# Characterisation and Functional Studies of the Lysosome-Associated Membrane Protein (LAMP-1) in Circulation

by

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## Addendum

1. In section 1.2 (page 15-17) the title of Table 1.1 should read "Classes of lysosomal storage disorders".

2. In section 1.2.1 (page 18, line 15), "(Table 1.3)" should be omitted.

3. In section 1.4.1.4 (page 37, line 15), "however it is clear that transport relies on the –G-Y-X-X-∅ motif …", insert ",where ∅ represents a hydrophobic residue." after 'motif'.

4. In section 2.2.4 (page 60, line 14), "antibodies were coupled to Affi-Gel 10 (Bio-Rad, CA, USA)…", insert "The coupling chemistry employed by these agarose supports is based on N-hydroxysuccinimide ester chemistry." after 'USA).'.

5. In section 5.2.4 (page 176, line 5), "The peptide masses of each sample obtained from APAF were searched against all…", insert the following paragraph before 'The peptide masses…':-

   "The method of peptide mass fingerprinting is the creation of peptides from endoproteinase digestion of proteins, in this case digestion by trypsin. The peptide masses are determined by mass spectrometry. Each protein generates unique (hence 'fingerprint') sets of peptide masses, and can thus be matched against a library of theoretical peptide masses generated from protein databases (i.e. SwissProt and TrEMBLE) [Wilkins Williams (1997) *J Theor Biol* 186(1):7-15.]."

6. In Figures 5.3-5.6 (pages 168-175), the Y-axes on the 2D gels figures are labelled "Molecular Weight (kDa)". These should read "Relative Molecular Mass ($M_r$)".

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## CHAPTER FOUR: Development of LAMP-1 Immunoassays and Determination of LAMP-1 Forms

# THESIS SUMMARY

Lysosome-associated membrane protein (LAMP-1), a major glycoprotein present in the lysosomal membrane, constitutes up to 50% of the membrane protein. Two forms of LAMP-1 have been reported. The full-length form, LAMP-1 (+Tail), contains a single membrane spanning domain, a short cytoplasmic tail and a lumenal domain. The truncated form, LAMP-1 (-Tail), contains only the lumenal domain. LAMP-1 is also expressed at the plasma membrane where it is reported to be the major molecule expressing the sialyl-Lewis X antigen. Cell surface LAMP-1 has been shown to interact with E-selectin and galectin and is proposed to function in cell-cell interactions. Soluble LAMP-1 in circulation was found to be elevated in the plasma from approximately 70% of lysosomal storage disorder (LSD) affected individuals and was proposed as a newborn screening marker for the detection of LSD. However, the functional role(s) of LAMP-1 in circulation are unclear. The aim of this study was to characterise and investigate the functional role of soluble LAMP-1 in circulation.

The two forms of LAMP-1 were produced in CHO and HT1080 cells. These were characterised and used to develop two immunoassays that distinguished between the LAMP-1 forms. Plasma from control and LSD affected individuals were assayed to determine which LAMP-1 form predominated. The interaction and aggregation properties of the different forms of LAMP-1 were investigated using the immunoassays as well as by size exclusion chromatography. The results showed that the form of LAMP-1 in plasma varied and only full length LAMP-1 formed aggregates.

Plasma proteins that interact with LAMP-1 were isolated using three LAMP-1 affinity columns made from the LAMP-1 (-Tail) produced by the CHO and HT1080 expression cell lines and the peptide sequence of the LAMP-1 cytoplasmic tail. Proteins were analysed by two-dimensional gel electrophoresis and peptide mass fingerprinting was used to identify these proteins. Transthyretin was shown to interact with the cytoplasmic tail of LAMP-1. Transthyretin exists as a homotetramer plasma protein and as such may play a role in the aggregation of LAMP-1 in circulation. The possible effects of aggregated forms of LAMP-1 on cell-cell interactions will be discussed.

# CANDIDATE'S DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Melissa H.Y. Chang

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***"For with You is the fountain of life;***

***In Your light we see light."***

***Psalm 36: 9***

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 1D; 2D | one dimensional; two dimensional |
| ADP | adenosine diphosphate |
| Amp | ampicillin |
| $Amp^R$ | β-lactamase (ampicillin resistance) gene |
| ATP | adenosine triphosphate |
| BCA | bicinchoninic acid |
| BMT | bone marrow transplantation |
| BSA | bovine serum albumin |
| CV | coefficient of variance |
| cDNA | complementary DNA |
| CHAPS | 3-{(3-cholamidopropyl)-dimethylammonio}-1-propanesulfonate |
| CHO-K1 | Chinese Hamster Ovary $DHFR^-$ cell line |
| cm; mm; μm; nm | centimetre(s); millimetre(s); micrometre(s); nanometre(s) |
| CMV promoter | cytomegalovirus constitutive expression promoter |
| DHFR | dihydrofolate reductase |
| DMEM | Dodecyl Modified Eagles Medium |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| DTT | 1,4-dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| ERT | enzyme replacement therapy |
| $Eu^{3+}$ | europium |
| FCS | foetal calf serum |
| FPLC | fast phase liquid chromatography |
| x g | times gravity force |
| g; mg; μg; ng | gram(s); milligram(s); microgram(s); nanogram(s) |
| G418 | gentimycin |
| HEPES | N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) |
| HRP | horse radish peroxidase |

| | |
|---|---|
| hr(s); min(s); sec | hour(s); minute(s); second(s) |
| HT1080 | human fibrosarcoma cell line |
| kb; bp | kilobase pair(s); base pair(s) |
| kDa | kilo-daltons |
| L; mL; μL | litre(s); millilitre(s); microlitre(s) |
| LAMP | lysosome-associated membrane protein |
| LB | Luria broth |
| LSD | lysosomal storage disorder(s) |
| mA | milli-amperes |
| MCS | multiple cloning site |
| MEM | minumum essential medium |
| M; mM; nM | moles per litre; millimoles per litre; nanomoles per litre |
| mRNA | messenger RNA |
| MW | molecular weight |
| $Neo^R$ | neomycin resistance |
| NP-40 | nonylphenoxy polyethoxy ethanol |
| OD | optical density |
| Ori | origin of replication |
| PANC | pancreatic carcinoma cell line |
| PBS | phosphate buffered saline |
| pI | isoelectric point |
| PMSF | phenylmethylsulfonyl fluoride |
| poly(A) | polyadenylation |
| PVDF | polyvinylidene difluoride |
| RER | rough endoplasmic reticulum |
| RNA | ribonucleic acid |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SF | skin fibroblast |
| SV40 poly(A) | simian virus 40 late polyadenylation signal |
| SV40 promoter | simian virus 40 promoter |
| TCA | trichloroacetic acid |
| Tris | Tris (hydroxymethyl) aminomethane |

| | |
|---|---|
| Tween-20 | polyoxyethylenesorbitan monolaurate |
| U | unit |
| UV | ultraviolet |
| V | volt(s) |
| w/v; v/v | weight per volume; volume per volume |

# CHAPTER ONE: Introduction

## 1.1 Lysosomes

The lysosome was first described by De Duve *et al.* (De Duve *et al.*, 1955) as a cytoplasmic particle associated with acid hydrolases. Today, this historical description forms the basis of the modern day definition of the lysosome. The accepted definition of lysosomes today is that they are vesicular compartments with a high concentration of lysosome-associated membrane proteins (LAMPs), mature dephosphorylated lysosomal enzymes, acid hydrolases, an absence of cation-independent mannose 6-phosphate receptor and an acid pH (Kornfeld and Mellman, 1989). Through uptake studies, lysosomes are now known to be the terminal compartments in the endocytic pathway (Figure 1.1) (Storrie, 1988).

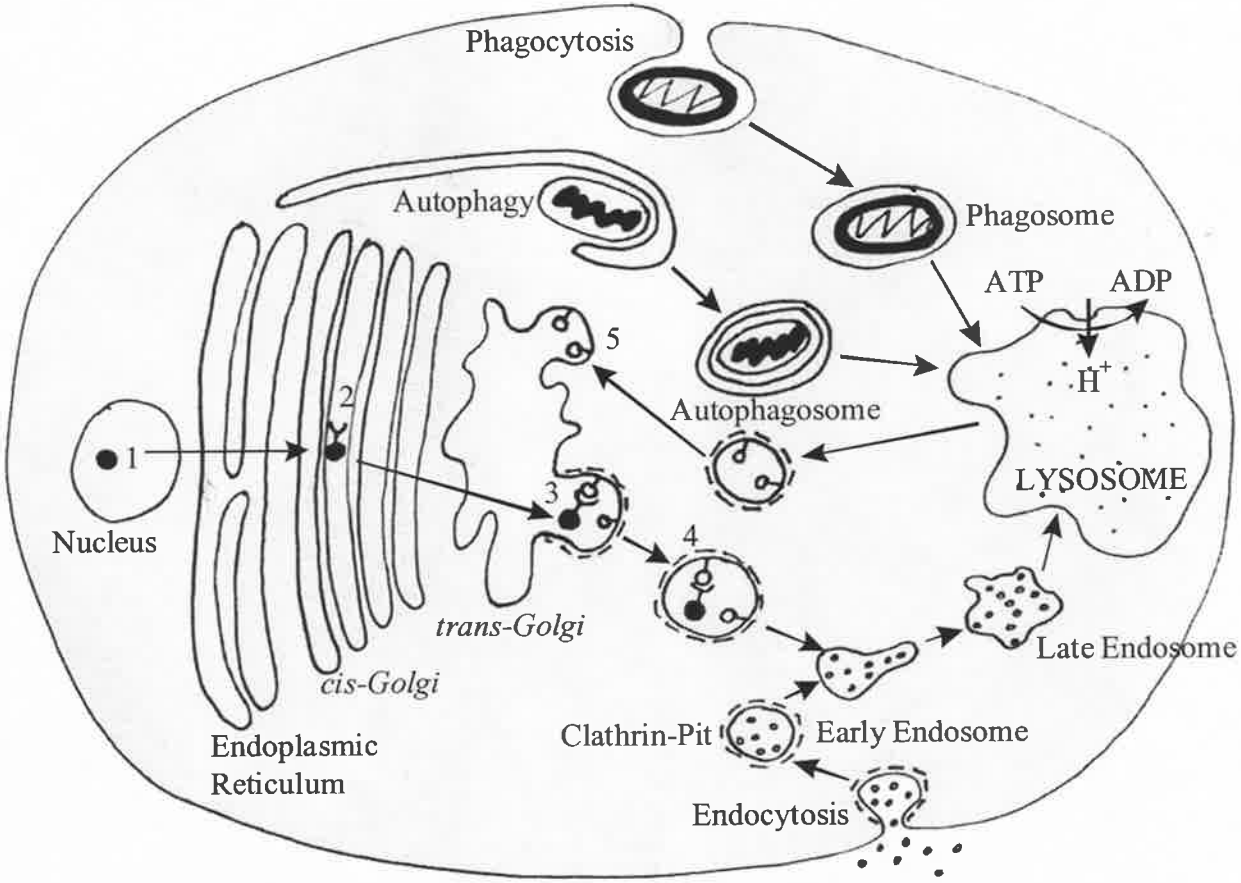
Lysosomes are ubiquitous intracellular organelles and are found within eukaryotic cells and yeast. The lysosomal membrane is a single phospholipid bilayer 7-10 nm thick which prevents other cellular constituents from degradation by containing its contents within the lumen of the lysosome (Winchester, 2001). The proteins that make up the lysosomal membrane, which includes several highly N-glycosylated proteins as well as various transporters, are important for lysosomal function.

### 1.1.1 Endocytic Trafficking

The trafficking of molecules through the endocytic system and eventually to the lysosome is complicated and is not completely understood. A large number of proteins

***Figure 1.1 Schematic representation of the endocytic system***

Macromolecules enter the lysosomes via three major pathways; endocytosis, phagocytosis and autophagy. Extracellular molecules are endocytosed into the cell in clathrin coated pits which are then transported to the lysosome via endosome vesicles. Products of ingestion (e.g. bacterium, food) are taken into the lysosome enclosed in vacuoles called phagosomes. Worn out organelles (e.g. mitochondria) are wrapped in a portion of the rough endoplasmic reticulum to form an autophagosome which is then delivered to the lysosome in a process called autophagy. Also shown in the diagram is the transport of a precursor lysosomal hydrolase from the nucleus (1) to the *cis*-Golgi network where a phosphate is attached to the mannose residue on the enzyme (2). In the *trans*-Golgi network, the mannose-6-phosphate on the enzyme is bound by a mannose-6-phosphate receptor (3). The receptor-ligand complex is endocytosed in clathrin coated pits (4), and travel to the lysosome via the endocytic pathway. The hydrolase dissociates from the receptor in the acidic environment. The receptors are recycled back to the Golgi complex (5).


Phagocytosis
Autophagy
Phagosome
ATP
ADP
$H^+$
5
Autophagosome
2
1
3
4
Nucleus
LYSOSOME
trans-Golgi
cis-Golgi
Late Endosome
Clathrin-Pit
Early Endosome
Endoplasmic
Reticulum
Endocytosis
Cell

are involved in this process, some of which are described below, however many have not been fully characterised. Lysosomes function as the cell's recycling center. Three major pathways lead macromolecules to the lysosome (Figure 1.1); clathrin-mediated endocytosis, phagocytosis and autophagy.

Endocytosis can be achieved by a fluid phase, an adsorptive, or a receptor mediated process. The first two processes of endocytosis are the basis for the take up of molecules into cells *in vitro*. Fluid phase endocytosis concerns any non-diffusable molecules present in the extracellular medium. Adsorptive endocytosis involves the nonspecific binding to plasma membrane of the compound that has to be taken up. Receptor-mediated endocytosis requires the presence in the plasma membrane of specific receptors that bind the molecule destined to be endocytosed (Wattiaux *et al.*, 2000). After internalisation from the plasma membrane, molecules are rapidly delivered to early endosomes, also known as sorting endosomes. Most of the soluble content of sorting endosomes is delivered to lysosomes for degradation, whereas the majority of membrane-bound proteins recycle back to the plasma membrane (van Kerkhof *et al.*, 2001).

Phagocytosis is central to the uptake and degradation of microorganisms as well as damaged or senescent cells (Arora *et al.*, 2000). Two types of cells have phagocytosis ability; "professional" phagocytic cells (macrophages and neutrophils) and nonphagocytic cells (epithelial and fibroblasts). In professional phagocytes, the phagocytic process is initiated by binding of particles to receptors on the plasma membrane, an event that subsequently generates a phagocytic signal (Kwiatkowska and Sobota, 1999). For example, after a macrophage has ingested a microorganism such as a

bacterium, the intracellular phagosome vacuole transforms into an acidic, hydrolase-rich phagolysosome, and the macrophage produces reactive oxygen species and reactive nitrogen species, which can kill microbes or inhibit their activities (Myers and Swanson, 2002). The transition from early phagosome to phagolysosome involves the fusion of endosomes and lysosomes with phagosomes (Jahraus *et al.*, 1998). Studies in fibroblasts of collagen degradation showed that the initial internalisation of collagen is a specific process that is mediated by the adhesive interactions between ligand and collagen receptors (Lee *et al.*, 1996). Arora *et al.* (Arora *et al.*, 2000) found that collagen degradation in fibroblasts closely resembled the vacuolar system in macrophages in that a fusion event occurs between endosome, lysosome and phagosome.

Autophagy is a central mechanism in cellular metabolism that cells use to degrade parts of their cytoplasm and organelles using lysosomal enzymes. The first step in autophagy is segregation of cytoplasm by a membrane cisterna, which forms a double or multiple membrane-bound vacuole called the autophagosome. It is presumed that autophagosomes fuse with endosomes and lysosomes, similar to the pathway taken by phagosomes. In the final step, the contents of autophagosomes are degraded by lysosomal enzymes (Klionsky and Emr, 2000). In mammalian cells, autophagy is activated by amino acid deprivation (Talloczy *et al.*, 2002).

Lysosomal proteins are synthesised on ribosomes associated with the rough endoplasmic reticulum (ER) as inactive precursors. As proteins are trafficked through the ER they undergo post-translational modifications including glycosylation, phosphorylation and proteolysis and are then sorted in the *trans*-Golgi network (TGN). Proteins that are regarded as residents of lysosomes can be directed from the TGN in at least two general

ways, the direct or the indirect way (Hunziker and Geuze, 1996). Newly synthesised lysosomal hydrolases have a mannose 6-phosphate tag that binds to mannose 6-phosphate receptors in the TGN. This receptor-ligand complex is then transported to late endosomes in clathrin-coated pits and then delivered to the lysosome. Alternatively, lysosome membrane proteins may first transit to the cell surface, then be endocytosed into early endosomes and subsequently traffick to lysosomes (Lippincott-Schwartz and Fambrough, 1986). In late endosomes/pre-lysosomes, ligands dissociate from the mannose 6-phosphates due to the acidic pH, and receptors are then recycled back to the TGN.

Protein structure and glycosylation is an important factor in endocytic trafficking. Oligosaccharides on proteins have a multifunctional role in the folding, stability, and targeting of glycoproteins. N-glycans, which are added during translocation of proteins in the endoplasmic reticulum, are used by the chaperones calnexin and calreticulin to retain glycoproteins within the endoplasmic reticulum until they are folded properly (Trombetta and Helenius, 1998). The N-glycans in soluble lysosomal proteins are then altered for recognition by the mannose 6-phosphate receptor (Kornfeld, 1987). The role of O-glycans may act as apical targeting signals for either the transmembrane or soluble form of the neurotrophin receptor (Yeaman *et al.*, 1997). Altschuler *et al.* (Altschuler *et al.*, 2000) studied the trafficking of the mucin-like MUC1 type I transmembrane protein and found that alterations in its O-glycan structure stimulated MUC1 endocytosis through clathrin-coated pits, indicating a role for O-glycan in endocytosis.

A cohort of proteins are involved in endocytic trafficking of molecules, however, only a few of these have been characterised. The GTP-binding protein dynamin is involved in

the formation of clathrin-coated endocytic vesicles at the plasma membrane (McNiven, 1998; Schmid *et al.*, 1998). Dynamin has also been localised at the *trans*-Golgi network (TGN), and its involvement in the budding of clathrin-coated and other vesicles from the TGN has been reported (Jones *et al.*, 1998). Moreover, dynamin is thought to assist in the recycling of mannose 6-phosphate receptors from endosomes to the TGN, by being responsible for the final scission of recycling vesicle buds from endosome tubules (Nicoziani *et al.*, 2000).

Endocytic trafficking events are in part governed by ras-related GTPases of the rab family. Rab proteins are 23-24 kDa in mass and tightly bound to membranes via C-terminal geranylgeranyl modifications (Novick and Zerial, 1997). Transport to late endosomes from the TGN of lumenal lysosome proteins liganded to mannose 6-phosphate receptors is regulated by rab9 (Lombardi *et al.*, 1993). Rab5a has been found to regulate early events in endocytosis as well as trafficking between endosomes and lysosomes (Bucci *et al.*, 1992; Rosenfeld *et al.*, 2001). While trafficking from sorting endosomes to lysosomes is governed by rab7 (Press *et al.*, 1998). Delivery of transferrin receptors from sorting endosomes to perinuclear recycling endosomes has been found to be regulated by Rab11 (Wilcke *et al.*, 2000).

Adaptor proteins (APs) are heterotetrameric complexes that facilitate cargo selection and coated vesicle budding from different membrane compartments. Four APs have been identified in mammals, and are composed of two large chains (α/γ/δ/ε and β1-4), a medium chain (μ1-4) and a small light chain (σ1-4) (Peden *et al.*, 2002; Nishimura *et al.*, 2002). Interaction with APs can involve both tyrosine-based sorting motifs (YXXX∅, where X can be polar residues and ∅ is a hydrophobic residue) as well as di-

leucine/acidic residue-containing motifs found in the cytoplasmic tail of transmembrane cargo (Bonifacino and Dell'Angelica, 1999; Kirchhausen, 1999). AP-2 mediate endocytosis through clathrin-coated pits at the cell surface (Kirchhausen, 1999), and AP-1 direct clathrin-dependent sorting of soluble enzymes from the TGN to the endosomal/lysosomal pathway (Le Borgne and Hoflack, 1998). AP-4 has been suggested to promote the basolateral targeting of a restricted set of proteins (Simmen *et al.*, 2002), and AP-3 directs cargo to lysosomes and specialised secretory lysosome-type compartments, such as melanosomes and platelets (Le Borgne *et al.*, 1998; Andrews, 2000).

### 1.1.2 Functions of the Lysosome

Once molecules enter the lysosome, the hydrolytic enzymes contained within the lysosome act in concert to degrade proteins, lipids, nucleic acids and polysaccharides. The lysosomal membrane also regulates the transport of these macromolecules as well as toxins, drugs, and heavy metals from the cytoplasm into the lysosome, and the release of digested molecules from the lysosomal lumen into the cytoplasm (Zhou *et al.*, 2001).

