvement of patients rather than a complete cure. This approach has not demonstrated any great improvements to central nervous system symptoms or joint problems. However, combination therapy of NB- DNJ and bone marrow transplantation in Sandhoff mice increased life expectancy further than with NB-DNJ alone and also increased the enzyme activity in brain synergistically (Platt et al., 1999). Therefore, it seems likely that combination therapies will now be examined with one therapy providing correction of systemic symptoms and a second therapy to tackle neurological or skeletal symptoms where required. Neurological symptoms could be targeted using bone marrow transplant, bone marrow gene therapy, cell implants which secrete the deficient enzyme or with herpes simplex virus or lentivirus vectors.

At present the transient expression and lower efficiency of LID-complex transfection make it unlikely that it will be more effective than viral vectors or enzyme replacement therapy. However it is a valuable tool for efficient transient expression *in vitro* providing higher transfection efficiencies than lipofection.

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Appendix 1.1

Disease	Defect
1. Sphingolipidoses	
A. Gangliosidoses	
G_{M1}- gangliosidosis	β-galactosidase
G_{M2}- gangliosidoses	
Tay-Sachs	hexosaminidase α subunit
Sandhoff	hexosaminidase β subunit
B1 variant	hexosaminidase α subunit
AB variant	activator protein deficiency
Galactosialidosis	cathepsin A
Saposins A,B,C,D deficiency	prosaposin or saposin
Farber disease	ceramidase
B. Leucodystrophies	
Krabbe	galactocerebrosidase
Metachromatic	arylsulphatase A
	saposin B
C. Visceral storage diseases	
Gaucher	β-glucocerebrosidase
	saposin C
Niemann-Pick types A and B	sphingomyelinase
Fabry	α-galactosidase
2. Glycoprotein Storage Diseases	
Fucosidosis	α-fucosidase
α- Mannosidosis	α-mannosidase
β- Mannosidosis	β-mannosidase
N- acetyl α- galactosaminidase deficiency	N-acetyl α-galactosaminidase
Aspartylglucosaminuria	N-aspartyl β-glucosaminidase

3. Mucopolysaccharidoses

I- Hurler, Hurler- Scheie and Scheie	α-iduronidase
II- Hunter	iduronate- sulphatase
IIIA- Sanfilippo A	heparin sulphamidase
IIIB- Sanfilippo B	N-acetyl α-glucosaminidase
IIIC- Sanfilippo C	Acetyl-CoA: α-glucosamide N-acetyl transferase
IIID- Sanfilippo D	N-acetyl glucosamine-6-sulphate sulphatase
IVA- Morquio A	N-acetyl galactosamine-6-sulphate sulphatase
IVB- Morquio B	β-galactosidase
VI- Maroteaux-Lamy	arylsulphatase B
VII- Sly	β-glucuronidase
IX- Hyaluronidase deficiency	hyaluronidase

4. Mucolipidoses

I- Sialidosis	α-neuraminidase
II- I-cell disease	N-acetyl glucosamine phosphoryl transferase
III- pseudo Hurler polydystrophy	N-acetyl glucosamine phosphoryl transferase
IV	Unknown

5. Other types

Wolman and cholesteryl ester storage disease	acid esterase (acid lipase)
Mucosulphatidosis	multiple sulphatase deficiencies
Pompe (GSD II)	α-1:4-glucosidase
Infantile Batten (CLN1)	Palmityl protein thioesterase
Late infantile Batten (CLN2)	pepstatin-insensitive protease
Juvenile Batten (CLN3)	protein defect unknown

Pycnodysostosis	cathepsin K

6. Transport defects

Sialic acid storage disease	free sialic acid transporter
Cystinosis	cystine transporter
Niemann-Pick disease type C	cholesterol transporter
Cobalamin deficiency type F	cobalamin transporter

Appendix 2.1

Clinical details for fucosidosis patients

The clinical symptoms listed in the table below are those which were found to be most prevalent in a review of 77 patients (Willems et al 1991) these include:- mental retardation (2), neurological deterioration (3), Coarse facies (4), growth retardation (5), recurrent infections (6), kyphoscoliosis (7), dysostosis multiplex (8), angiokeratoma (9), joint contractures (10), seizures (11), visceromegaly (12), hearing loss (13), hernia (14) and loss of visual acuity (15). Symptoms were regarded as being absent if their presence was not clearly indicated in the clinical details provided and in published reports. This could cause a bias toward the under- reporting of symptoms where they might not have been noticed, recorded or investigated.