The acidic lumen of the lysosome results from the action of a vacuolar proton pump (V-type $H^+$-ATPase), found in the lysosomal membrane, that couples hydrolysis of ATP in the presence of magnesium to the translocation of protons (Forgac, 1999; Sun-Wada *et al.*, 2002). A pH of $\leq 5$ is maintained which creates an environment in which the lysosomal enzymes can degrade the macromolecules into their simple components, such as amino acids, monosaccharides, and fatty acids (Storrie, 1988; Kornfeld and Mellman, 1989; Lehninger *et al.*, 1993). These simple monomeric structures are then transported into the cytosol to be recycled into new cellular components or are further catabolised

(Lehninger *et al.*, 1993). Passive diffusion of small molecules with molecular weights of up to 200 kDa is possible as a method of transport of these monomeric digestion products, alternately transport can occur by substrate-specific transporters found within the lysosomal membrane (Winchester, 2001). Transporters that have been identified include those that transport amino acids (Lloyd, 1996), oligopeptides (Zhou *et al.*, 2000) and monosaccharides (Lloyd, 1996; Verheijen *et al.*, 1999). The function of the lysosome is derived from the collective contribution of these transporters together with the various lysosomal luminal and membrane proteins.

In dendritic cells, the major histocompatibility complex class II molecule (MHC II) are targeted to late endosomes and lysosomes where they reside unproductively with internalised antigens. After exposure to microbial products or inflammatory mediators, endocytosis is downregulated, the expression of co-stimulatory molecules is enhanced, and newly formed immunogenic MHC II-peptide complexes are transported to the cell surface (Inaba *et al.*, 2000). MHC II then present the peptide antigen to $CD4^+$ T helper cells to initiate an immune response and antibody production (Raviprakash *et al.*, 2001). In Chow *et al.* (Chow *et al.*, 2002), dendritic cell lysosomes were shown to be able to transfer selective components, such as MHC II, to the plasma membrane by forming tubules that extend from the lysosome to fuse directly with the plasma membrane in a process called lysosome exocytosis.

In many cells, including activated monocytes, lysosomes function as secretory organelles (Page *et al.*, 1998) and release hydrolytic enzymes and other proteins in a regulated manner similar to exocytosis (Rodriguez *et al.*, 1997). Andrei *et al.*, (Andrei *et al.*, 1999) showed that after lysosomes fuse with the plasma membrane, the secretion of

the cytokine, interleukin 1, which is a mediator of inflammation, is driven by exogenous ATP and by hypotonic conditions in the cell medium.

Recently it was reported that specific granules in eosinophils may be lysosomes or lysosome-like organelles (Persson *et al.*, 2002). Eosinophils are cells that participate in allergic inflammatory responses capable of ingesting microbes, upon activation they release their granule content onto the surface of multicellular targets such as parasites or to the extracellular environment (Blom *et al.*, 1992). It is highly probable that the lysosomal characteristics of specific granules play a key role in the host defence functions of eosinophils.

#### *1.1.2.1 Cell Adhesion Molecules*

Adhesion molecules are directly involved in the pairing of many receptors and their ligands and transmit signals that direct specific effector functions in a variety of cellular events. There are at least 6 families of adhesion molecules, cadherins, CD44, integrins, selectins, immunoglobulin superfamily (IgSF), and galectins.

The cadherin transmembrane proteins are $Ca^{2+}$-dependent, and through interaction with catenins, are involved in tissue formation and help maintain tissue architecture in adult animals (Yoshida-Noro *et al.*, 1984; Ozawa *et al.*, 1989).

The major ligand for CD44 is hyaluronan, however fibronectin, collagen and sulfated proteoglycan are also CD44 ligands. CD44 is involved in cell-cell and cell-matrix interactions and has been implicated to act in processes such as lymphocyte homing, hematopoiesis, tumour progression, lymphocyte activation, pattern formation in

embryogenesis, signal transduction and inflammation (Gunthert *et al.*, 1995; Ruiz *et al.*, 1995; Stauder *et al.*, 1995).

Integrins are transmembrane glycoproteins found predominantly on the surface of leukocytes that mediate cell-cell and cell-substratum interactions (Haas and Plow, 1994) in processes such as inflammation, cellular growth, differentiation, junction formation and polarity (Albelda and Buck, 1990). Ligands for integrins include bacterial and viral proteins, coagulation and fibrinolytic factors, complement proteins and cellular counter-receptors, as well as members of the IgSF (Haas and Plow, 1994).

The selectin family of proteins are $Ca^{2+}$-dependent and mediate the initial attachment of flowing leukocytes to the blood vessel wall during the capture and rolling step of the inflammatory adhesion mechanism. Selectins play an important role in the immune system by accumulating leukocytes, a process essential for effective host defence to infection and injury. Ligands for selectins are fucosylated carbohydrates, particularly structures containing the sialyl-Lewis X antigen (section 1.4.1.1) (von Andrian *et al.*, 1993).

The immunoglobulin superfamily (IgSF) is the most abundant family of cell surface molecules, accounting for 50% of leukocyte surface glycoproteins. Members of this family act as ligands for selectin-mediated adhesion of leukocytes, these include Intercellular Adhesion Molecule-1 and -2 (ICAM-1, ICAM-2), and Vascular Cell Adhesion Molecule-1 (VCAM-1) (Shimizu *et al.*, 1992).

Galectins are a family of mammalian proteins that bind to galactose-containing glycoconjugates in a $Ca^{2+}$-independent manner (Barondes *et al.*, 1994). Galectin-3 (formerly called Mac-2 antigen) is reported to be involved in development, oncogenesis, inflammation, and cell-cell interaction (Barondes *et al.*, 1994; Kaltner and Stierstorfer, 1998; Perillo *et al.*, 1998). It is expressed in the extracellular space, at the cell surface and is also secreted from the cells (Sato *et al.*, 1993). Galectin-3 has particular affinity for poly-N-acetyllactosaminoglycans and has been shown to associate with lysosome-associated membrane proteins (LAMP-1 and LAMP-2) (section 1.4.1) on the cell surface (Dong and Hughes, 1997; Feuk-Lagerstedt *et al.*, 1999) and was found in the lens, interacting with the junction-forming intrinsic fibre cell membrane protein MP20 (Gonen *et al.*, 2000).

## 1.2 Lysosomal Storage Disorders

Lysosomal storage disorders (LSD) are a group of approximately 50 distinct genetic deseases, each one resulting from a deficiency of a particular lysosomal enzyme, activator protein, transporter protein, or proteins involved in lysosomal biogenesis. A dysfunction in one of these proteins results in a blockage in the lysosomal system, leading to the storage of undegraded or partially degraded substrates in the lysosome. In 1963, Hers (Hers, 1963) reported that a deficiency in a lysosomal α-glucosidase, which was responsible for the hydrolysis of glycogen, led to the storage of the substrate and was responsible for the disorder known as Pompe disease. From this observation, Hers predicted that acid α-glucosidase deficiency was the first of a number of LSD (Hers, 1963).

A common feature of all LSD is the accumulation of substrates, resulting in the increase in number and size of lysosomes within the cell from approximately 1% to as much as 50% of the total cellular volume (Figure 1.2). In general, LSD affects young children and have a progressive degenerating nature. Symptoms include central nervous system dysfunction, skeletal abnormalities, coarse facial features, organomegaly, and corneal clouding (Neufeld and Meunzer, 1995).

On the basis of the stored substrate or undegraded materials, the LSD can be classified into ten groups. The disorders that fall into each of the ten classes are listed in Table 1.1, together with the deficient enzymes or proteins and the substrates stored in each disorder. Also listed in Table 1.1 are the methods used for the first step in diagnosing (or screening) of these diseases, before more specific tests are carried out on positively screened individuals. All LSD described are autosomal recessive with the exception of Danon disease, Fabry disease and MPS II, which are X-linked.

### 1.2.1 Newborn Screening for LSD

The devastating effects of LSD are obvious and impact on both the patient and families involved. Moreover, LSD generally affects young children who often die before adolescence. With the seriousness of this group of disorders and having a combined incidence as low as 1:5,000 births, the impact of LSD on the health systems is significant.

Newborn screening enabling early detection of LSD has several potential advantages including the ability to offer counseling to parents from an early stage, the option of prenatal diagnosis in subsequent pregnancies, and the continuous monitoring of the

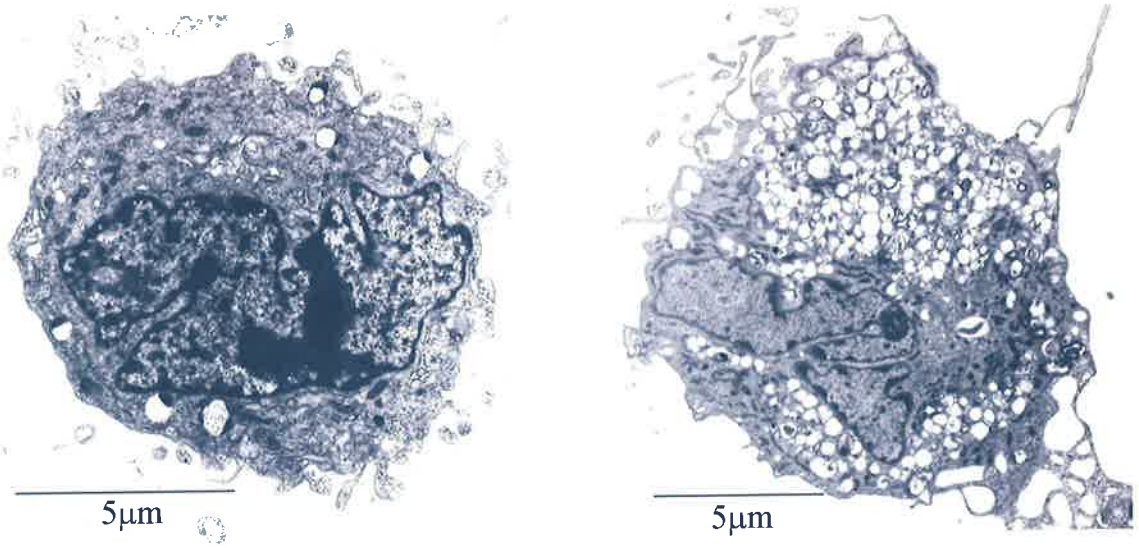



***Figure 1.2 Skin Fibroblast from Unaffected and LSD Affected Individuals***

Electron micrographs of skin fibroblast sections taken from a control individual (left) and an I-Cell LSD patient (right). The enlargement and accumulation of acidic vacuoles, caused by lysosomal substrate storage, is a characteristic biochemical feature of LSD affected individuals.

**Table 1.1 Lysosomal storage disorders and their prevalence in Australia**

| Disorder | Enzyme/Protein Deficiency | Substrates Stored | Screening Test |
|---|---|---|---|
| **1. Mucopolysaccharidoses (MPS)** | | | |
| MPS I (Hurler, Scheie) | α-L-iduronidase | Dermatan sulphate; heparan sulphate | Urine GAGs |
| MPS II (Hunter) | iduronate-2-sulfatase | Dermatan sulphate; heparan sulphate | Urine GAGs |
| MPS IIIA (Sanfilippo A) | glucosamine-N-sulfatase | Heparan sulphate | Urine GAGs |
| MPS IIIB (Sanfilippo B) | α-N-acetylglucosaminidase | Heparan sulphate | Urine GAGs |
| MPS IIIC (Sanfilippo C) | AcCoA: α-GlcN-N-acetyltransferase | Heparan sulphate | Urine GAGs |
| MPS IIID (Sanfilippo D) | N-acetylglucosamine-6-sulfatase | Heparan sulphate | Urine GAGs |
| MPS IVA (Morquio A) | N-acetylgalactosamine-6-sulfatase | Keratan sulphate | Urine GAGs |
| MPS IVB (Morquio B) | β-D-galactosidase | Keratan sulphate | Urine GAGs |
| MPS VI (Maroteaux-Lamy) | N-acetylgalactosamine-4-sulfatase | Dermatan sulphate | Urine GAGs |
| MPS VII (Sly) | β-D-glucuronidase | Dermatan sulphate; heparan Sulphate | Urine GAGs |
| MPS IX | Hyaluronidase | Hyaluronic acid | None |
| **2. Mucolipidoses** | | | |
| Mucolipidosis (Sialidosis I) | neuraminidase | Sialyloligosaccharides | Urine sialic acid |
| Mucolipidosis type II and III (I-Cell) | phosphotransferase | Glycolipids; oligosaccharides | Urine oligosaccharides |
| Mucolipidosis type IIIA | phosphotransferase | Glycolipids; oligosaccharides | Urine oligosaccharides |
| Mucolipidosis type IIIC | transferase-δ-sub-unit | Glycolipids; oligosaccharides | Urine oligosaccharides |
| Mucolipidosis type IV | unknown | Unkown | None |
| **3. Glyco-proteinoses** | | | |
| α-Mannosidosis | acid α-D-mannosidase | α-mannosides | Urine oligosaccharides |
| β-Mannosidosis | β-D-mannosidase | β-mannosides | Urine oligosaccharides |
| Aspartylglucosaminuria | aspartylglucosaminidase | Aspartylglucosamine | Urine oligosaccharides |
| Fucosidosis | α-L-fucosidase | α-fucosides; glycolipids | Urine oligosaccharides |
| Schindler disease | α-N-acetylgalactosaminidase | N-acetylgalactosaminides; Glycolipids | Urine oligosaccharides |
| **4. Peptides** | | | |
| Pycnodysostosis | cathepsin K | Various bone proteins | X-Ray |

**Table 1.1 Continued**

| Disorder | Enzyme/Protein Deficiency | Substrates Stored | Screening Test |
|---|---|---|---|
| **5. Multiple Enzyme Deficiencies** | | | |
| Multiple sulphatase deficiency | multiple sulphatase enzymes | Sulphatides; glycolipids; glycosaminoglycans | Urine glycosaminoglycans |
| Galactosialidosis | neuraminidase; β-galactosidase protective protein | Oligosaccharides; sialic acid | Urine oligosaccharides |
| **6. Monosaccharide, Amino Acids and Monomer** | | | |
| Cobalamin deficiency type F | cobalamin transporter | Cobalamin | Methylmalonc aciduria; Homocystinuria |
| Cystinosis | cystine transporter | Cystine | Renal tubular disease |
| Sialic Acid Storage disorder (Salla Disease) | sialic acid transporter | Sialic acid; glucuronic acid | Urine oligosaccharides |
| **7. Lipid** | | | |
| Niemann-Pick type C | Unknown | Cholesterol; sphingomyelin | Filipin staining of cultured cells |
| Wolman disease | acid lipase | Cholesterol esters | None |
| **8. S-acylated Proteins – Ceroid Lipofuscinosis (CLN, Batten's disease)** | | | |
| CLN I (infantile) | palmitoyl-protein thioesterase | Palmitoylated proteins; saposin A; Saposin D | Histology |
| CLN II (late infantile) | pepstatin insensitive carboxypeptidase | Subunit C mitochondrial ATP Synthase | Histology |
| CLN III (juvenile) | membrane protein | Subunit C mitochondrial ATP Synthase | Histology |
| CLN IV (adult, Kuf's disease) | Unknown | Subunit C mitochondrial ATP Synthase | Histology |
| CLN V (late infantile, Finnish variant) | membrane protein | Subunit C mitochondrial ATP Synthase | Histology |
| CLN VI (late infantile variant) | Unknown | Subunit C mitochondrial ATP Synthase | Histology |
| CLN VII (late infantile variant) | Unknown | Unknown | Histology |
| CLN VIII (progressive epilepsy with Mental retardation) | membrane protein | Subunit C mitochondrial ATP Synthase | Histology |

**Table 1.1 Continued**

| Disorder | Enzyme/Protein Deficiency | Substrates Stored | Screening Test |
|---|---|---|---|
| **9. Glycogen Storage Disorders** | | | |
| Glycogen Storage disease IIA (Pompe Disease) | α-D-glucosidase | Glycogen | ECG characteristic |
| Glycogen Storage disease IIB (Danon Disease) | LAMP-2 | Cytoplasmic debris and glycogen | None |
| **10. Sphingolipidoses** | | | |
| Fabry disease | α-galactosidase A | α-galactosylsphingolipids; oligosaccharides | None |
| Farber disease | acid ceramidase | Ceramide | None |
| Gaucher disease | β-glucosidase | Glucoceramide | None |
| Gaucher disesease[a] | saposin C | Glucoceramide | None |
| GM 1-gangliosidosis | β-galactosidase | GM 1-gangliosides; Oligosaccharides; glycolipids; Keratan sulphate | Urine oligosaccharides |
| GM 2-gangliosidosis | GM2 activator | GM2-gangiosides, glycolipids | None |
| Krabbe disease | galactosylceramidase | Galactoceramides | None |
| Metachromatic Leukodystrophy | galactose-3-sulfatase | Sulphatides | None |
| Metachromatic Leukodystrophy[a] | saposin B | GM 1-gangliosides; Oligosaccharides; glycolipids; Keratan sulfate; sulphatides | None |
| Niemann-Pick disease (A and B) | sphingomyelinase | Sphingomyelin | None |
| Sandhoff disease | β-hexosaminidase (β subunit) | GM 2-gangliosides; Oligosaccharides | None |
| Tay-Sachs disease type 1 | β-hexosaminidase (α subunit) | GM 2-gangliosides; Oligosaccharides | None |
| Tay-Sachs disease type AB | β-hexosaminidase activator protein | GM 2-gangliosides | None |

[a]Variant form of the disease. Table adapted from Wraith (2002) (Wraith, 2002).

child's progress by clinicians (Meikle *et al.*, 1997). The greatest benefit of early diagnosis, and therefore treatment, of LSD will be in the increased efficacy of current and proposed LSD therapies (section 1.2.3). Early diagnosis, before the onset of irreversible damage, is particularly important for those LSD involving the central nervous system and bone pathologies.

If screening for LSD is to be cost effective, a single procedure is required which will detect all LSD. Meikle *et al.* (Meikle *et al.*, 1997) proposed that the concentration of certain lysosomal proteins would be elevated as a result of storage and that these proteins would be suitable diagnostic markers for use in a newborn screening assay to detect all LSD. Using time-resolved fluorescence immunoassays, lysosome-associated membrane proteins, LAMP-1 and LAMP-2 (section 1.4.1), were demonstrated to be potential markers (Meikle *et al.*, 1997; Hua *et al.*, 1998). LAMP levels were found to be elevated in the plasma from approximately 72% of LSD patients, with the LAMP-2 concentration in plasma being four times higher than LAMP-1 (Table 1.3). As a result of the inability of LAMPs to detect all LSD patients, saposins A, B, C and D were evaluated as potential additional markers for LSD. Saposin C was found to be elevated in the plasma from 61% of LSD patients, some of which were not elevated with LAMPs (Chang *et al.*, 2000). Thus the use of LAMP-1 and saposin C as screening markers in a single assay could potentially be useful for the newborn screening of LSD.

Newborn screening for LSD is currently under development in the Lysosomal Diseases Research Unit (Women's and Children's Hospital, SA, Australia). The proposed strategy for the newborn screening of LSD will involve a primary screening assay to detect elevations in LAMP-1 and saposin C. Blood spots from the same Guthrie card of

those identified to be in the high risk population will then undergo a second tier screen using tandem mass spectrophotometry to identify specific storage products for each LSD (Meikle *et al.*, 1999a).

### 1.2.2 Diagnosis of LSD

Except for those cases with a family history of the disease, LSD is brought to attention only after the presentation of clinical symptoms. The diagnosis of LSD is frequently a difficult task, involving a complex range of laboratory assays performed on urine and blood samples, measuring for specific enzyme activities and substrate levels. In some cases tissue cultures are required when a large amount of material is needed. Electron microscopy of tissue biopsies is also used as part of the diagnostic procedure (Kolodny and Fluharty, 1995; Meikle *et al.*, 1997). Even after these tests the precise diagnosis of the specific LSD is difficult due to the overlapping phenotypes that can exist within the disorders (Hopwood, 1991). Moreover, these methods are time consuming, expensive and invasive, which have prompted for the development of better, more rapid and definitive procedures.

Pompe disease is characterised by a deficiency of lysosomal acid α-glucosidase. Currently the clinical diagnosis of this disease involves taking muscle biopsies and cultured fibroblasts and is confirmed by the virtual absence of α-glucosidase activity in infantile onset Pompe, or a marked reduction in enzyme activity in juvenile and adult onset Pompe (Shin *et al.*, 1985; Ausems *et al.*, 1999). However, a simple and less invasive method of diagnosing Pompe has been evaluated where dried blood spots are measured for α-glucosidase activity by using a sensitive immune-capture assay (Umapathysivam *et al.*, 2001). Preliminary assays performed on dried blood spots taken

from newborn and adult controls, Pompe-affected individuals and obligate heterozygotes showed great promise with the assay showing a sensitivity and specificity of 100% for the identification of Pompe-affected individuals. This method of diagnosis may also prove useful for the development of a newborn screening program (section 1.2.1).

Another development in high throughput LSD diagnosis is the use of tandem mass spectrometers for the detection and quantification of storage material in samples taken from individuals, such as plasma or urine (Mills *et al.*, 2002; Whitfield *et al.*, 2002). By using electrospray ionisation-tandem mass spectrometry, Whitfield *et al.* (Whitfield *et al.*, 2002) showed that there was an increased 16:0-glucosylceramide/16:0-lactosylceramide ratio and elevated concentrations of LAMP-1 and saposin C in the plasma of patients with Gaucher disease compared to unaffected individuals. Moreover, Gaucher patients displaying the highest 16:0-glucosylceramide/16:0-lactosylceramide ratios and saposin C and LAMP-1 levels were among the most severely affected in the study group (Whitfield *et al.*, 2002). This indicates that the use of tandem mass spectrometry is not only useful for diagnosis of LSD but may also be useful in the prediction of disease severity and monitoring patients undergoing therapy (section 1.2.3). Currently the quantification of molecules by tandem mass spectrometers are limited due to the lack of internal standards (Whitfield *et al.*, 2000; Whitfield *et al.*, 2001). However, suitable internal standards can be synthesised and the use of these novel internal standards have been proven to be able to quantify globotriaosylceramide, a storage product, in plasma taken from Fabry patients (Mills *et al.*, 2002). Diagnosis of some LSD can take months or even years, with some adult patients going undiagnosed. The recent developments in the identification of protein markers and advancements in

mass spectrometry techniques hold promise for better screening procedures and earlier diagnosis of LSD, which will allow for therapy to be administered at an earlier stage.

### 1.2.3 Treatments for LSD

In recent years treatments for several LSD have become possible. New treatment protocols are being developed with the increased knowledge of the underlying cause of the specific disorders, and the availability of animal models, together with development of new technologies. Animal models are of particular importance for the testing of new therapies, such as bone marrow transplantation, enzyme replacement, and gene transfer (Neufeld and Meunzer, 1995).

The first treatment available for LSD was that for Cystinosis. The majority of Cystinosis patients die because of kidney failure. Cysteamine, a weak base, is readily taken up into the lysosome where it reacts with the storage product, cystine, forming a cystine-cysteamine compound. This compound can then be transported out of the lysosome through another transporter, effectively clearing the lysosome of cystine (Reuser *et al.*, 1994). Patients treated early and adequately with cysteamine have improved renal function and a normal life span, as long as they maintain treatment throughout their entire life (Markello *et al.*, 1993). More recently drug based therapy has been trialed for other LSD. Drugs that slow the rate of formation of accumulating glycolipids are being developed and one of them, OGT-918 (N-butyldeoxynojirimycin), an inhibitor of glycosphingolipid biosynthesis, have been evaluated for the treatment of several LSD including Tay Sachs (Platt *et al.*, 1997), Sandhoff (Jeyakumar *et al.*, 1999) and Gaucher disease (Cox *et al.*, 2000). Prevention of substrate storage in the brain was observed when mice models of Tay-Sachs disease were treated with this compound, showing that

the drug effectively crosses the blood brain barrier (Platt *et al.*, 1997). In Sandhoff disease, mice treated with N-butyldeoxynojirimycin showed delayed onset of symptoms and also increased the life expectancy by 40% (Jeyakumar *et al.*, 1999). In Gaucher patients, 100 mg of OGT-918 administered orally tree times daily for 12 months resulted in improvements in haematologic parameters and the mean liver and spleen volumes were significantly lowered by 12% and 19%, respectively (Cox *et al.*, 2000). However, several side effects exist for OGT-918. In mice OGT-918 have shown to cause a reversible cellular depletion in lymphoid organs (Platt *et al.*, 1997). In Gaucher patients, 2 of the 28 patients experienced a peripheral neuropathy that was reversed with discontinuation of the drug and diarrhoea was the most frequent side effect displayed in 80% of the patients (Cox *et al.*, 2000). The potential toxicity factors of OGT-918 will need to be further investigated to establish whether or not OGT-918 can provide a safe alternative treatment for patients who are unable or unwilling to receive enzyme replacement therapy (ERT).

Bone marrow transplantation (BMT) has been shown to be an effective therapy for several LSD. MPS I patients who have undergone BMT have shown improvement in their somatic features (Neufeld and Meunzer, 1995). Gaucher patients treated with BMT have also had favourable outcomes, resulting in the arrest of neurologic effects of the disease (Beutler and Grabowski, 1995). Factors that affect the outcome of BMT include the type of disorder, donor, and preparative regiment, the degree of clinical involvement, and the age at the time of transplantation (Neufeld and Meunzer, 1995).

De Duve (De Duve, 1964) first suggested that replacement of the defective enzyme with exogenous enzyme might be a successful treatment for LSD. The type 1 Gaucher

disease phenotype is expressed entirely as the result of changes in macrophages, and with no primary central nervous system involvement. This disorder was thus an attractive candidate for enzyme replacement therapy (ERT) and was the first LSD to be successfully treated with macrophage-directed ERT. Normal β-glucosidase was targeted to the mannose-specific receptor found on macrophages, which lead to reduction in organomegaly, haematological recovery and skeletal improvements (Barton *et al.*, 1991; Zimran *et al.*, 1995; Weinreb *et al.*, 2002). Industrial-scale production of human placental enzyme, modified to expose covered N-acetylglucosamine and mannose residues, has made available sufficient material for prolonged clinical administration of large amounts of acid β-glucosidase (Beutler and Grabowski, 1995). ERT is now also available for Fabry disease in some countries (Schiffmann *et al.*, 2001; Eng *et al.*, 2001; Desnick *et al.*, 2002). To date ERT has been the most successful therapeutic approach for LSD. The use of recombinant enzymes purified from expression systems and the availability of numerous animal models has made ERT a possibility for various other LSD. ERT clinical trials are currently being carried out on MPS I, MPS VI (Kakkis *et al.*, 2001; Kakkis, 2002) and Pompe disorders (Amalfitano *et al.*, 2001) with promising results. The current use of ERT is limited to those LSD that do not involve the central nervous system, as the enzymes cannot cross the blood brain barrier.

The positive outcome of ERT in LSD patients gives optimism for gene replacement therapy (GRT) in which a sustained enzyme source would be provided. The application of somatic gene transfer techniques to treat selected inherited metabolic diseases, including several LSD, is currently an area of intense investigation. This treatment aims to replace defective genes, or at least insert a normal gene to allow a cell to make functional proteins (Gieselmann, 1995). LSD involving the central nervous system are

particularly hard to treat due to the difficulty of therapeutic enzymes or drugs crossing the blood brain barrier, however this may be overcome by GRT. The best methods for the targeting of genes into cells is still being assessed. Several trials have been carried out using retroviral-mediated gene transfer to introduce full-length lysosomal enzyme cDNA into cultured LSD affected fibroblasts (Schuchman and Desnick, 1995). Results show that the accumulated substrates were reduced to a normal level in these cells. Moreover, relatively high efficiency of transfer of the human acid β-glucosidase and β-glucuronidase cDNA into the bone marrow of Gaucher and MPS VII mice, respectively, has been accomplished. Adeno-associated viral vector-mediated gene transfer of the α-galactosidase A cDNA was used to treat Fabry mice (Jung *et al.*, 2001). An increase of the α-galactosidase A enzyme was observed in the liver of treated mice and continued to improve up to 6 months post treatment. A 40-60% correction of the stored globotriaosylceramide protein was maintained in the liver and other organs with no signs of toxicity (Jung *et al.*, 2001). Taken together these results showed evidence of sustained long-term expression of the enzymes and clearance of the stored substrates from the spleen and liver. Intravenous injection of the β-glucuronidase gene using adeno-associated virus neonatally into LSD affected mice prevented storage in the brain as well as other organs, proving that the efficacy of therapies depend on early treatment (Daly *et al.*, 1999b; Daly *et al.*, 1999a). Recently Martiniuk *et al.* (Martiniuk *et al.*, 2002) reported on using a method whereby subcellular-sized particles are accelerated to high velocity to carry DNA into cells. This method was used to introduce the α-glucosidase gene into Pompe cell lines and *ex vivo* into peripheral blood cells, resulting in an increase in enzyme activity in all cell types (Martiniuk *et al.*, 2002). The Pompe mouse model was used to further test the efficacy of this method of gene therapy.

Muscle weakness in the hind and forelimbs was reversed, suggesting that particle delivery of genes may be a safe and effective treatment for LSD (Martiniuk *et al.*, 2002).

Positive results from the various drug, ERT and GRT trials have brought about certain optimism for the development of definite treatments for the remaining LSD that currently do not have any specific treatments, and it is anticipated that effective therapies will be available for most LSD patients within the next 5 to 10 years.

## 1.3 Lysosomal Luminal Proteins

The diverse range of proteins contained within the lumen of the lysosome (section 1.1) is responsible for the lysosome's ability to degrade a broad spectrum of macromolecules. These acid hydrolases include proteases, glycosidases, sulphatases, phosphatases and lipases (Hopwood and Brooks, 1997). Approximately 53 distinct lysosomal luminal proteins (Table 1.2) have been characterised and sequenced. Lysosomal luminal proteins also include stabilisation and activator proteins.

## 1.4 Lysosomal Membrane Proteins

The lysosomal membrane plays a vital role in containing the hydrolytic environment required for the degradation of macromolecules by numerous acid hydrolases (section 1.1.2) (Fukuda *et al.*, 1988). It is impermeable to the macromolecules themselves and to intermediate products of catabolism and therefore require the assistance of lysosomal membrane proteins, some of which act as transporters.

Due to difficulties involved in the isolation of functional membrane proteins, only a few of the lysosomal membrane proteins have been sequenced. All lysosomal membrane

proteins are believed to be glycoproteins as those that have been sequenced were found to be heavily glycosylated (Storrie, 1988; Peters and von Figura, 1994). A large number of the unsequenced proteins have been functionally characterised as transporters of small molecules including amino acids, calcium, sulphate, phosphate and monosaccharides (Hopwood and Brooks, 1997). There are approximately 35 different characterised lysosomal membrane proteins (Table 1.2) with sizes ranging from 15 to 200 kDa (Storrie, 1988).

The rat ATP-binding transporter 2 (ABC2) protein was cloned by Zhou *et al.* (Zhou *et al.*, 2001) and found to localise specifically in the lysosome membranes of oligodendrocytes in the rat brain. ABC2 was thus identified as a novel lysosome-associated membrane protein. ABC transporter proteins function specifically by coupling the hydrolysis of ATP to the transmembrane flux of molecles such as ions, sugars or polysaccharides, steroids, phospholipids, amino acids or peptides and proteins as well as of chemotherapeutic drugs (Zhou *et al.*, 2001).

### 1.4.1 LAMP-1 and LAMP-2

Two lysosomal glycoproteins that have been characterised are the lysosome-associated membrane protein-1 and –2 (LAMP-1 and LAMP-2). LAMP-1 and LAMP-2 are type-I membrane proteins and are major constituents of the lysosomal membrane, making up approximately 0.1-0.2% of the total cell proteins. Consequently, LAMP-1 and LAMP-2 are the best characterised lysosomal membrane proteins (Carlsson and Fukuda, 1989).

## Table 1.2 Lysosomal luminal and membrane proteins

| Lysosomal Luminal Proteins | Lysosomal Membrane Proteins |
|---|---|
| α-Galacotsidase A | Acetyl-CoA: α-glucosaminide Acetyltransferase |
| α-Galactosidase B (α-N-acetylgalactosaminidase) | Acid sugar transporter (sialic) |
| α-Glucosidase | Anionic amino acid transporter |
| α-L-fucosidase | ATP-binding cassette transporter 2 (ABC2) |
| α-L-iduronidase | Branched and aromatic amino acid transporter |
| α-mannosidase | Calcium ion transporter |
| α-microglobulin/inter-α-trypsin inhibitor | Cationic amino acid transporter |
| α-N-acetylglucosaminidase | CD-M6PR |
| Arylsulfatase A | Cholesterol transporter |
| Arylsulfatase D | CI-M6PR |
| Aspartylglucosaminidase | CLN3 Protein |
| β-Galactosidase | Cobalimine transporter |
| β-Galactosidase-related protein | Cysteine transporter |
| β-Glucocerebrosidase (acid-β-Glucosidase) | Cystine transporter (cystinosin) |
| β-Glucuronidase | DC-LAMP-1/TSC403 |
| β-hexosaminidase -alpha subunit | Endolyn |
| β-hexosaminidase -beta subunit | Folypolygluatamate transporter |
| β-mannosidase | $H^+$-ATPase |
| Cathepsin A (Lysosomal protective protein) | LAMP-1 (CD107a) |
| Cathepsin B | LAMP-2 (CD107b) |
| Cathepsin C | LAMP-3 (LIMPI; CD63) |
| Cathepsin D | LIMPII (lysosomal integral membrane protein) |
| Cathepsin H | Lysosomal acid phosphatase (LAP) |
| Cathepsin K | Lysosomal-associated multitransmembrane Protein |
| Cathepsin L | Macrosialin (CD68) |
| Cathepsin L2 | MBR 39 |
| Cathepsin S | N-acetylated hexose transporter |
| Ceramidase | Neutral monosaccharide transporter |
| Deoxyribonuclease II | Nucleoside transporter |
| Di-N-acetylchitobiase | Phosphate ion transporter |
| Galactocerebroside β-galactosidase (Galactocerebrosidase) | Proline transporter |
| Glucosamine 3-sulfatase | Small dipeptide transporter |
| Glucuronate 2-sulfatase | Sulphate transporter |
| Iduronate 2-sulfatase | Syntaxin 7 |
| IP-30 (γ-interferon-inducible protein) | Taurine transporter |
| Lysosomal acid lipase | |
| Lysosomal carboxypeptidase C | |
| Lysosomal protective protein (Cathepsin A) | |
| N-acetylgalactosamine 4-sulfatase (arylsulfatase B) | |
| N-acetylgalactosamine 6-sulfatase (Galactose 6-sulfatase) | |
| Neuromindase (sialidase) | |
| Nonsecretory ribonuclease | |
| Palmityol protein thioesterase | |
| Prostatic acid phosphatase | |
| Saposin A | |
| Saposin B | |
| Saposin C | |
| Saposin D | |
| Sialidase (neuraminidase) | |
| Sphingolipid acivator protein 3 | |
| Sphongomyelinase (sphingomyelin phophodiesterase) | |
| Sulphamindase | |
| Tartrate-resistant acid phophatase | |

### *1.4.1.1 Structure of LAMPs*

Both LAMP-1 and LAMP-2 are structurally similar, sharing approximately 36.7% homology in their amino acid sequence (Figure 1.3). Each has a polypeptide core of approximately 42 kDa (Figure 1.4). LAMP-1 and LAMP-2 consist of 389 and 382 amino acids, respectively, and a major portion of these (350 residues) are found on the lumenal side of the lysosomal membrane (Carlsson *et al.*, 1993). This intralumenal domain is connected to a single transmembrane domain, which is extended to a short cytoplasmic tail.

The lumenal domain is extensively glycosylated with N- and O-linked carbohydrate chains, some of the N-linked oligosaccharides are terminated with poly-N-acetyllactosamine chains. LAMP molecules are unique for having a high proportion of poly-N-acetyllactosamine, which is composed of (Galβ1→4GlcNAcβ1→3)$_n$(N-acetyllactosamine) repeating units. The termini of poly-N-acetyllactosamines often contain unique structures such as the sialyl-Lewis X antigen, which consists of NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAc. The carbohydrate constitutes 55-65% of the total mass in the LAMP molecules giving them a total molecular weight of 100-120 kDa (Fukuda, 1991).

By digesting the precursor form of LAMP-1 and LAMP-2 with endo-β-N-acetylglucosaminidase F and endo-β-N-acetylglucosaminidase H for increasing periods of time, it was shown that these proteins contained 18 and 16 potential N-glycosylation sites, respectively (Carlsson *et al.*, 1988; Fukuda *et al.*, 1988). These N-glycosylation sites are clustered into two domains, separated by a hinge-like structure enriched with proline and serine (LAMP-1) and proline and threonine (LAMP-2). The two domains

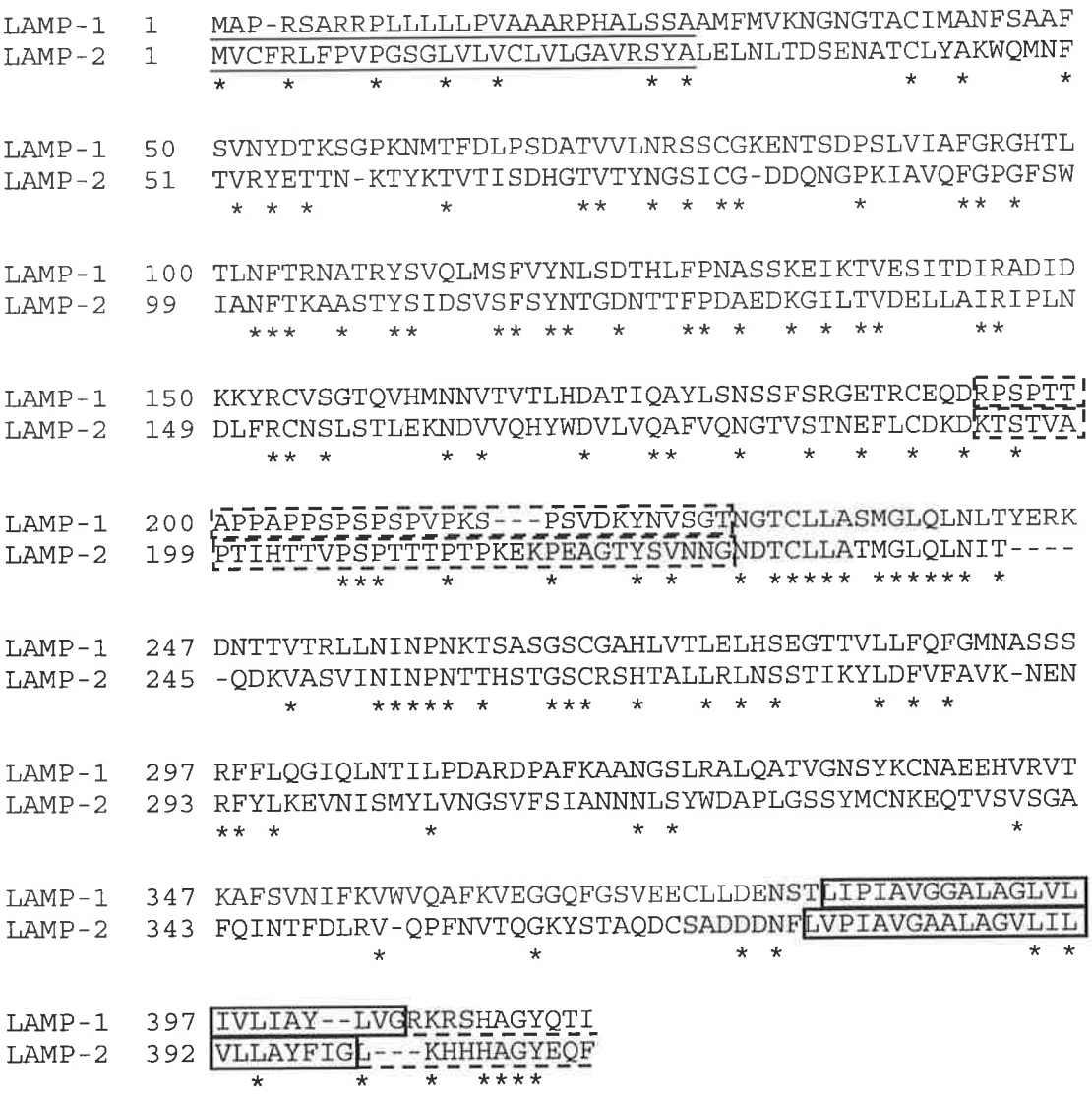



***Figure 1.3 Alignment of Human LAMP-1 and LAMP-2 Amino Acid Sequences***

The amino acid sequence of LAMP-1 and LAMP-2 were compared and gaps (-) were introduced to optimise the alignment. LAMP-1 shares 36.7% amino acid sequence homology with LAMP-2. Conserved residues are shown (*). Signal peptides are underlined; hinged regions are enclosed in dashed boxes; transmembrane regions are enclosed in solid boxes; cytoplasmic tails are underlined with dashes. Figure adapted from WebANGIS (http://www.angis.org.au/pbin/WebANGIS/wrapper.pl), best-fit program.