Patients' initials	Ethnic origin	Current age (yr.)	Onset age and clinical symptoms														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
S.B.	Asian	15	1.5	Y	Y	Y	Y	-	Y	-	N	Y	-	N	Y	-	N
M.B.	Austrian	10	-	Y	Y	Y	-	-	-	-	Y	Y	-	-	-	-	Y
C.L.o	British	47	5	Y	Y	Y	-	-	Y	-	Y	Y	-	-	-	-	Y
C.L.e**	British	7	**	Y	-	-	-	-	-	-	-	-	-	-	Y	-	-
A.M.	Bangladeshi	16d	4	Y	Y	Y	-	Y	Y	-	Y	Y	Y	-	-	-	-
G.S.	Austrian	3d	0.5	Y	Y	Y	-	Y	Y	Y	-	-	Y	Y	-	-	-
D.P.	Korean	22	-	Y	-	-	-	-	-	-	Y	-	-	-	-	-	-
O.B.*	Russian	29	-	-	-	-	-	-	-	-	Y	-	-	-	-	-	-
H.A.	Arabian	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S.D.	Asian	11	2	Y	Y	Y	Y	-	-	-	Y	-	-	-	-	-	-
Z.T.	Turkish	15	-	Y	-	Y	-	-	-	-	-	Y	-	-	Y	-	Y
M.C.	British	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* In some atypical patients subsequent studies revealed that there was no mutation in the gene.

** This patient was diagnosed when asymptomatic after the diagnosis of an older sibling.

1. onset age 2. mental retardation 3. neurological deterioration 4. coarse facies 5. growth retardation 6. recurrent infections 7. kyphoscoliosis 8. dysostosis multiplex 9. angiokeratoma 10. joint contractures 11. seizures 12. visceromegaly 13. hearing loss 14. hernia 15. loss of visual acuity

'-' is unknown, 'Y' is yes, 'N' no and 'd' is the age of death.

Appendix 3.1

Patients' initials	Phenotype	Enzyme activity*	Relative intensity of 50-51kDa band on Western blotting	Q/R281 polymorphic phenotype	Mutation in α-fucosidase gene
S.B.	Severe	0.2 (lymphoblastoid)	86% (lymphoblastoid)	A/A	IVS5+1
M.B.	Severe	0.4 (fibroblast)	72% (fibroblast)	G/G	E113fs
C.Lo.	Mild	0.3 (fibroblast)	-	G/G	L405R
C.Le.	Severe	0.3 (lymphoblastoid)	64% (lymphoblastoid)	A/G	G18X/?
M.M.	Severe	-		A/G	W183X
A.M.	Moderate/ severe	1.0 (fibroblasts)	5% (fibroblast)	A/A	P5R andY330fs
G.S.	Severe	0.3 (fibroblasts)	8% (fibroblast)	A/A	N329Y
D.P.	-	-	-	G/G	W228X
O.B.	Atypical	75 (plasma) [a]	-	A/A	Pseudodeficiency
H.A.	-	0 (leukocytes) [a]	-	A/A	W36G
S.D.	-	0 (leukocytes) [a]	-	A/A	I221fs
Z.T.	-	0.3 (fibroblasts)	-	G/G	V153fs
A.A	-	-	-	A/A	-
M.C.	Atypical	15 (leukocyte) [a] 49 (plasma) [a]	-	-	P5R polymorphism

*Normal ranges: 27-229 nmol/h/mg protein in fibroblasts, 30-253nmol/h/mg protein in lymphoblastoid cells, 30-189 nmol/h/mg protein in leukocytes and 170-500 nmnol/h/ml plasma.

Information in red provided by this study ([a] by another laboratory) and in blue confirmed in this study having been investigated previously.