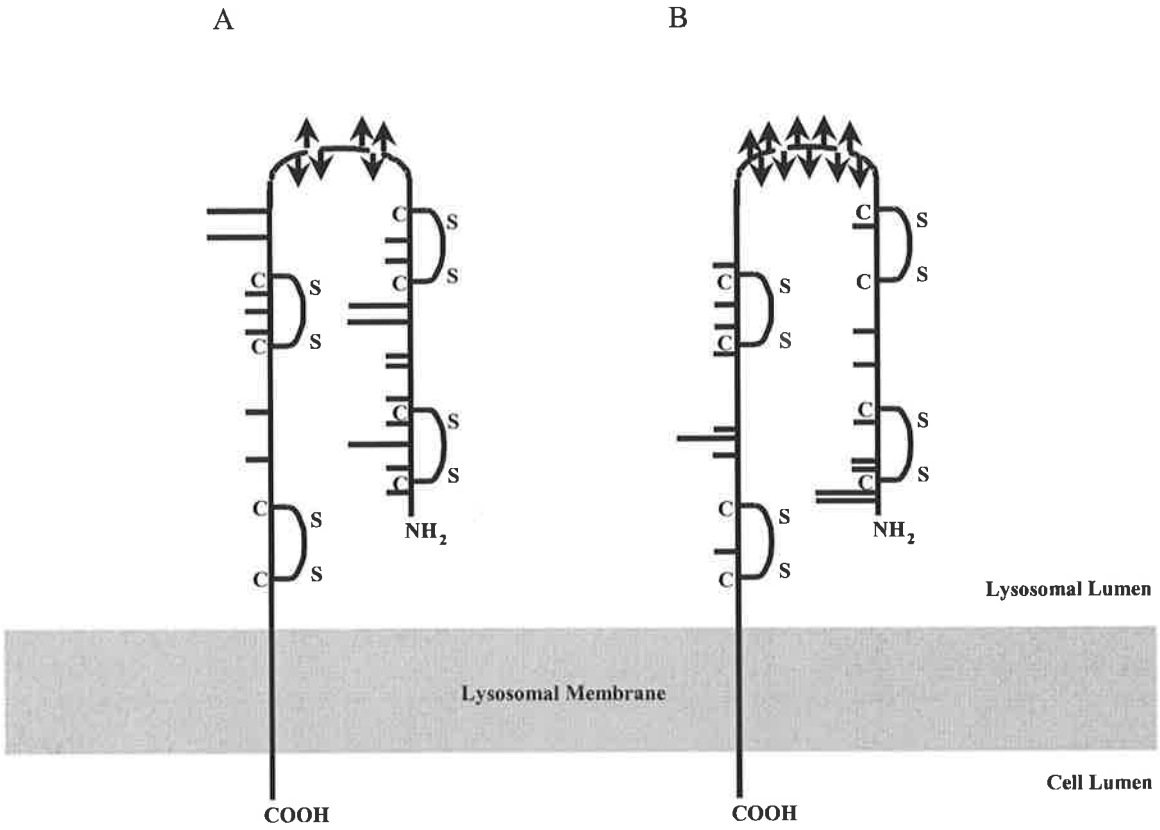



***Figure 1.4 Schematic representation of lysosome-associated membrane proteins***

Panel A, LAMP-1; panel B, LAMP-2

— N-linked oligosaccharides; → O-linked oligosaccharides; —— polylactosamine repeat units; C cystine; S S disulphide bonds.

on each side of the hinge region are homologous (Fukuda, 1991; Gough and Fambrough, 1997), each containing two disulfide loops. Sequence analysis revealed the localization of O-linked oligossacharides exclusively to the hinge-like regions of both LAMPs isolated from human chronic myelogenous leukemia cells, LAMP-1 having 6 sites and LAMP-2 having 10 sites (Carlsson *et al.*, 1993).

The transmembrane domain of LAMPs consists of 24 amino acid residues that are predominantly hydrophobic and 11 amino acids make up the short cytoplasmic tail (Figure 1.3) (Fukuda *et al.*, 1988). The targeting of LAMPs to the lysosome is dependant upon the C-terminal five residues of the cytoplasmic tail, which conform to the sequence –G-Y-X-X-∅, where ∅ is a hydrophobic residue (Guarnieri *et al.*, 1993; Honing and Hunziker, 1995; Gough and Fambrough, 1997). Mutations within the cytoplasmic tail may either disrupt lysosomal targeting completely, resulting in accumulation at the cell surface, or alter the relative use of the direct versus indirect pathway of lysosomal targeting (sections 1.1.1 and 1.4.1.2) (Williams and Fukuda, 1990; Harter and Mellman, 1992; Guarnieri *et al.*, 1993; Akasaki *et al.*, 1995; Honing and Hunziker, 1995). Through mutagenesis experiments, it was found that the –G-Y-X-X-∅ motif is recognised as a lysosomal targeting signal when it involves: 1) the presence of a tyrosyl (Williams and Fukuda, 1990), preceded by a critical glycyl residue (Honing and Hunziker, 1995); 2) the presence of this motif at the C terminus (Guarnieri *et al.*, 1993); 3) the occurrence of this C-terminal signal at a narrowly restricted distance from the membrane (Rohrer *et al.*, 1996); 4) the size of the C-terminal residue side chain; and 5) the hydrophobicity of the side chain residues (Gough *et al.*, 1999).

Despite the similarity of the LAMPs, comparison of the amino acid sequences of LAMP-1 and LAMP-2 from different species revealed that the LAMP-1 from one species is more homologous to LAMP-1 from another species than to LAMP-2 of the same species. This suggests that LAMP-1 and LAMP-2 diverged relatively early in their evolution, however their structures have been conserved following their divergence. The difference in LAMP-1 and LAMP-2 can be more appreciated by the fact that LAMP-1 and LAMP-2 are encoded by separate genes, located on different chromosomes, 13q34 and Xq24-25, respectively, further confirming their evolutionary history (Fukuda, 1991; Sawada *et al.*, 1993). The genes for human, chicken and mouse LAMP-1 and LAMP-2 each contain 9 exons (Zot and Fambrough, 1990; Sawada *et al.*, 1993; Hatem *et al.*, 1995). Comparison of the exon organization of human LAMP-1 and LAMP-2 genes revealed that these two proteins utilize the same exon phase in corresponding introns (Sawada *et al.*, 1993).

The LAMP-1 gene encodes for a single transcript (Zot and Fambrough, 1990), while the LAMP-2 gene encodes for three transcripts with alternatively spliced 3' exons, LAMP-2a, LAMP-2b and LAMP-2c (Gough *et al.*, 1995; Hatem *et al.*, 1995; Konecki *et al.*, 1995).

#### *1.4.1.2 Regulation of Expression of LAMPs*

Most genes encoding lysosomal proteins have been reported to possess "housekeeping" type promoters where the GC content is high, TATA box sequence is absent, and potential binding sites for transcription factors are present. These proteins appear to be constitutively expressed in all cells, providing a basic lysosomal function. LAMP-1 is believed to be a constitutively expressed, housekeeping protein (Zot and Fambrough,

1990; Sawada *et al.*, 1993). However, upon induction of melanogenisis in a range of cells, LAMP-1 mRNA and protein expression was shown to be increased (Jimbow *et al.*, 1994). The expression of LAMP-2 is uniquely regulated at the level of transcription. The LAMP-2 gene contains a strong promoter and a Kpn I repeat sequence 5' upstream that suppresses the activity of the promoter (Sawada *et al.*, 1993).

In the study of LAMP-1 and LAMP-2 regulation, sucrosome storage and LSD-affected skin fibroblasts were used to demonstrate that these proteins were regulated at the transcriptional level (Karageorgos *et al.*, 1997; Isaac *et al.*, 2000). LAMP-1 was greatly up-regulated in both sucrosome (6 to 8-fold) (Isaac *et al.*, 2000) and LSD model cell lines (up to 7-fold) (Karageorgos *et al.*, 1997), these increases were accompanied by an increase in mRNA levels, indicating that expression of LAMP-1 is regulated at a pre-translational level. The $t_{1/2}$ of LAMP-1 mRNA from control (11 hr) and sucrose-treated cells (14 hr) suggests that the observed increase in mRNA encoding LAMP-1 in sucrose-treated fibroblasts was not due to an increase in the stability of the mRNA but due to an increase in the rate of transcription (Isaac *et al.*, 2000). LAMP-2 mRNA was also observed to be elevated 2-fold in storage-affected cells, suggesting pre-translational regulation. Correction of storage, in both systems, resulted in the rapid decline of the mRNA to basal levels, with a slower decrease in the levels of LAMP-1 and LAMP-2 proteins. Cells responded to correction by transporting LAMP-1 out into the cell medium and the degradation of LAMP-1 (Karageorgos *et al.*, 1997). These observations would indicate that both LAMP-1 and LAMP-2 are regulated pre-translationally. Contradicting this is a study by Andrejewski *et al.* (Andrejewski *et al.*, 1999) where mice deficient for LAMP-1 were generated to investigate the physiological role of this protein. Western analysis of LAMP-1 deficient and heterozygote tissues revealed that

the LAMP-2 protein was up-regulated without an increase in the level of LAMP-2 mRNA or $t_{1/2}$ of the protein, suggesting that LAMP-2 regulation is post-translational. Thus the site of regulation remains uncertain.

The three isoforms of LAMP-2 protein are encoded by a single gene, indicating that LAMP-2a, LAMP-2b and LAMP-2c arise by alternative splicing (Hatem *et al.*, 1995). mRNA from several tissues of adult chicken were analysed on Northern blots, probing with labelled DNA encoding for regions common to LAMP-2a, LAMP-2b and LAMP-2c (Hatem *et al.*, 1995). It was found that LAMP-2 was regulated in a tissue-specific manner, with LAMP-2c detected in the brain, colon, heart, skeletal muscle, gizzard, spleen, kidney and liver. LAMP-2a was detected in the brain and heart, while LAMP-2b was found only in the brain (Hatem *et al.*, 1995). The LAMP-2 splice variants also display quantitatively different subcellular distributions, that has been shown to be due to differences in the carboxyl terminal residue of the cytoplasmic tail (Gough and Fambrough, 1997). Thus regulation of LAMP-2 expression is dependant on tissue type and its distribution is governed by its cytoplasmic tail sequence.

#### *1.4.1.3 Distribution of LAMPs*

Other than in the lysosomal membrane, LAMPs have been detected on the plasma membrane of cells in a differentiation dependent (Mane *et al.*, 1989; Saitoh *et al.*, 1992) and activation or cell type dependent manner. LAMPs have been detected on the surface of activated platelets (Febbraio and Silverstein, 1990; Silverstein and Febbraio, 1992; Kannan *et al.*, 1996), phagocytes (Dahlgren *et al.*, 1995; Persson *et al.*, 2002), macrophages (Watarai *et al.*, 2002), cytotoxic T lymphocytes (Peters *et al.*, 1991), myelomonocytic leukemia cells (Mane *et al.*, 1989), osteoclasts (Akamine *et al.*, 1993),

metastatic tumour cells (Saitoh *et al.*, 1992; Chakraborty *et al.*, 2001), and carcinoma cells (Amos and Lotan, 1990; Kunzli *et al.*, 2002).

Lichter-Konecki *et al.* (Lichter-Konecki *et al.*, 1999) reported on murine LAMP-2 transcripts and their prevalent expression patterns during development. All three LAMP-2 transcripts were found to be expressed in neural crest derived ganglia, particularly LAMP-2b. However, LAMP-2a and LAMP-2c expression differed in mesoderm or endoderm derived mesenchymal and epithelial tissues. LAMP-2c was prevalent in mesenchyme early in development, in limb connective tissue, and in lung parenchyma. LAMP-2a was prevalent in liver, pancreas, and in differentiating kidney epithelium, and became increasingly prominent in the epithelial lining of the digestive and the respiratory tract during development. These observations support the concept that LAMP-2 expression is tissue and cell type specific.

Recently it was found that an isoform of LAMP-1 exists called M150 (Prasad *et al.*, 2002). M150 was isolated from the surface of activated macrophages as a 150 kDa protein and was shown to possess co-stimulatory activity capable of stimulating T cell proliferation (Agrewala *et al.*, 1994). M150 is a uniquely glycosylated form of LAMP-1 that is produced only in activated macrophages, thus the distribution of LAMP-1 is activation dependent. It is thought that upon activation, LAMP-1 is selectively modified post-translationally into a molecule with co-stimulatory potential (Prasad *et al.*, 2002).

#### *1.4.1.4 Processing and Trafficking of LAMPs*

Pulse chase studies and fractionations on Percoll density gradients of LAMP molecules in several cell types have been performed to determine the biosynthetic transport of

these glycoproteins. Newly synthesised LAMPs utilise two cellular pathways to reach the lysosome from the trans-Golgi network (section 1.1.1): 1) a direct pathway in which the LAMPs are targeted directly to a late-endosome/lysosome compartment and 2) an indirect pathway in which the LAMPs move to an early endosome/plasma membrane compartment first and then are internalised and delivered to lysosomes. The majority of studies were on LAMP-1, however in most experiments LAMP-2 was also analysed and gave the same results as LAMP-1 in terms of transport pathways or kinetics.

LAMP-1 is first synthesised as a core polypeptide of approximately 42 kDa which is then co-translationally modified by the addition of asparagine-linked oligosaccharides to yield an endo-β-N-acetylglucosaminidase H (Endo H) sensitive precursor glycoprotein of about 92 kDa in the rough endoplasmic reticulum (RER) (D'Souza and August, 1986). The protein is further processed in the Golgi, a majority of the high-mannose glycans are converted to complex-type oligosaccharides with some containing poly-N-acetyllactosamines resulting in the mature 120 kDa molecule. In pulse chase experiments, a 30 min incubation with tunicamycin, which inhibits the addition of asparagine-linked oligosaccharides to nascent polypeptide chains during transport through the RER, resulted in the 42 kDa core polypeptide increasing to 43 kDa, suggesting that O-linked oligosaccharides were also added in the Golgi (D'Souza and August, 1986).

The $t_{1/2}$ for LAMP-1 transport to the trans-Golgi was found to be similar in rat hepatocytes (Akasaki *et al.*, 1995), rat NRK cells and mouse macrophages ($t_{1/2}$ = 13-15 mins). While the $t_{1/2}$ was significantly greater in mouse 3T3 cells (D'Souza and August, 1986) and dog MDCK cells ($t_{1/2}$ = 25 mins) (Nabi *et al.*, 1991) and human HL-60 cells

($t_{1/2}$ = 35 mins) (Carlsson and Fukuda, 1992). The difference observed in the $t_{1/2}$ for LAMP-1 transport to the trans-Golgi from one cell type to another is correlated to the varying amounts of poly-N-acetyllactosamines added to LAMP-1 (Nabi and Rodriguez-Boulan, 1993; Akasaki *et al.*, 1995).

Meikle *et al.* (Meikle *et al.*, 1999b) compared the $t_{1/2}$ for LAMP-1 processing from the RER to the Golgi apparatus in Pompe-affected and unaffected skin fibroblasts and found that the trafficking of LAMP-1 in Pompe cells was significantly retarded. There was an increase in $t_{1/2}$ for Pompe-affected cells (25 min) compared to control (17 min) suggesting a delay in glycoprocessing. This slower processing through the ER and the Golgi may represent a secondary effect of the spatial congestion observed in these cells as a result of the lysosomal storage of Pompe disease (Meikle *et al.*, 1999b).

The transport route of LAMPs from the TGN to endosomes and lysosomes is unclear, however it is clear that transport relies on the –G-Y-X-X-∅ motif at the C-terminus of the proteins' cytoplasmic tail (section 1.4.1.1). Unlike lysosomal hydrolases, LAMP-1 and LAMP-2 are not transported from the TGN by the same vesicles as the mannose 6-phophate receptors (section 1.1.1) (Karlsson and Carlsson, 1998). The signal motif on the cytoplasmic tail of LAMPs interacts with clathrin adaptor protein complexes (AP-1 and AP-2) and the adaptor-like protein complex (AP-3), which mediate the sorting into coated vesicular carriers (section 1.1.1) (Harter and Mellman, 1992; Honing and Hunziker, 1995; Le Borgne and Hoflack, 1998; Bonifacino and Dell'Angelica, 1999; Nishimura *et al.*, 2002). As AP-3 does not appear to bind to LAMP-1 and LAMP-2 with great affinity, it is not known whether the interaction of AP-3 with LAMPs is specific (Honing *et al.*, 1998). However, over expression of LAMPs in HeLa and NRK cells was

shown to promote AP-3 recruitment on cell membranes, while inhibition of the synthesis of the μ3 subunit of AP-3 led to a selective misrouting of LAMPs to the cell surface (Le Borgne *et al.*, 1998). This suggests that LAMPs interact with AP-3 specifically and is required for the proper transport of LAMPs to the lysosome. The alternatively spliced variants of LAMP-2 have different cytoplasmic tails, these differences include different amino acids within the targeting signal which may effect its targeting. LAMP-2a and LAMP-2b showed much higher levels of cell surface expression than LAMP-2c, suggesting that the cytoplasmic tail influence whether the protein is targeted to the cell surface or to the lysosome (Gough and Fambrough, 1997). Moreover, LAMP-2a and LAMP-2c were found to be deficient in their ability to be endocytosed from the cell surface, suggesting that AP-2 may not efficiently recognise LAMP cytoplasmic tails that have a valine in the carboxyl terminal position. Thus differences in the recognition of LAMP cytoplasmic tails by intracellular trafficking machinery at the level of the TGN, the plasma membrane, and the endosomal compartment may also affect the targeting of LAMPs (Gough and Fambrough, 1997).

Carlsson and Fukuda (Carlsson and Fukuda, 1992) demonstrated that the majority of LAMP-1 in human HL-60 cells is directly transported to lysosomes by a fast pathway with $t_{1/2}$ of 60 min. A minor part of LAMP-1 in HL-60 cells was shown to follow the longer pathway with $t_{1/2}$ of more than 2 hr where the protein is transported out to the cell surface, internalised and eventually delivered to the lysosome. These observations are now accepted as being the routes of LAMP transport and have been confirmed by Akasaki *et al.* (Akasaki *et al.*, 1995) and Rosenfeld *et al.*, (Rosenfeld *et al.*, 2001). The majority of newly synthesised LAMP-1 in rat hepatocytes was shown to be transported to the lysosome with a $t_{1/2}$ of 85 min. It was also shown that the maximal expression of

LAMP-1 at the cell surface (2% of total labelled LAMP-1) occurred after 60-80 min of chase, after which the amounts decreased, indicating that the molecules were internalised (Akasaki *et al.*, 1995). The $t_{1/2}$ for internalisation was found to be 13 min, with a delivery rate of 30 min of the internalised LAMP-1 to lysosomes. Thus it is clear that LAMPs follow the direct pathway of transport to the lysosome, with a small portion following the indirect pathway.

In human skin fibroblast cells, the $t_{1/2}$ for the trafficking of LAMP-1 to the lysosome was 100 min and 200 min in Pompe-affected cells (Meikle *et al.*, 1999b). These observations together with those described above suggest that trafficking of LAMPs is dependent on cell type and cell stage. It is also evident that in LSD-affected cells LAMPs have altered trafficking and turnover.

A significant proportion of soluble LAMP-1 was found within the lysosome representing approximately 25% of the total lysosomal LAMP-1, with similar observations for LAMP-2 (Jadot *et al.*, 1996; Meikle *et al.*, 1999b). Western analysis of this soluble LAMP-1 showed that this form of LAMP-1 did not contain a cytoplasmic tail (Meikle *et al.*, 1999b). The release of these soluble LAMPs may be the result of proteolytic cleavage of the transmembrane domain and the cytoplasmic tail. A similar mechanism has been reported for the type II Golgi protein β-galactoside α-2,6-sialyl transferase (Weinstein *et al.*, 1987).

LAMP-1 is also secreted from the cell without trafficking first to the lysosomes, representing 8.5% of newly synthesized LAMP-1 in human skin fibroblasts (Meikle *et al.*, 1999b). This form of LAMP-1 retains its cytoplasmic tail and transmembrane

domain, indicating that secretion was not the result of proteolytic processing of the membrane bound form, as was seen in the soluble form of LAMP-1 within the lysosome (Meikle *et al.*, 1999b). Moreover, soluble LAMP-1 and LAMP-2 have been detected in circulation (Meikle *et al.*, 1997; Hua *et al.*, 1998). The mechanism by which LAMP-1 may be secreted from the cell, particularly with the membrane span intact, remains unclear. The proteolytic degradation of LAMPs may be a possibility for LAMPs secreted without the membrane domain. Alternatively, spliced forms of LAMP-1 in which different transmembrane domains exist may also result in its secretion. The differentially glycosylated LAMP-1 protein, M150, reported by Prasad *et al.* (Prasad *et al.*, 2002), suggests that variants of LAMP-1 may exist (section 1.4.1.3). Yet another possibility is lysosome exocytosis where LAMPs are selective released by lysosomes, such as that shown for MHC II or Interleukin 1 (section 1.1.2). Kima *et al.*, (Kima *et al.*, 2000) showed that the presence of cell surface LAMP-1 in Chinese hamster ovary cells enhanced calcium-triggered lysosome exocytosis, monitored by the secretion of β-hexosaminidase. As this mechanism require specific activation of the cell and presumably only proteins that are essential for the activity are released, it implies that soluble LAMPs in circulation may have specific functions.

### *1.4.1.5 Functions of LAMPs*

Although LAMP-1 and LAMP-2 share many structural features, the fact that they are encoded by two separate genes (section 1.4.1.1) suggests that they have distinctly separate functions. Andrejewski *et al.* (Andrejewski *et al.*, 1999) showed that LAMP-2 was up-regulated to compensate for LAMP-1 deficiency in gene knockout mice. In LAMP-2 deficient mice an increase in mortality was seen, thus while LAMP-2 can compensate for LAMP-1, LAMP-1 does not appear to be able to compensate for the

deficiency of LAMP-2 (Tanaka *et al.*, 2000). When both glycoproteins were deficient the result was lethal (Andrejewski *et al.*, 1999). Taken together these observations suggest that LAMP-1 and LAMP-2 have similar or overlapping as well as different functions. The *in vivo* functions of LAMPs still remains unclear, however several possibilities exist.

### *1. LAMPs Protect Themselves and the Lysosomal Membrane from Degradation*

As LAMP-1 and LAMP–2 are present in large amounts, it is thought that they form a nearly continuous carbohydrate coat lining the lumenal surfaces of lysosomal membranes (Fukuda, 1991). Thus as integral proteins, LAMPs may play a role in creating a barrier to lysosomal hydrolases, protecting themselves and the membranes from destruction by hydrolases (Neiss, 1984; Kornfeld and Mellman, 1989; Fukuda, 1991; Sawada *et al.*, 1993). The heavy glycosylation of LAMPs is essentially the reason for this theory. As protease resistance has been observed for glycoproteins in general, glycosylation is thought to be important for the protection of the LAMP polypeptide cores from proteolytic degradation within the lysosome which would in turn protect the lysosomal membrane (Fukuda, 1991). The function of O-linked carbohydrate in LAMP molecules may protect the hinge region, which is otherwise susceptible to intralumenal proteases (Carlsson *et al.*, 1993). Evidence that the asparagine-linked oligosaccharides of LAMPs protect against proteolysis was achieved by Endo H treatment, which removed asparagine-linked glycans from fully folded LAMP-1 and LAMP-2 protein *in vivo*. This resulted in the rapid degradation of the LAMPs and subsequent depletion of these proteins within cells (Kundra and Kornfeld, 1999). However, several studies have shown that LAMPs are not essential for lysosomal membrane integrity. In Andrejewski *et al.* (Andrejewski *et al.*, 1999), where LAMP-1 deficient mice were studied, there did

not seem to be any abnormality in size, distribution, and shape of the lysosomal compartment in LAMP-1 deficient cells. It is possible that the proposed function that LAMPs help to protect the membranes from hydrolases was compensated for by the up-regulation of LAMP-2 in these cells. The only physiological change in these LAMP-1 deficient mice was in the brain where astrogliosis and altered cathepsin-D distribution was observed. As very little LAMP-2 is detectable in mouse brain, it may be that LAMP-1 deficiency cannot be compensated for by an increase of LAMP-2 and hence the observed alterations. However, depletion of both LAMP-1 and LAMP-2 within cells by Endo H treatment did not alter the endosomal/lysosomal pH, osmotic stability, density and cell viability, suggesting that LAMPs are not involved in keeping membrane integrity (Kundra and Kornfeld, 1999). Therefore, while the high level of glycosylation does appear to protect the LAMP polypeptide from protease activity the LAMPs are not required to protect the lysosomal membrane.

### 2. *LAMPs Protect Cells Against Infection*

Lin *et al.* (Lin *et al.*, 1997) investigated the effect of infection of human epithelial cells by the pathogenic *Neisseria* spp *in vitro*. *Neisseria meningitidis* and *Neisseria gonorrhoeae* are the cause of bacterial meningitis and gonorrhoea, respectively. It is thought that *Neisseria* enter cell by a phagocytic mechanism, the precise nature of the invasion process is unknown. It was found that the rate of LAMP-1 degradation was increased due to cleavage of LAMP-1 by the *Neisseria* IgA1 protease, which recognised the IgA1-like hinge region in LAMP-1 that is rich in proline and serine. LAMP-1 levels were reduced 20-50% in infected cultures, thus Lin *et al.* (Lin *et al.*, 1997) proposed that reducing the level of LAMP-1 would modify lysosomes in such a manner as to promote intracellular bacterial survival and growth. As the cleavage of LAMPs is the only

method for *Neisseria* to survive, it may be possible that LAMPs have a role in the stabilisation and/or maturation of phagosomes.

### 3. *LAMPs Assist in the Fusion of Membranes*

Rodriguez *et al.* (Rodriguez *et al.*, 1997) showed that triggering an increase in intracellular calcium ion levels with calcium ionophores lead to the exposure of the luminal domain of LAMPs on the cell surface as a result of lysosome fusion with the plasma membrane. Moreover, during phagocytosis LAMPs become incorporated into the membrane of the phagosomes (section 1.1.1) (Persson *et al.*, 2002).

The protozoan parasite *Trypanosoma cruzi* has been reported to invade cells by binding to sialic acid residues on the surface of host cells (Ming *et al.*, 1993). Shortly after interaction with host cells, *T. cruzi* trigger signalling cascades that produce elevations in host cell cytoplasmic calcium ions and cyclic AMP concentrations. These events are directly correlated with lysosome-mediated parasite invasion and exocytosis of lysosomes (Rodriguez *et al.*, 1999). Cell surface LAMP-1 was shown to facilitate the interaction of lysosome derived vesicles with the plasma membrane and *T. cruzi* invasion. LAMPs expressed at the cell surface with a mutant cytoplasmic tail did not enhance infection. Thus the sialylated lumenal domain was not sufficient to promote *T. cruzi* entry, instead it was the cytoplasmic tail of LAMP-1 that was the important factor (Kima *et al.*, 2000). This suggests that intracellular interactions are key for the parasite invasion-promoting effect. Kima *et al.* (Kima *et al.*, 2000) proposed that LAMP-1 molecules associate with each other through their cytoplasmic domain, and such interactions could facilitate docking of lysosomes at the plasma membrane and potentiate calcium-triggered fusion. Taken together, these findings suggest that LAMPs

may assist in fusion events involving lysosome derived vesicles by interacting with each other through their cytoplasmic domains.

### 4. *LAMPs Mediate Immune Responses*

Increased cell surface LAMP expression on peripheral blood lymphocytes has been shown to correlate with platelet activation in patients with the autoimmune diseases scleroderma and systemic lupus erythematosus (Holcombe *et al.*, 1993; Holcombe *et al.*, 1994). Kannan *et al.* (Kannan *et al.*, 1996) demonstrated that LAMP-2 can be expressed at the cell surface of T cells, monocytes, and $CD56^+$ cells (principally natural killer, NK, cells) in addition to B cells *in vitro*. Moreover, it was shown that LAMP-2 was one of the important mediators of peripheral blood mononuclear cells adherence to endothelium. Evidence of the involvement of LAMP-1 in the regulation of T cell responses came in the finding that M150 was a unique subset of LAMP-1, which has a distinct glycosylation pattern restricted to macrophages (Prasad *et al.*, 2002). M150 was shown to be capable of driving differentiation of naive T cells into the Th1 subset and stimulating Th1-specific responses (Agrewala *et al.*, 1994; Prasad *et al.*, 2002). It has therefore been proposed that LAMPs may be immunologically important molecules involved in the regulation of peripheral blood mononuclear cell migration, as well as in mediating inflammation and immune response of patients with autoimmune diseases.

### 5. *LAMPs are Involved in Development*

The LAMP-1 and LAMP-2 double knockout mice that resulted in embryonic lethality (Andrejewski *et al.*, 1999; Peters and von Figura, 1994) and the differential and tissue specific-dependent distribution pattern of LAMP-2 (section 1.4.1.3) (Lichter-Konecki *et al.*, 1999) suggests that LAMPs may play an important role in development, particularly

LAMP-2. LAMP-2 deficient mice showed an increase in mortality with 50% dying between 20 and 40 days of age, while mice that survived were smaller in size (Tanaka *et al.*, 2000).

### 6. *LAMP-2 Deficiency Causes Danon Disease and LAMP-2 is Involved in Autophagy*

LAMP-2 deficiency in humans is the cause of an LSD, Danon disease (section 1.2) (Nishino *et al.*, 2000), characterised clinically by cardiomyopathy, myopathy and variable mental retardation. Mutations in the coding sequence of LAMP-2 was shown to be the cause of Danon disease (Saftig *et al.*, 2001). The disease affects males predominantly with affected females often manifesting milder phenotypes. Because male-male transmission has not been described, the inheritance of Danon disease is considered to be X-linked (Saftig *et al.*, 2001). However, in a recent case, a mutation carried by an affected son and daughter was not detected in their mother, indicating germline mosaicism (Takahashi *et al.*, 2002). The pathological hallmark of the disease is intracytoplasmic vacuoles containing autophagic material and glycogen in skeletal and cardial muscle cells. In LAMP-2 deficient mice, the animal model for Danon disease (Saftig *et al.*, 2001), there is extensive accumulation of autophagic vacuoles in many tissues including liver, pancreas, spleen, kidney and skeletal and heart muscle. There was also a decrease in the rate of degradation of long-lived proteins from 1.8% $h^{-1}$ in control hepatocytes to 0.95% $h^{-1}$ in LAMP-2 deficient hepatocytes. The degradation of long-lived proteins occurs mainly by autophagy (section 1.1.1). It is thought, therefore, that LAMP-2 is involved in the process of fusion of autophagic vacuoles with endosomes/lysosomes, which provide the acid hydrolases required for degradation or a

function in the maturation of the autophagolysosomes into actively digesting organelles (Tanaka *et al.*, 2000).

### 7. *LAMPs Facilitate the Transport of Molecules to and from the Lysosome*

LAMPs have also been suggested to act as a receptor for the selective import of macromolecules from the cytoplasm into the lysosome for subsequent degradation, or chaperone-mediated autophagy (Cuervo and Dice, 1996; Cuervo and Dice, 1998). The function of LAMP-2 as a transporter of molecules for lysosomal degradation was evident in Chinese hamster ovary cells where over expression of human LAMP-2 increased the activity of proteolysis in the lysosome of transfected cells. Moreover, in LAMP depleted cells, transport of endocytosed material to dense lysosomes were delayed (Kundra and Kornfeld, 1999). A closer examination of the phenotype of LAMP-2 deficient mice using cultured hepatocytes showed that the recycling of the mannose 6-phosphate receptor was impaired which lead to the mistargeting of some lysosomal enzymes (Eskelinen *et al.*, 2002). This observation suggests that LAMP-2 may be involved in mannose 6-phosphate receptor trafficking. Since LAMP-2 does not interact directly with the mannose 6-phosphate receptor, Eskelinen *et al.* (Eskelinen *et al.*, 2002) suggested that LAMP-2 may participate in chaperone-mediated transport of recycling promoting factors from the cytoplasm to the endosomal lumen.

Jadot *et al.* (Jadot *et al.*, 1997) demonstrated the ability of LAMP-2 to aggregate by forming a supramolecular complex with lipids called lipid rafts. Components associated with these sphingolipid-cholesterol-rich microdomains include cholesterol and GM1 gangliosides (Watarai *et al.*, 2002). Such aggregations were optimally formed under conditions of mild acid pH (pH 4.5-5.0), comparable to that found in the lysosome. It

has been suggested that lipid rafts could be important for the regulation of protein traffic to and from the lysosomes, as it would prevent leaking of lysosomal proteins from lysosomes involved in vesicular transport (Buckmaster *et al.*, 1988; Cherukuri *et al.*, 2001). It is therefore possible that the concentration of LAMP-2 in lipid rafts may facilitate the chaperone-mediated autophagy by providing a LAMP-2 rich, membrane environment.

### *8. LAMPs Facilitate Cell-Cell Interactions*

LAMP-1 and LAMP–2 are the major carriers of polylactosaminoglycans, which are enriched in the sialyl-Lewis X antigen. This structure serves as ligands for adhesion proteins (section 1.1.2.1) present on various cells and platelets (Carlsson *et al.*, 1988; Foxall *et al.*, 1992). This suggests that cell surface LAMPs may function to provide ligands for binding to P-, E-, and L-selectin (Sawada *et al.*, 1994). LAMPs have also been shown to be substrates for cell surface β-1,4-galactosyltransferase, which recognises terminal N-acetylglucosamine residues (Maillet and Shur, 1993). The poly-N-acetyllactosamines on LAMPs serve to bind lectins, particularly galectin-3 (Inohara and Raz, 1994; Woynarowska *et al.*, 1994; Feuk-Lagerstedt *et al.*, 1999; Kunzli *et al.*, 2002). Thus LAMPs may be important mediators of cellular adhesion through interactions of their carbohydrate chains with carbohydrate receptors (Gough and Fambrough, 1997). Studies of the distribution of murine LAMP-2 transcripts showed that all LAMP-2 transcripts were detected in tissues undergoing apoptosis during development requiring phagolysosome involvement. In addition, LAMP-2a and LAMP-2c transcripts were observed in epithelium and mesenchyme during the time of epithelial-mesenchymal interaction, mesenchymal-epithelial transformation, and

branching. These distribution patterns indicate a possible involvement of LAMP-2 in cell-cell or cell-extracellular matrix interaction (Lichter-Konecki *et al.*, 1999).

### 9. *LAMPs Facilitates Tumour Metastasis*

LAMP-1 and LAMP-2 are present in relatively small amounts on the surface of normal cells, however highly metastatic tumour cells show an increase level of cell surface LAMPs compared to low metastatic tumour cells.

The tetrasaccharide sialyl-Lewis X antigen is displayed on the terminus of oligosaccharides and is expressed on the cell surface. The sialyl-Lewis X antigen has also been found in elevated levels on the surface of a number of tumour cells (Magnani *et al.*, 1982; Fukushima *et al.*, 1984; Ohyama *et al.*, 1999). LAMP-1 and LAMP-2, the major carriers of polylactosamines able to display the sialyl-Lewis X antigen, were also found in greater levels on highly metastatic colonic carcinoma tumour and melanoma cells than poorly metastatic cells (Saitoh *et al.*, 1992; Ohyama *et al.*, 1999; Chakraborty *et al.*, 2001). LAMP-1 and LAMP-2 levels were also increased in human pancreatic carcinoma tissue in comparison with normal tissue (Kunzli *et al.*, 2002). Moreover, high metastatic cells, as compared to their low metastatic counterparts, bind more efficiently to activated human endothelial cells that express E-selectin and this adhesion can be inhibited by soluble LAMP-1 or soluble leukosialin that contain the sialyl-Lewis X antigen (Sawada *et al.*, 1994). Soluble forms of leukosialin and LAMP-1 may therefore be useful as therapeutic agents for the inhibition of E-selectin-mediated binding to tumour cells.

Through flow cytometry experiments, Sarafian *et al.* (Sarafian *et al.*, 1998) showed the presence of cell surface LAMPs in A2058 (human metastasising melanoma), HT1080 (human fibrosarcoma) and CaCo-2 (human colon-adenocarcinoma) cells. There was strong binding of A2058 to recombinant galectin-3, and was enhanced when expression of cell surface LAMPs was increased by the addition of butyrate. These observations suggest that LAMPs may participate in the complex process of tumour invasion and metastasis by providing the ligand structure, sialyl-Lewis X antigen, for interaction with adhesion molecules present on endothelial cells and platelets (Sarafian *et al.*, 1998). Cell invasion is the process of movement, penetration or infiltration of cells into adjacent tissues (Woolley, 1984), and is what distinguishes benign from malignant cells.

Although there have been many speculations on the functions of intracellular LAMPs and LAMPs expressed on the cell surface, the question remains as to the functions of extracellular soluble LAMPs. In particular, what do LAMPs do in circulation?

## 1.5 Aims and Significance

As the function(s) of LAMPs are not fully known, particularly soluble LAMPs found in circulation, it is uncertain to what extent, if any, the elevated LAMP levels observed in LSD patients are contributing to the pathology of this group of disorders and what mechanisms may be involved. The focus of this thesis is on LAMP-1, with the aim to characterise and investigate the interaction and potential function(s) of soluble LAMP-1 in circulation.

Characterisations of the function(s) of LAMP-1 in circulation will be critical for a complete understanding of the relevance of elevated LAMP levels in LSD and their use

as newborn screening markers for LSD. In addition, this study may help to further elucidate the role of LAMPs in cancer and other disease states.

# CHAPTER TWO: MATERIALS AND METHODS

## 2.1 MATERIALS

See Appendices

## 2.2 METHODS

### 2.2.1 Expression of Full Length and Truncated LAMP-1

The constructions of the full length and truncated LAMP-1 expression vectors as well as the transfection of these vectors into HT1080 and PANC cells were performed by C. Hua and E. Melville (Women's and Children's Hospital, SA, Australia). At the same time, I used the same methods to construct and transfect full length and truncated LAMP-2 expression vectors into HT1080 and PANC cells. However, the work on LAMP-2 was stopped due to time constraints and the cloning and expression of LAMP-1 was then taken over by M. Chang. As the focus of this thesis is on LAMP-1, the preparation of LAMP-2 expression constructs is not reported.

Full length human LAMP-1 cDNA (2455 bp) was generously provided by M. Fukuda (La Jolla Cancer Research Foundation, CA, USA) inserted into the *Eco RI* site of plasmid Bluescript (Fukuda *et al.*, 1988). The LAMP-1 cDNA was excised from the pBluescript-LAMP-1 vector with restriction enzymes *Eco RI* and *Hind II* and blunt end ligated into the *Eco RI – Eco RV* site of the expression vector, pFRNEE.2 (Bielicki *et al.*, 1998). For truncated LAMP-1, the transmembrane coding and cytoplasmic tail sequence of the LAMP-1 cDNA were removed (at 1335 bp) by digesting with *Eco RI*

and *Sau 3AI* restriction endonucleases. The *Sau 3AI* rectriction cut of the truncated LAMP-1 cDNA was filled in to create a blunt end by incubating (12°C, 15 min) with deoxynucleotide triphosphates (100 μM) and T4 DNA polymerase (5 U). The truncated LAMP-1 cDNA was then blunt end ligated into the *Eco RI – Eco RV* site of vector pFRNEE.2.

To construct the full length LAMP-1 expression vector, the LAMP-1 cDNA was isolated from the pFRNEE.2-LAMP-1 vector by digestion with *Eco RI a*nd *Not I* and ligated into the *Eco RI* and *Not I* cloning site of the expression vector, pGRE-neo.N-dhfr, constructed by T. Litjens (Women's and Children's Hospital, SA, Australia). To construct the truncated LAMP-1 expression vector, truncated LAMP-1 cDNA was digested from the pFRNEE.2-LAMP-1 (-Tail) vector with *Eco RI* and *Not I* and ligated into the *Eco RI* and *Not I* cloning site of pGRE-neo.N-dhfr. Both expression vectors were calcium phosphate transfected (section 2.2.1.9) into HT1080 and PANC cells.

#### *2.2.1.1 Restriction Endonuclease Digestion of Plasmids*

Digestion of plasmid DNA was carried out using conditions suggested by the manufacturers of each restriction endonuclease, unless otherwise stated.

#### *2.2.1.2 Agarose Gel Electrophoresis*

Electrophoresis of DNA, for analytical purposes, was carried out using 1% agarose gels in horizontal submerged gel electrophoresis tanks (Eastman Kodak Company, CT, USA). DNA samples were prepared in 1x agarose gel loading buffer (Appendix 3A). Gels were electrophoresed (100 V) in 1x tris buffered ethylene diamine tetraacetic acid (TBE) running buffer (Appendix 3A) until the bromophenol blue dye indicator had

migrated to a sufficient distance to ensure adequate separation of the DNA fragments. The gels were stained in ethidium bromide (10 mg/L, 5–10 min) and briefly washed under running water. Gels were visualised on an UV transilluminator (Polaroid Ltd., MA, USA) and photographed using Polaroid land film 667 (Polaroid Corp., MA, USA). Semi-log plots of the molecular weight marker Spp1 digested with *Eco RI* (Appendix 1) versus the distance travelled was used to determine the molecular weights of DNA fragments.

#### *2.2.1.3 Preparation of the Plasmid Vector for Ligation*

The expression vector, pGRE-neo.N-dhfr, was a modification of Promega's pCI-neo Mammalian Expression Vector (Promega Corporation, NSW, Australia). The human cytomegalovirus (CMV) promoter in pCI-neo was removed and replaced with an SV40 promoter, new cloning sites were also added and an amplification gene, dihydrofolate reductase (DHFR), was included. pGRE-neo.N-dhfr (3 μg) was linearised with 20 U of *Not I* and *Eco RI* in 1x Buffer H (37°C, overnight) at a final reaction volume of 40 μL. Analysis of the digested DNA was by agarose gel electrophoresis (section 2.2.1.2). In order to prevent the DNA from re-ligating, the 5'-terminal phosphate group was removed by treating 100 ng of the DNA with 10 U of alkaline phosphatase in 1 x AP Buffer. The reaction mix (100 μL) was incubated (37°C, 30 min), extracted with phenol/chloroform (1:1) and centrifuged (13,000 x g, 4°C, 5 min). The aqueous phase was retained and 1 M sodium acetate added to achieve a final concentration of 0.3 M. The DNA was precipitated with 3 volumes of 100% (v/v) ethanol (-70°C, 3 hrs). The DNA was pelleted by centrifugation (13,000 x g, 4°C, 15 min), washed with 70% (v/v) ethanol, centrifuged (13,000 x g, 4°C, 10 min), air dried and then resuspended in TE buffer (20 μL) (Appendix 3A).

#### *2.2.1.4 Isolation of Full Length and Truncated LAMP-1 cDNA*

The transfer vector, pFRNEE.2 (5 μg), containing either the full length or truncated LAMP-1 cDNA, was digested with 20 U *Not I* and *Eco RI* in 40 μL 1x Buffer H (37°C, overnight) and analysed by agarose gel electrophoresis (section 2.2.1.2). The remaining DNA was electrophoresed on an agarose preparative gel then viewed and excised on an UV transilluminator at minimum intensity. The DNA was purified from the gel using buffers provided in BIO 101's The GENECLEAN II® Kit (BIO 101 Inc., CA, USA).

Purification of DNA utilising The GENECLEAN II® Kit involved mixing the excised gel piece with NaI solution (3 volumes) and incubating at 50°C until the gel had melted, mixing at 1 min intervals. 'Glassmilk' (5 μL) was added to the melted gel solution and incubated (20°C, 5 min), mixing at 1 min intervals. The silica beads were then pelleted by centrifugation (13,000 x g, 20°C, 30 sec) and washed with New Wash buffer (3 x 500 μL). Elution of the DNA from the silica beads was carried out by resuspending and incubating the beads in 15 μL TE buffer (50°C, 5 min) (Appendix 3A). The beads were pelleted by centrifugation (13,000 x g, 20°C, 5 sec) and the supernatant containing the DNA was retained.

#### *2.2.1.5 Ligation of LAMP-1 cDNA and the Plasmid Vector*

The full length and truncated LAMP-1 cDNAs were ligated with the linearised expression vector, pGRE-neo.N-dhfr, by combining the fragments in a 1:1 molar ratio with 0.5 U of T4 DNA ligase (Bresatec, Adelaide, Australia) and a final concentration of 1x ligation buffer. The 20 μL ligation mix was incubated at 20°C, overnight.

### *2.2.1.6 Heat Shock Transfection of E. coli*

Plasmid DNA were amplified in *E. coli* strain MC1061 by heat shock transfection. MC1061 cells were made competent by inoculating 1 L of LB broth (Appendix 3B) with 5 mL of an overnight culture, grown from a single colony, and incubating at 37°C, with shaking, until an $OD_{600}$ of 0.2 was reached. The cells were cooled on ice (10 min) then pelleted by centrifugation (2,500 x g, 0°C, 10 min). Cells were resuspended in ½ volume (approximately 250 mL) of ice-cold 50 mM $CaCl_2$, 10 mM PIPES/HCl, pH 6.6, 15% (v/v) glycerol, and incubated on ice (30 min). Cells were then repelleted and resuspended in a $^1/_{20}$ volume (approximately 25 mL) of the same buffer, snap frozen (1 mL aliquots) in liquid nitrogen and stored at –70°C.

The ligation mix (10 µL) was added to 200 µl of thawed competent MC1061 cells and incubated on ice (10 min) then heat shocked (37°C, 5 min). Pre-warmed LB broth (200 µL) was added and incubated (37°C, 30 min) to allow the cells to recover from the heat shock. Cells were pelleted by centrifugation (2,000 x g, 4°C, 2 min), then 200 µL of the supernatant discarded and the pellet was resuspended in the remaining 250 µl and plated onto LB-Amp plates (Appendix 3B), containing 100 µg/mL ampicillin. Plates were incubated overnight at 37°C.

### *2.2.1.7 Purification of Plasmid Vectors*

To obtain plasmid vector from large numbers (> 20) of individual MC1061 clones, individual colonies from LB-Amp plates were picked with a toothpick and dropped into 1 mL of LB broth containing 100 µg/mL ampicillin. Cultures were grown with vigorous shaking (37°C, 5 hrs), then 0.5 mL of each culture was pelleted by centrifugation (2,000

x g, 4°C, 30 sec). Cell pellets were resuspended in 100 µL of 2.5 M LiCl, 50 mM Tris/HCl, pH 8.0, 4% (v/v) Triton X-100, 62.5 mM EDTA. To extract the plasmid DNA, phenol/chloroform (1:1) was added (100 µL), with vigorous vortexing for 5 sec. After centrifugation (13,000 x g, 4°C, 5 min), 90 µL of the aqueous phase was vortex mixed with 60 µL of isopropanol then centrifuged (13,000 x g, 4°C, 5 min). The resulting pellet was washed with 70% (v/v) of ice-cold ethanol, air-dried and redissolved in 20 µL TE buffer (Appendix 3A).

Large-scale purification of the plasmid DNA was performed using Bresatec's BRESApure™ Plasmid Midi Kit (Bresatec, SA, Australia) and the buffers and solutions provided in the kit as follows. Cells from an overnight culture of recombinant MC1061 clones (100 mL) were pelleted by centrifugation (6,000 x g, 4°C, 10 min) then resuspended in ice-cold BPR buffer (4 mL) containing RNase A (100 µg/mL). BPL buffer (4 mL) was added, mixed by inversion and incubated (20°C, 5 min). BPN buffer (4 mL) was then added, mixed by inversion, centrifuged (15,000 x g, 4°C, 10 min) and the supernatant retained. A BRESApure column was equilibrated with 10 mL of BPQ buffer after which the supernatant was applied onto the column. The column was washed twice with 10 mL of BPW buffer before eluting the plasmid DNA with 5 mL of BPE buffer. The eluted DNA was precipitated with 3.5 mL of isopropanol and pelleted by centrifugation (15,000 x g, 4°C, 30 min). The resulting pellet was washed with 2 mL of 70% (v/v) ethanol, air-dried, then redissolved in 100 µL of TE buffer (Appendix 3A).

### *2.2.1.8 Quantification of DNA*

DNA purified by BRESApure™ Plasmid Midi Kit was quantified by diluting it 1:100 in water, measuring its absorbance at 260 nm and using the following formula:

$OD_{260}$ = 1.0 for 50 μg/mL solution of DNA (Sambrook *et al.*, 1989).

### *2.2.1.9 Calcium Phosphate Transfection of Human Cell Lines*

The protocol used for calcium phosphate transfection of human fibrosarcoma cells (HT1080) was based largely on the established method given in Ausubel *et al.* (Ausubel *et al.*, 1997), with slight alterations (T. Litjens, personal communication). Cells to be transfected were grown to confluence in 10 cm diameter culture dishes. Plasmid DNA samples (5 μg per transfection) for transfection were precipitated with 3 μL of 1 M sodium acetate/acetic acid, pH 5.0, 26 μL of 95% (v/v) ethanol (final volume of 39 μL) and pelleted by centrifugation (13,000 x g, 20°C, 5 min). The DNA pellet was washed with 95% (v/v) ethanol (30 μL), centrifuged (13,000 x g, 20°C, 3 min), and the pellet resuspended in water (450 μL), finally 2.5 M $CaCl_2$, (50 μL) was added. The DNA/$CaCl_2$ complex solution (500 μL) was added drop-wise to a tube containing 2 x HEPES-buffered saline (500 μL) (Appendix 3A) while vortexing continuously. The mixture was vortexed for a further 30 sec then left at 20°C for 60 sec. The DNA mixture was then added drop-wise to the dish containing the cells to be transfected. After an overnight incubation in a 37°C, 5% $CO_2$, humidified incubator, the medium from the cell lines was replaced with fresh medium and the cells were incubated for a further 48 hrs.

#### *2.2.1.10 Harvesting of Cells*

Cells were harvested by removing the medium and washing the cells once with Dulbecco's phosphate-buffered saline (PBS). CHO-K1 and HT1080 cells were then trypsinised by adding enough 20% (v/v) trypsin-versene solution (CSL Ltd., Australia) to cover the cells and incubating (20°C, 5 min). For PANC and skin fibroblasts, neat trypsin was used. Cells were dislodged from the surface of the flask by tapping the sides of the flask with the palm of the hand. Harvested cells were then split into flasks containing fresh media.

#### *2.2.1.11 Selection of Recombinant Clones*

Dishes containing confluent transfected cell lines were harvested (section 2.2.1.10). The harvested cells were split 1:3, 1:10, 1:30 and 1:100, in duplicates, into fresh 10 cm diameter culture dishes containing selection medium (Appendix 3B). A 1:3 and 1:10 split of control cells (wild type HT1080 or PANC) were also carried out into selection medium. Cells were incubated in a 37°C, 5% $CO_2$, humidified incubator for 10 days. Transformed clones appeared on the plates as colonies, these were washed once with PBS then visually selected and picked by placing cloning rings over the colonies to prevent contamination from neighbouring colonies. After the colonies had been harvested, they were transferred to individual wells of a 96 well plate and incubated for approximately 7 days then transferred into 24 well plates. Three day condition media and cell homogenates (section 2.2.2) were obtained from wells containing confluent clones and wild type cells (control), and were analysed for recombinant LAMP-1 levels (section 2.2.11).

#### *2.2.1.12 Cloning of Cells*

To ensure a clonal cell line of CHO-K1, HT1080 and PANC expressing human LAMP-1, the highest expressing cells were cloned by limiting dilution. The cells were grown to confluence in a 25 $cm^2$ flask, harvested and transferred into a tube containing 1 mL of selection medium. The cells were counted, then diluted into selection medium to achieve a final concentration of 8 cells/mL and pipetted into two 96 well plates (200 µL/well). Plates were incubated at 37°C, 5% $CO_2$ (humidified) until clones could be visualised.

#### *2.2.1.13 Amplification of Expression*

To select for clones with the highest copy number of LAMP-1 DNA and thus increase the expression of LAMP-1, cells were grown in media containing methotrexate (MTX). MTX concentration was increased from 200 nM to 25 µM (for CHO-K1), doubling the concentration approximately every 4 days. LAMP-1 levels in the medium and cell lysates were quantified by the LAMP-1 immunoassay (section 2.2.11). Cells were then continued to be grown in the MTX concentration in which the cell lines expressed the highest LAMP-1 levels.

#### *2.2.1.14 Freezing Down of Cells*

Clones positively expressing LAMP-1 were grown to confluence in 75 $cm^2$ flasks. Each flask was harvested (section 2.2.1.10), and the harvested cells were washed twice with PBS. After resuspending gently in 1 mL of selection medium containing 10% (v/v) DMSO, cells were pipetted into freezing vials, snap frozen then stored in liquid nitrogen.

### 2.2.2 Preparation of Cell Lysates

Cells were harvested (section 2.2.1.10), washed with PBS and resuspended in PBS containing 1% (v/v) NP40 (1 mL per 75 $cm^2$ flask). Cells were lysed by six freeze/thaw cycles and the cell debris pelleted by centrifugation (13,000 x g, 4°C, 5 min).

### 2.2.3 Protein Determination

Protein determination was carried out using the established micro assay, bicinchoninic acid method of Smith, *et al.* (Smith *et al.*, 1985). Bovine serum albumin was used as the assay calibrator. Samples were read on a Ceres 900 HDI plate reader (Bio Teck Instruments Inc., VT, USA).

### 2.2.4 Preparation of Affinity Columns

For the preparation of affinity columns, proteins were coupled to Affi-Gel 15 whilst antibodies were coupled to Affi-Gel 10 (Bio-Rad, CA, USA). The desired quantity of Affi-Gel slurry was washed on a vacuum flask with ice cold water for injection (10 volumes), followed by coupling buffer (0.1 M $NaHCO_3$/NaOH, pH 8.5, 10 volumes). The slurry was transferred to a chromatography column and the protein to be coupled was diluted (1-10 mg/mL) in coupling buffer to an equal volume as that of the gel. The diluted protein or antibody was added onto the column containing the Affi-Gel and mixed on a rotator (4°C, overnight). The protein/antibody bound Affi-Gel column was then washed with coupling buffer until the gel was free of reactants, as indicated by the absorbance at 280 nm. The column was then equilibrated with PBS (pH 7.4), washed with elution buffers 1 M NaCl/PBS, 0.1 M $H_3PO_4$/NaOH (pH 2.5), pre elution buffer 10 mM $NaH_2PO_4$/NaOH (pH 6.5), and 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5). Columns were stored in 0.02% sodium azide/PBS at 4°C when not in use.

### 2.2.5 Purification of LAMP-1 Proteins

The recombinant full length LAMP-1 was purified from CHO and HT1080 cells, and truncated LAMP-1 from the media as described below.

#### *2.2.5.1 Purification of Full Length LAMP-1*

CHO and HT1080 cells expressing full length LAMP-1 were grown in 6 x 1700 $cm^2$ roller bottles until confluent, harvested with 20% (v/v) trypsin (20 mL) and washed with PBS (2 x 40 mL). Total cellular membranes were prepared by resuspending cells in 20 mL of PBS and adding NaCl to a final concentration of 1 M. Protease inhibitors (1 μg/mL leupeptin, 1 μM pepstatin and 200 μM PMSF) were then added to the cell suspension. Cells were placed in ice and broken by forcing through a 26-gauge needle (x10). Cells were then clarified by centrifugation (13,000 x g, 4°C, 10 min) and the resulting supernatant retained. The cell pellet was resuspended in PBS (5 mL) and was again forced through a 26-gauge needle (x10) then clarified (13,000 x g, 4°C, 10 min). The forcing of cells through the needle and clarification process was repeated 6 times. The supernatant was pooled after each clarification step. The membrane containing the full length LAMP-1 in the collected supernatant was pelleted by ultracentrifugation (100,000 x g, 4°C, 60 min). LAMP-1 was solubilised from the total cellular membrane by resuspending the membrane pellet in 1% (w/v) 3-{(3-cholamidopropyl)-dimethylammonio}-1-propanesulfonate (CHAPS)/PBS (20 mL). Protease inhibitors were added to the membrane suspension with NaCl (1 M). The membrane suspension was then dounce homogenised (x10) and subjected to six freeze/thaw cycles. The homogenate was clarified by ultracentrifugation (100,000 x g, 4°C, 60 min). The supernatant containing the full length LAMP-1 was retained and dialysed against PBS (1

L, 4°C, overnight). Dialysed supernatant was affinity purified over an anti-LAMP-1 monoclonal antibody affinity column (section 2.2.5.3).

### *2.2.5.2 Purification of Truncated LAMP-1*

CHO and HT1080 cells expressing truncated LAMP-1 were grown to confluence in two-tier cell factories with 250 mL of media. Cells were conditioned with serum free media (200 mL, 6-7 days), grown back to confluence on media with 1% (v/v) foetal calf serum (FCS) and conditioned again with serum free media. Condition media was collected and ultracentrifuged (100,000 x g, 60 min). Media were pooled until 1 L had been collected. The pooled media was then concentrated (10x) in a hollow fibre concentrator (Amicon, MA, USA). The media was subjected to octanoic precipitation by the slow addition of octanoic acid whilst vigorously stirring for 30 mins, as described in Harlow and Lane (Harlow and Lane, 1988). Octanoic acid precipitated media was clarified by centrifugation (6,000 x g, 15 min) and dialysed against PBS (4°C, overnight). Dialysed media was further concentrated (10x) by ultrafiltration over an Amicon™ YM10 membrane (Amicon, MA, USA). The concentrated media was then affinity purified over an anti-LAMP-1 monoclonal antibody affinity column (section 2.2.5.3).

### *2.2.5.3 Affinity Purification of LAMP-1*

Solubilised full length LAMP-1 from CHO and HT1080 cell membranes and truncated LAMP-1 from CHO and HT1080 media were affinity purified over columns of Affi-Gel 10 (Bio-Rad, CA, USA) coupled with either anti-LAMP-1 monoclonal antibody 4F5 (3 mg/mL) or BB6 monoclonal antibody (10 mg/mL) (section 2.2.4). Full length or truncated LAMP-1 solution was applied onto the column at a flow rate of 0.25 mL/min.

The column was washed with PBS until all unbound protein was removed as determined by absorbance at 280 nm. The LAMP-1 protein was then eluted with 0.1 M $H_3PO_4$/NaOH, pH 2.5, at 0.25 mL/min. Eluted LAMP-1 proteins were dialysed against PBS (4°C, overnight).

### 2.2.6 Production and Purification of Anti-LAMP-1 Antibodies

Five anti-LAMP-1 antibodies were used in this study; BB6, 4F5 and 1B1 monoclonal antibodies, and rabbit anti-LAMP-1 and rabbit anti-LAMP-1 (Tail) polyclonal antibodies. The BB6 monoclonal antibody was produced by S. Carlsson (University of Umea, Sweden) (Carlsson and Fukuda, 1989). The 4F5 monoclonal antibody was produced as described by Meikle *et. al.* (Meikle *et al.*, 1997). The production of rabbit anti-LAMP-1 and rabbit anti-LAMP-1 (Tail) polyclonal antibodies have been previously described (Dahlgren *et al.*, 1995; Meikle *et al.*, 1999b). The anti-LAMP-1 monoclonal antibody clone 1B1 was produced by E. Ravenscroft (Women's and Children's Hospital, SA, Australia). The 1B1 anti-LAMP-1 monoclonal antibody generated against truncated LAMP-1 purified from the media of the CHO expression cell line (section 2.2.5.2). Female Balb/C mice were immunised according to the schedule and method described in Meikle *et al.* (Meikle *et al.*, 1997). Spleen cells of mice immunised with LAMP-1 were harvested and fused with P3.653 myeloma cells as described by Zola and Brooks (Zola and Brooks, 1982).

#### *2.2.6.1 Purification of Anti-LAMP-1 Polyclonal Antibodies*

Rabbits immunised with LAMP-1 were bled and serum containing the polyclonal antibody against LAMP-1 was collected. The serum was centrifuged (13,000 x g, 4°C, 5 min) and filtered through a 0.2 μm filter. A 5 mL Hitrap™ protein G column

(Pharmacia Biotech, Uppsala, Sweden) was washed with 0.1 M $H_3PO_4$/NaOH, pH 2.5, and then equilibrated with PBS. Rabbit serum was diluted 1:1 with PBS and applied at a flow rate of 0.5 mL/min, the column washed with PBS until all unbound protein was removed as determined by absorbance at 280 nm. The bound antibodies were eluted with 0.1 M $H_3PO_4$/NaOH, pH 2.5, and collected in 1 mL fractions. Eluted fractions were then neutralised with a 1:10 volume of 1M $Na_2HPO_4$, pH 9.1 and the $OD_{280}$ determined. Fractions containing anti-LAMP-1 polyclonal antibody were pooled and dialysed against PBS (4°C, overnight). The dialysed antibody was filtered through a 0.2 μm filter and purified on a LAMP-1 affinity column.

Rabbit anti-LAMP-1 polyclonal antibody was affinity purified over an Affi-Gel 15 affinity column (section 2.2.4) containing truncated LAMP-1 purified from the media of the CHO expression cell line (section 2.2.5.2). The polyclonal antibody was applied onto the column at a flow rate of 0.25 mL/min. The column was washed with PBS until all unbound protein was removed as determined by absorbance at 280 nm. The polyclonal antibody was then eluted with 0.1 M $H_3PO_4$/NaOH, pH 2.5, at 0.25 mL/min. Eluted antibody were dialysed against PBS (4°C, overnight).

Rabbit anti-LAMP-1 (Tail) polyclonal antibody was affinity purified on a matrix of a synthetic LAMP-1 cytoplasmic tail peptide (CLVGRKRSHAGYQTI) (5 mg) coupled to Sepharose, agarose gel (5 mL) (Mimotopes, Melbourne, Australia). The conditions of purification were as described above.

### 2.2.6.2 *Purification of Anti-LAMP-1 Monoclonal Antibodies*

Anti-LAMP-1 monoclonal antibody was produced by growing hybridoma cells in dialysis tubing, suspended in a tissue culture flask containing RPMI/10% (v/v) FCS media (diacults). The diacult media (125 mL) was changed twice a week for 3 weeks and incubated at 37°C in a 5% $CO_2$ humidified incubator. Diacult cells were harvested by centrifugation (2,000 x g, 10 min), the supernatant retained and filtered through a filter (0.2 μm). Anti-LAMP-1 monoclonal antibodies in the diacult supernatant were purified over a 5 mL Hitrap™ protein G column using the conditions as described above (section 2.2.6.1).

### 2.2.6.3 *Concentration and Storage of Antibodies*

Monoclonal and polyclonal antibodies were concentrated by ultrafiltration over an Amicon™ YM10 membrane or in an Amicon™ Centricon 10 Concentrator (Amicon Inc., MA, USA). Concentrated antibodies were clarified by centrifugation (13,000 x g, 4°C, 10 min) and stored 4°C with sodium azide (0.02%).

### 2.2.6.4 *Quantification of Anti-LAMP-1 Antibodies*

Monoclonal and polyclonal antibodies were quantified based on their absorbance at 280 nm using the following formula:

$OD_{280}$ = 1.4 for 1 mg/mL protein (Harlow and Lane, 1988).

### 2.2.6.5 *Europium Labelling of Anti-LAMP-1 Antibodies*

Purified monoclonal and polyclonal anti-LAMP-1 antibodies were labelled with $Eu^{3+}$ using the DELFIA® $Eu^{3+}$-labelling kit following the manufacturer instructions (Wallac, North Ryde, Australia). Labelled antibodies were purified on a Superose 12 (1 x 30 cm),

fast phase liquid chromatography (FPLC) column (Pharmacia, Uppsala, Sweden) by E. Ravenscroft (Women's and Children's Hospital, SA, Australia). The column was equilibrated with 50 mM Tris/HCl, pH 7.8, 0.9% (w/v) NaCl and the samples applied at a flow rate of 0.5 mL/min. BSA was added to purified labelled antibodies to a final concentration of 0.1%, lyophilysed and stored at –20°C.

### 2.2.7 Epitope Mapping of Anti-LAMP-1 Antibodies

To identify epitopes that the anti-LAMP-1 antibodies recognise, a peptide library was generated from the LAMP-1 amino acid sequence (Mimotopes, Melbourne, Australia). The method for the synthesis of peptides for epitope mapping are described in Rodda and Tribbick (Rodda and Tribbick, 1996). The peptides were each 15 amino acids in length and biotinylated with an –SGSG- tetrapeptide spacer to prevent steric hindrance of the binding of antibody to the peptide. Each peptide had an overlap of 7 amino acids with the previous peptide. A total of 52 peptides were synthesised, representing the entire amino acid sequence of LAMP-1.

To epitope map anti-LAMP-1 antibodies, 96 well microtitre plates were coated with streptavidin diluted in water (5 μg/mL). The plates were frozen and placed in a lyophiliser overnight. Non-specific absorption of the plate was blocked by incubating each well with 200 μL of blocking buffer (1% (w/v) BSA, 0.1% (v/v) Tween 20/PBS) into each well (20°C, 60 min). Plates were washed (x4) with 0.1 % (v/v) Tween 20/PBS. Each of the 52 LAMP-1 biotinylated peptides were dispensed into each well (55 pmol/100 μL) and incubated on a plate shaker (60 min). Plates were washed and 100 μL of anti-LAMP-1 monoclonal antibody (1 μg/mL) or polyclonal antibody (10 μg/mL), diluted in 0.1% (w/v) sodium azide, 0.1 % (v/v) Tween 20/PBS was added. Plates were

washed (x6) with DELFIA® wash buffer. Bound antibody was then detected with either $Eu^{3+}$-labelled rabbit anti-mouse-IgG or $Eu^{3+}$-labelled sheep anti-rabbit-IgG (Silenus Lab., Hawthorn, Australia), diluted in DELFIA® assay buffer (0.1 μg/mL, 100 μL/well). Plates were incubated on a plate shaker (20°C, 60 min), then washed (x6) with DELFIA® wash buffer. DELFIA® enhancement solution (200 μL) was then added to each well, shaken (20°C, 10 min) and the fluorescence read on a WALLAC 1234 DELFIA® Research Fluorometer (Wallac, North Ryde, Australia).

### 2.2.8 Characterising Expressed LAMP-1

HT1080 expression cell lines producing full length and truncated LAMP-1 were characterised by immunofluorescence staining and flow cytometry.

#### *2.2.8.1 Immunofluorescence Staining*

To characterise the expression of full length and truncated LAMP-1 in the HT1080 expression cell lines, cells were grown in 2 chambered slides (37°C in a 5% $CO_2$ humidified incubator, 24 hr). Cells on the slide were washed (x2) with PBS (500 μL) and fixed by incubating with 1% (v/v) formaldehyde/PBS (200 μL, 4°C, 30 min), then washing with methanol (2 x 500 μL). Cells were washed (x2) with PBS then blocked with 10% (v/v) heat inactivated FCS/PBS (200 μL, 3 x 15 min). The cells were permeated and the primary antibody was bound by incubating with a 1:1 mixture of 1.5 mg/mL digitonin/PBS and primary antibody (20°C, 3 hr). Cells were then washed with PBS (3 x 5 min) and incubated in the dark (4°C, 1 hr) with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse-IgG secondary antibody (Silenus) which had been absorbed against 10 mg/mL ovalbumin/BSA affigel. Cells were washed with PBS (3 x 5

min), the chambers were then removed from the slide, and coverslips were mounted in 50% (v/v) glycerol containing 2% (v/v) diazabicyclo(2.2.2.)octane (DABCO). Cells were examined using an Olympus BX40 (Olympus Optical Co., Tokyo, Japan) epifluorescent microscope and photos taken with Cytovision photo-capturing software (Applied Imaging Co., CA, USA) at 100x magnification.

#### *2.2.8.2 FACScan Analysis*

To specifically analyse the cell surface expression of LAMP-1 in the expression cell lines, flow cytometry was performed. Cells were harvested and resuspended with 0.5% (w/v) BSA/PBS at a final concentration of 2.5 - 5 x $10^6$ cells/mL in FACScan tubes. Non-specific binding of primary antibodies was blocked by the addition of Intragam (20 µL) in each tube of cells, vortex mixed and incubated (4°C, 10 min). Cells were pelleted by centrifugation (6,000 x g, 4°C, 1 min) and resuspended in 0.5% BSA (1 mL), this was repeated twice. Cells were incubated with either FITC-conjugated anti-sialyl Lewis X CD15s monoclonal antibody (Pharmingen, CA, USA) or unlabelled BB6 monoclonal primary antibody (100 µg/mL, 4°C, 60 min). Cells were washed and blocked with Intragam as before. For cells labelled directly with conjugated antibodies, analysis was carried out after the primary incubation and wash steps. Where secondary antibodies were required, cells were resuspended and incubated with 10 µL (1:20 dilution) of rabbit anti-mouse-phycoerythrin (PE)-conjugated antibody (4°C, 30 min). After a final wash with 0.5% (w/v) BSA (2 x 1 mL), labelled cells were resuspended in 0.5% (w/v) BSA (500 µL) and analysed using the Cellquest software (Becton Dickinson, NJ, USA).

To check for background fluorescence the following negative primary antibodies were used; FITC conjugated IgM antibody (for CD15s antibody directly conjugated to FITC) and unlabelled IgG (for IgG antibody, BB6) (Pharmingen, CA, USA).

### 2.2.9 Size Exclusion Chromatography

A Superose 12 FPLC column (1 x 30 cm) (Pharmacia Biotech, Uppsala, Sweden) was used to evaluate LAMP-1 aggregation and to identify the LAMP-1 form present in normal and LSD affected plasma. Size exclusion chromatography was carried out on a Bio-Rad Duo Logic Flow system (Bio-Rad, CA, USA). The Superose 12 column was equilibrated with PBS. Samples were applied at a flow rate of 0.5 mL/min, eluted with PBS and collected in 1 mL fractions.

### 2.2.10 Identifying Proteins that Interact with LAMP-1 in Circulation

Plasma proteins that interact with LAMP-1 were isolated by applying plasma over three LAMP-1 affinity columns. Two columns contained truncated LAMP-1 produced by the CHO and HT1080 expression cell lines coupled to Affi-Gel 15 (section 2.2.4), and a third column was prepared by coupling a synthetic LAMP-1 cytoplasmic tail peptide coupled agarose gel (Mimotopes, Victoria, Australia).

#### *2.2.10.1 Isolating Plasma Proteins that Interact with LAMP-1*

A pre-column consisting of BSA coupled to Affi-Gel 15 was connected directly to each of the three LAMP-1 columns (section 2.2.10). Plasma (10 mL) collected from healthy individuals was applied onto the BSA-coupled Affi-Gel 15 column at a flow rate of 0.5 mL/min. The pre-cleared plasma was then allowed to flow through the LAMP-1 column and recirculated back onto the BSA and LAMP-1 columns (x5) (Figure 2.1). After

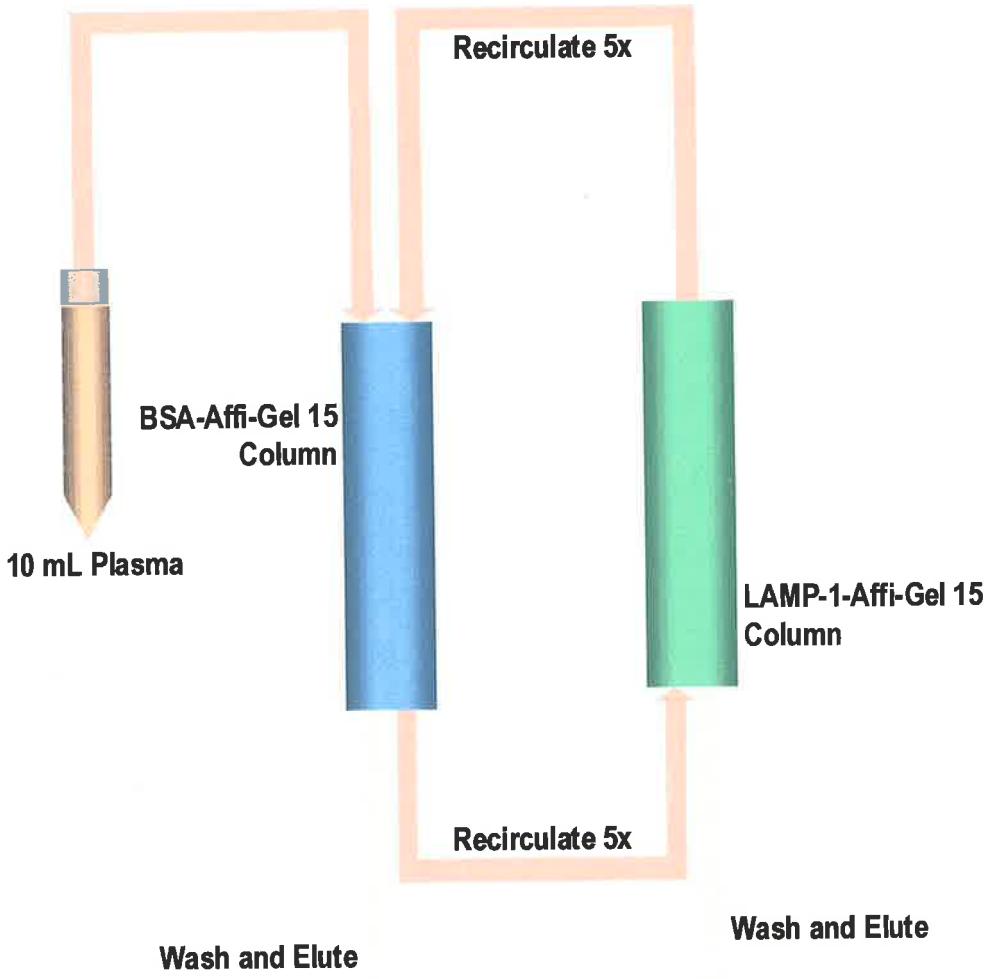



***Figure 2.1 Isolating Plasma Proteins that Bind to LAMP-1 by Affinity Chromatography***

Plasma (10 mL) was applied onto the BSA pre-clear column connected to a LAMP-1 column at a flow rate of 0.5 mL/min, then recirculated (5x). The columns were washed with PBS then seperated and plasma proteins were eluted with 1 M NaCl/PBS followed by 0.1 M $H_3PO_4$/NaOH (pH 2.5). The columns were cleaned with 10 mM $NaH_2PO_4$/NaOH (pH 6.5) and 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5).

recirculation, the sample was washed through with PBS at a flow rate of 1 mL/min until all unbound protein was removed as determined by absorbance at 280 nm. Plasma proteins were eluted with elution buffers 1 M NaCl/PBS and 0.1 M $H_3PO_4$/NaOH (pH 2.5) and collected in 1 mL fractions. To clean the LAMP-1 columns of remnant plasma proteins, columns were washed with pre elution buffer (10 mM $NaH_2PO_4$/NaOH, pH 6.5) followed by 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5). Eluted plasma proteins were analysed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and 2D gel electrophoresis (section 2.2.13).

### 2.2.11 LAMP-1 Immunoassays

LAMP-1 in samples was identified and quantified using a sandwich-type immunoassay, whereby primary antibodies against LAMP-1 were used to capture the protein and a secondary antibody, coupled to $Eu^{3+}$, was used to detect the amount of protein captured.

#### *2.2.11.1 Preparation of Truncated HT1080 LAMP-1 Standard*

Truncated LAMP-1 standard was obtained from affinity purified 6-7 day condition media of HT1080 truncated LAMP-1 expression cells (section 2.2.5). The affinity purified truncated LAMP-1 was then diluted in DELFIA® assay buffer at a stock concentration of 2 mg/L, aliquoted into 100 µL fractions and stored at -20°C.

#### *2.2.11.2 Preparation of Skin Fibroblast Membrane Full Length LAMP-1 Standard*

Full length LAMP-1 standard was obtained from a membrane preparation of skin fibroblast cells. Skin fibroblasts were grown to confluence in 15-20 x T75 flasks, and harvested with neat trypsin (2 mL per flask). Cells were resuspended in PBS (10 mL), NaCl added to a final concentration of 1 M and protease inhibitors (1 µg/mL leupeptin,

1 μM pepstatin and 200 μM PMSF) were added. Cells were lysed by six freeze/thaw cycles. Lysed cells were then dounce homogenised (x10) and the membranes pelleted by ultracentrifugation (100,000 x g, 4°C, 60 min). LAMP-1 was solubilised from the total cellular membrane pellet by resuspending in 1% (w/v) CHAPS/PBS (500 μL). Protease inhibitors were added and the membrane suspension was dounce homogenised (x10). LAMP-1 was isolated by ultracentrifugation (100,000 x g, 15 min). The supernatant containing the full length LAMP-1 was retained and stored at -20°C.

The concentration of the full length skin fibroblast LAMP-1 standard was determined with the BB6 LAMP-1 immunoquantification assay (section 2.2.11.3), using the truncated HT1080 LAMP-1 as a calibrator. Western analysis (section 2.2.14) was also carried out on the skin fibroblast full length LAMP-1 standard and compared to the truncated HT1080 LAMP-1 standard to estimate the concentration. The skin fibroblast LAMP-1 standard was diluted in DELFIA® assay buffer at a stock concentration of 2 mg/L. This was aliquoted into 100 μL fractions and stored at -20°C.

#### *2.2.11.3 Two-Step LAMP-1 Immunoassay*

For the quantification and detection of both full length and truncated LAMP-1, 96 well microtitre plates were coated with BB6 monoclonal antibody (5 μg/mL) diluted in 0.1 M $NaHCO_3$ (100 μL/well). Plates were coated overnight at 4°C and then washed (x1) with DELFIA® wash buffer. Samples and standards were diluted in DELFIA® assay buffer and added to the wells (100 μL/well), shaken (20°C, 10 min) and incubated (4°C, overnight). Plates were then washed (x6) with DELFIA® wash buffer. $Eu^{3+}$-labelled rabbit anti-LAMP-1 polyclonal antibody (0.2 mg/L) was added to the wells (100

μL/well) and incubated (37°C, 4 hrs). Plates were washed (x6) with DELFIA® wash buffer and DELFIA® enhancement solution (200 μL) was added to each well. Plates were shaken (20°C, 10 min) and the fluorescence read on a WALLAC 1234 DELFIA® Research Fluorometer (Wallac, North Ryde, Australia).

LAMP-1 concentrations in the samples assayed were calculated using a spline fit of the calibration curves. All LAMP-1 calibration curves, and samples were assayed in duplicate. For each LAMP-1 assay, the corresponding LAMP-1 calibration curve was located across the first row of the microtitre plate.

#### *2.2.11.4 One-Step Full Length LAMP-1 Immunoassay*

To specifically detect full length LAMP-1 in samples, plates were coated with anti-LAMP-1 (Tail) polyclonal antibody (5 μg/mL) diluted in 0.1 M $NaHCO_3$ (100 μL/well). Plates were coated overnight at 4°C and then washed (x1) with DELFIA® wash buffer. Samples and standards were diluted in DELFIA® assay buffer and added to the wells (50 μL/well) together with $Eu^{3+}$-labelled rabbit anti-LAMP-1 polyclonal antibody (0.4 mg/L, 50 μL/well), shaken (10 min at 20°C) and incubated (4°C overnight). Plates were washed (x6) with DELFIA® wash buffer and DELFIA® enhancement solution (200 μL) was added to each well, shaken (20°C, 10 min) and the fluorescence read on a WALLAC 1234 DELFIA® Research Fluorometer (Wallac, North Ryde, Australia).

### 2.2.12 Trichloroacetic Acid-Deoxycholate Precipitation of Proteins

To concentrate proteins from dilute solutions, trichloroacetic acid-deoxycholate (TCA-Doc) precipitation was carried out prior to SDS-PAGE analysis (section 2.2.13).

Dilute protein samples (up to 1 mL) were mixed with sodium deoxycholate (4 µL of a 2% stock solution) and incubated on ice (15 min). Trichloroacetic acid was then added to a final concentration of 6% and incubated on ice (30 min). Proteins were pelleted by centrifugation (13,000 x g, 15 min) and washed in 1% (v/v) triethylamine/acetone (200 µL). Proteins were then pelleted (13,000 x g, 15 min) and washed in diethylether (200 µL), pelleted again and air dried.

### 2.2.13 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the methods of Laemmli (Laemmli, 1970) on 12.5% acrylamide gels (80 x 100 x 0.75 mm). Samples were mixed with SDS loading buffer (Appendix 3A) containing 5% (v/v) mercaptoethanol and heated (95°C, 10 min). The polyacrylamide gels were run at a constant voltage (200 V) in a Hoefer minigel electrophoresis unit (Hoefer Scientific Instruments, CA, USA). Molecular weight of proteins were determined using semi-log plots of BenchMark™ Protein Ladder Standards or BenchMark™ Prestained Protein Ladder (Appendix 1) (Invitrogen™, Melbourne, Australia) versus the migration distance travelled.

#### *2.2.13.1 Two Dimensional Gel Electrophoresis*

Samples to be electrophoresed were prepared as follows. Samples were incubated with Lysis Buffer 1 (50 µL, 37°C, 30 min) (Appendix 3A). Lysis Buffer 2 (46 µL) (Appendix 3A) and urea (72 mg) were added to the sample with Pharmolyte 3-10 (1 µL) (Bio-Rad, CA, USA). The sample was then centrifuged (13 000 rpm, 15 min) and Rehydration Solution added (Appendix 3A) (80 µL).

In the first dimension, samples were applied onto Immobiline$^{TM}$ DryStrips (pH 3-10, 7 cm) (Amersham Pharmacia Biotech, NJ, USA) in the rehydration apparatus (Bio Technology Services, SA, Australia), incubating on a rocking platform (20°C, overnight). Isoelectric focussing of the sample was carried out on a flat bed Metaphor II apparatus (Pharmacia LKB, Uppsala, Sweden) using the following program:

Step 1: gradient 0-300 V, 1 min

Step 2: constant 300 V, 3 hr

Step 3: gradient 300-3500 V, 5 hr

Step 4: constant 3500 V, 13 hr

Step 5: gradient 3500-200 V, 30 min

Step 6: constant 200 V, 4 hr

Following isoelectric focussing, immobuline strips were incubated in Equilibration Solution 1 (Appendix 3A) (5 mL, 20°C, 15 min). The strips were then transferred into Equilibration Solution 1 that had been heated to 95°C and incubated (5 mL, 1 min) on a rocking platform. Strips were incubated in Equilibration Solution 2 (Appendix 3A) (5 mL, 20°C, 15 min). The strips were then fitted onto 4-20% mini 2D gradient gels (Bio-Rad, CA, USA) and the samples electrophoresed (180 V, 60 min) in a Bio-Rad Miniprotean II electrophoresis unit (Bio-Rad, CA, USA).

#### *2.2.13.2 Silver Staining of SDS-Polyacrylamide Gels*

Silver staining of SDS-polyacrylamide gels was performed according to the method of Chattaway and Barritt (1994), with minor modifications. Briefly, gels were incubated in fixative solution (40% (v/v) ethanol, 10% (v/v) acetic acid) for 60 min, then incubated with periodic acid solution (25 mM periodic acid, 25% (v/v) ethanol, 5% (v/v) acitic

acid) for 5 min and washed with water (3 x 10 min). The gels were then placed in incubation solution (0.5 M sodium acetate, 13 mM sodium thiosulphate, 0.13% (v/v) gluteraldehyde, 33% (v/v) ethanol) overnight, washed in water (3 x 10 min) and placed in silver nitrate solution (12 mM silver nitrate, 0.05% (v/v) formaldehyde) for 60 min. Following a brief rinse with water (2 min), the gels were placed in developing solution (0.24 M sodium carbonate, 0.05% (v/v) formaldehyde) for 15 min or until the protein bands reached the desired intensity. Development was stopped by placing the gels in stopping solution (40 mM EDTA) for 10 min. The gels were then washed in water (2 x 5 min) and incubated in preserving solution (10% (v/v) glycerol for 12.5% acrylamide gels or 70% (v/v) glycerol for 4-20% gradient gels) for 30 min and air dried between cellophane at 20°C.

#### *2.2.13.3 Coomassie Staining of SDS-Polyacrylamide Gels*

Following SDS-PAGE, gels were incubated in fixative solution (40% (v/v) ethanol, 10% (v/v) acetic acid) for 60 min. The fixed gels were stained with Brilliant Blue G-Colloidal stain (Sigma Chemical Co., MO, USA) according to the method described on the manufacture's instructions.

### 2.2.14 Western Analysis

Following SDS-PAGE, gels were transferred to NEN™ Life Science Products PolyScreen® PVDF Transfer Membrane (0.2 μM) (NEN™ Life Science Products Inc., MA. USA) with transfer buffer (200 mM Glycine, 25 mM Tris, 20% (v/v) methanol) in a Hoefer mini blot transfer apparatus (Hoefer Scientific Instruments, CA, USA). Transfer was carried out at a constant current (250 mA) for 70 min. Transferred membranes were blocked (20 mM Tris/HCl, 3% (w/v) BSA, 0.05% (v/v) Tween 20, pH

7.4) for 60 min. Membranes were then washed (3 x 10 min) in 100 mL wash solution (20 mM Tris/HCl, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.4). Each membrane was incubated with 10 mL of primary antibody (rabbit anti-LAMP-1 polyclonal antibody) (1 μg/mL), diluted in the wash solution (4°C, overnight). Membranes were then washed (3 x 10 min) with the wash solution and incubated with 10 mL of the secondary polyclonal antibody, Silenus® HRP-conjugated sheep anti-rabbit (Silenus Lab., Hawthorn, Australia), diluted 1:1000 in the wash solution (20°C, 60 min). The membranes were washed in the wash solution (3 x 10 min) then in PBS (1 x 10 min).

Development of the membranes was carried out in a dark room using Renaissance® NEN™ Life Science Products Western Blot Chemiluminescence Reagents (NEN™ Life Science Products Inc., MA, USA) and Kodak X-Omat™ diagnostic films (Eatman Kodak Co., NY, USA) as per manufacturer's instructions.

# CHAPTER THREE:

# Expression and Characterisation of LAMP-1

## 3 Introduction and Aims

The lysosome-associated membrane protein (LAMP-1) exists in two forms, the full length form, LAMP-1 (+Tail), and the truncated form, LAMP-1 (-Tail). LAMP-1 (+Tail) has an extensively glycosylated lumenal domain, a membrane spanning domain and also a short cytoplasmic tail. LAMP-1 (-Tail) contains only the lumenal domain. The mechanism by which LAMP-1 (-Tail) is produced is not known. It may result from the proteolytic degradation of LAMP-1 as part of the normal turnover of this protein within the lysosome (Meikle *et al.*, 1999b). LAMP-1 at the plasma membrane is reported to be the major molecule expressing the sialyl-Lewis X antigen on the cell surface. The sialyl-Lewis X antigen is found on the polylactosaminoglycans of the LAMP proteins and provides ligand structures for cell-adhesion molecules.

A Chinese hamster ovary (CHO-K1) cell line expressing LAMP-1 (-Tail) was previously established (Meikle *et al.*, 1999b). However, CHO cells have been reported to produce different post-translational modifications in recombinant human proteins, where the proteins have fewer carbohydrate side chains (Van Hove *et al.*, 1996; Tsujioka *et al.*, 1998). In order to study LAMP-1 and its interactions we aimed to express and characterise recombinant LAMP-1 (±Tail) in a human cell line in addition to the CHO cell line. Human fibrosarcoma (HT1080) and human pancreatic (PANC) cells were transfected with LAMP-1 and the best cell line was chosen. LAMP-1 (+Tail)

was expressed in the cell membrane whereas LAMP-1 (-Tail) was secreted into the cell media.

LAMP-2 (±Tail) were also expressed in HT1080 and PANC cells, however, as LAMP-1 is to be used for the newborn screening of LSD (section 1.2.1) it was our interest to further investigate LAMP-1 rather than LAMP-2. For this reason the expression of LAMP-2 (±Tail) has not been reported and only work done on LAMP-1 have been discussed.

## 3.1 Expression of LAMP-1 (±Tail) in HT1080 and CHO-K1 Cells

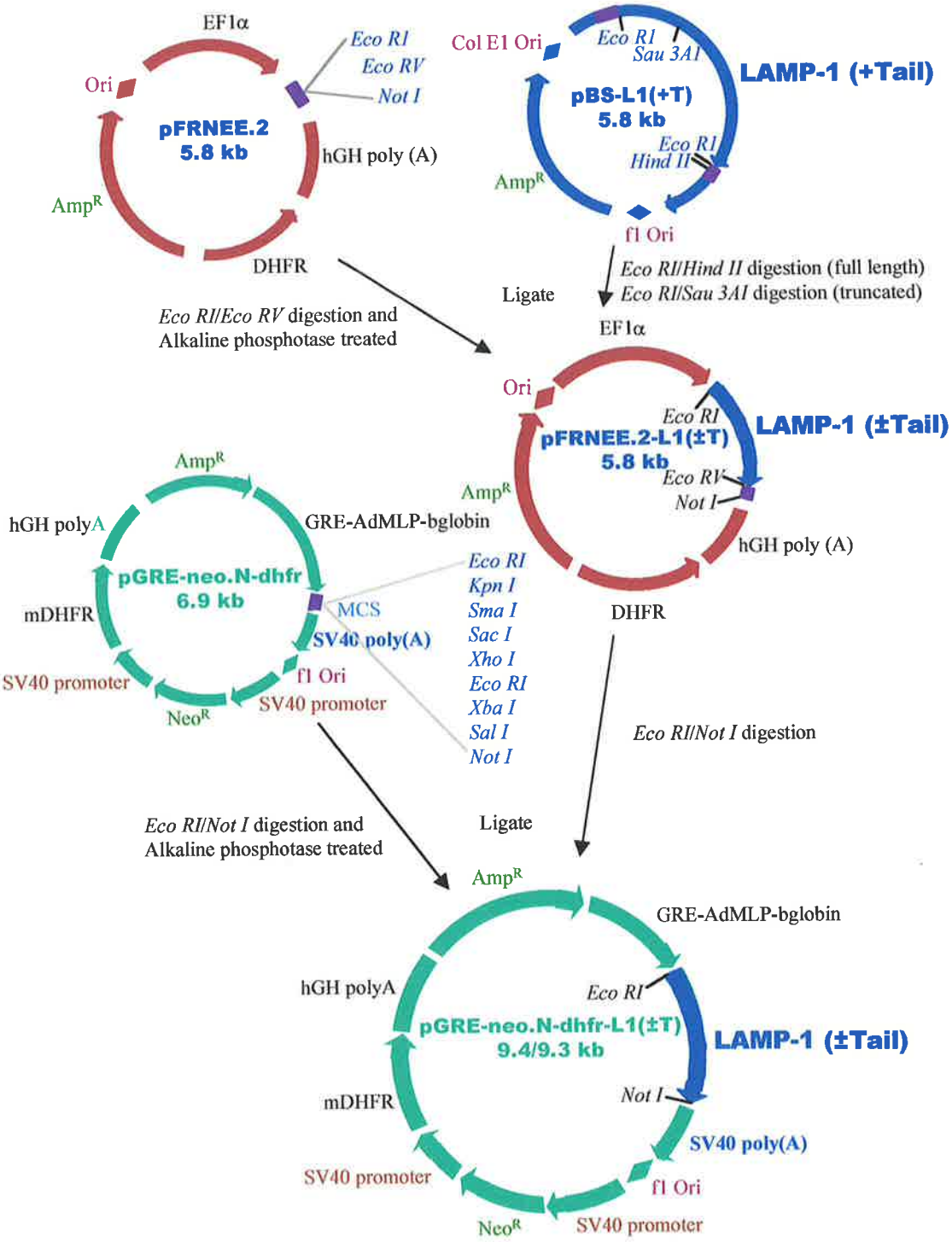
### 3.1.1 Construction of LAMP-1 (±Tail) Expression Vector

The full length and truncated LAMP-1 expression vectors, pGRE-neo.N-dhfr-LAMP-1 (+Tail) and pGRE-neo.N-dhfr-LAMP-1 (-Tail) respectively, were derived by ligating the LAMP-1 (+Tail) and LAMP-1 (-Tail) cDNA with the expression vector, pGRE-neo.N-dhfr (section 2.2.1). This work was carried out by C. Hua and E. Isaac (Women's and Children's Hospital, SA, Australia). Figure 3.1 outlines the construction of the recombinant vectors. LAMP-1 (+Tail) cDNA was isolated from the pBluescript-LAMP-1 vector by digestion with *Eco RI* and *Hind II* and blunt end ligated into the *Eco RI* and *Not I* site of pFRNEE.2. LAMP-1 (-Tail) was isolated from the pBluescript-LAMP-1 vector by digestion with *Eco RI* and *Sau 3AI*, which cleaved LAMP-1 (+Tail) at the membrane spanning domain (1335 bp), and blunt end ligated into the *Eco RI* and *Not I* site of pFRNEE.2. This expression vector was electroporated into CHO-K1 cells (Meikle *et al.*, 1999b). LAMP-1 (±Tail) cDNA was then isolated with *Eco RI* and *Not I* from pFRNEE.2-LAMP-1 (±Tail) vectors and inserted into the *Eco RI* and *Not I* site of pGRE-neo.N-dhfr, this was calcium phosphate transfected into HT1080 and PANC cells (section 2.2.1.9). The pGRE-neo.N-dhfr vector was used as it was shown to give highest recombinant protein expression in mammalian cells (T. Litjens, unpublished data).

The DHFR gene in the pFRNEE.2 and pGRE-neo.N-dhfr expression vectors encodes for the dihydrofolate reductase enzyme that converts folate to tetrahydrofolate, which is necessary for purine biosynthesis (Simonsen and Levinson, 1983; Lewin, 1994). The DHFR

***Figure 3.1 Construction of pGRE-neo.N-dhfr-LAMP-1 (±Tail) Expression Vectors***

LAMP-1 (+Tail) cDNA was excised with *Eco RI/Hind II* and LAMP-1 (-Tail) was excised with *Eco RI/Sau 3AI* from pBluescript. After ligation with pFRNEE.2, LAMP-1 (±Tail) cDNA were isolated with *Eco RI* and *Not I* and ligated into the *Eco RI* and *Not I* restriction sites of linearised pGRE-neo.N-dhfr to produce the pGRE-neo.N-dhfr-LAMP-1 (±Tail) vectors.


EF1α
Eco RI
Eco RV
Not I
Ori
pFRNEE.2
5.8 kb
hGH poly (A)
Amp^R
DHFR
Col E1 Ori
Eco RI
Sau 3AI
LAMP-1 (+Tail)
pBS-L1(+T)
5.8 kb
Eco RI
Hind II
Amp^R
f1 Ori
Eco RI/Hind II digestion (full length)
Eco RI/Sau 3AI digestion (truncated)
Ligate
Eco RI/Eco RV digestion and
Alkaline phosphotase treated
EF1α
Ori
Eco RI
LAMP-1 (±Tail)
pFRNEE.2-L1(±T)
5.8 kb
Eco RV
Not I
Amp^R
hGH poly (A)
DHFR
Amp^R
hGH polyA
GRE-AdMLP-bglobin
pGRE-neo.N-dhfr
6.9 kb
mDHFR
MCS
SV40 poly(A)
f1 Ori
SV40 promoter
SV40 promoter
Neo^R
Eco RI
Kpn I
Sma I
Sac I
Xho I
Eco RI
Xba I
Sal I
Not I
Eco RI/Not I digestion
Eco RI/Not I digestion and
Alkaline phosphotase treated
Ligate
Amp^R
GRE-AdMLP-bglobin
hGH polyA
Eco RI
pGRE-neo.N-dhfr-L1(±T)
9.4/9.3 kb
LAMP-1 (±Tail)
Not I
mDHFR
SV40 poly(A)
SV40 promoter
f1 Ori
Neo^R
SV40 promoter

gene is useful as it can also be used to amplify the gene of interest, that is LAMP-1. By slowly increasing the concentration of MTX, a potent inhibitor of the DHFR enzyme, DHFR is amplified to cope with the increased level of MTX and subsequently LAMP-1 is also co-amplified with DHFR (Ioannou *et al.*, 1992; Van Hove *et al.*, 1996).

LAMP-1 in the pFRNEE.2-LAMP-1 vector is constituvely expressed. However, in the pGRE-neo.N-dhfr-LAMP-1 vector, the dexamethosone (DEX) inducable SV40 promoter governs transcription of the DHFR gene and as the LAMP-1 cDNA is on the same transcript, LAMP-1 expression is also induced.

G418 is an aminoglycoside, an analogue of neomycin, gentamycin and kanamycin. It acts to block protein synthesis in mammalian cells by interfering with ribosomal function (Southern and Berg, 1982). G418 was used for the selection of clones containing the pFRNEE.2-LAMP-1 and pGRE.neo.N-dhfr-LAMP-1 expression vectors. The expression vectors consists of the aminoglycoside phosphotransferase gene (depicted in Figure 3.1 as $Neo^R$) which confers resistance to G418. Therefore, clones that express the $Neo^R$ gene are able to detoxify G418 and survive in its presence.

### 3.1.2 Selection of the Highest Expressing Cells

Positively transformed clones were selected by growing the cells in selection media containing 570 mg/L of G418 antibiotic. In addition to the G418 selection pressure, CHO-K1 was grown in α-minimum essential media (α-MEM) minus nucleosides. CHO-K1 is a DHFR negative cell line and thus cannot survive in the absence of exogenous nucleosides, however clones that contain the expression vector can. HT1080 and PANC cell lines were grown in MEM media containing G418. Positively

transfected clones were selected and grown to confluence in 24 well plates. At this time fresh media (500 μL) was added to each well of transfected CHO-K1 cells and fresh media (500 μL) containing 4 μM DEX was added to each well of transfected HT1080 and PANC cells to induce LAMP-1 expression. After 3 days, the condition media was collected from CHO-K1 and, HT1080 and PANC cells and the cells harvested into 50 μL of lysis buffer (1% (v/v) NP-40/PBS). These were analysed for LAMP-1 expression using the BB6 2-step assay, which detects both forms of LAMP-1 (section 4.3). Approximately 95% of the total LAMP-1 (+Tail) expressed by the clones was in the cell lysates whereas for LAMP-1 (-Tail) approximately 95% was secreted into the media. Cells were cloned by limiting dilution and frozen down for storage. The highest CHO-K1 clone expressing LAMP-1 (+Tail) was CHO L1+T7. CHO L1+T7 expressed LAMP-1 (+Tail) at an average of 6.6 μg/mg total cell protein. The highest CHO-K1 clone expressing LAMP-1 (-Tail) was CHO L1-T40, expressing LAMP-1 at an average of 2 mg/L. LAMP-1 was undetectable in wild type CHO cells and media. The highest HT1080 LAMP-1 (+Tail) clone was HT1080 F+T1, expressing at 2.1 μg/mg of total cell protein, compared to 0.5 μg/mg in wild type HT1080 cells. The highest HT1080 LAMP-1 (-Tail) clone was HT1080 F-T7, expressing at 3.1 mg/L, compared to 0.02 mg/L in the media of wild type HT1080 cells.

PANC cells were found to be slow growing, taking twice as long to reach confluence as compared to CHO-K1 and HT1080 cells. Moreover, expression levels of LAMP-1 (±Tal) were very low with LAMP-1 (-Tail) being expressed at 0.5 mg/L and LAMP-1 (+Tail) at less than 2 μg/mg. Thus HT1080 cells were chosen over PANC cells as our LAMP-1 expression system.

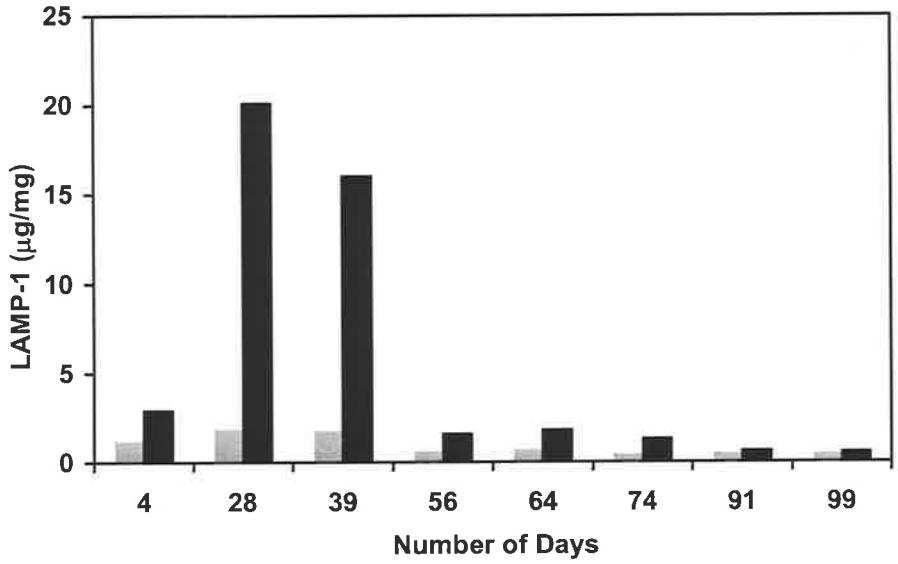
### *3.1.2.1 Effects of MEM and Iscoves Media on LAMP-1 Expression by HT1080 Cells*

Expression levels of LAMP-1 were examined for HT1080 grown in MEM as compared to that grown in Iscoves media. Iscoves contains higher sugar levels and as such may enhance expression of recombinant proteins. Cells were grown in either MEM or Iscoves and conditioned for 3 days, after which LAMP-1 in the cells and media were assayed. For HT1080 expressing LAMP-1 (-Tail), cells were conditioned in serum free media. HT1080 cells grown in MEM expressed slightly higher levels (1.1 mg/L) of LAMP-1 (-Tail) in the media as compared to that grown in Iscoves (0.9 mg/L), thus HT1080 cells expressing LAMP-1 (-Tail) was kept in MEM media. For HT1080 expressing LAMP-1 (+Tail), cells were grown in media supplemented with 10% (v/v) FCS. HT1080 cells grown in MEM expressed significantly lower levels of LAMP-1 (+Tail) in the cells (0.7 μg/mg) compared to that grown in Iscoves media (1.9 μg/mg), thus HT1080 cells expressing LAMP-1 (+Tail) was grown in Iscoves media.

### *3.1.2.2 Effects of Dexamethasone on HT1080 LAMP-1 Cell Expression*

To determine the extent of the effects of adding DEX to the media of HT1080 expression cell lines, cells were grown in the presence of DEX and LAMP-1 expression was assayed and compared to cells grown without DEX. Without DEX, expression of LAMP-1 was the same as that for wild type HT1080. The addition of DEX to cell selection media increased expression of LAMP-1. DEX was capable of inducing expression of LAMP-1 (+Tail) up to 10-fold (Figure 3.2A) and LAMP-1 (-Tail) up to 50-fold as compared to cells that had not been induced with DEX (Figure 3.2B). However, expression levels of LAMP-1 (+Tail) in HT1080 cells dropped after long periods of exposure to DEX (Figure 3.2A). The expression of LAMP-1 (-Tail) in the

A




B

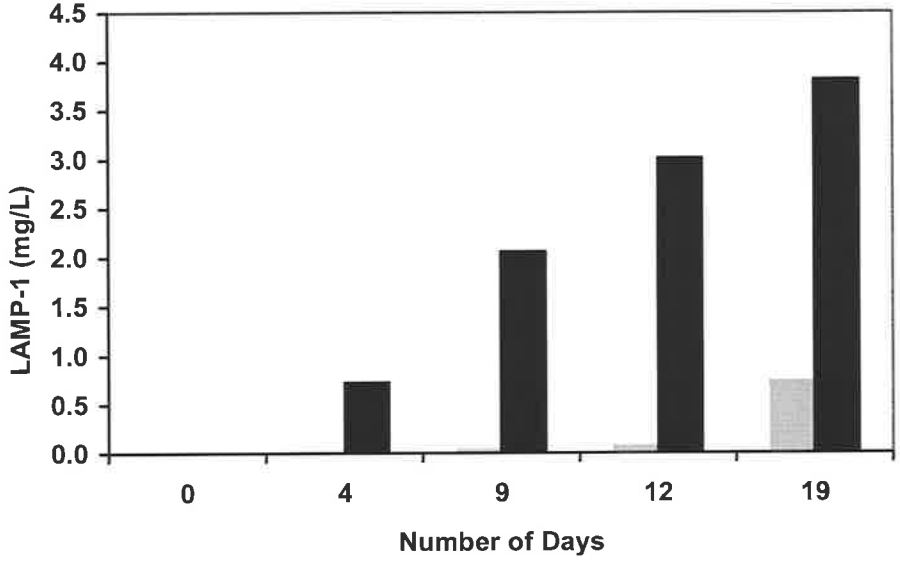



***Figure 3.2 Effects of Dexamethosone on LAMP-1 Expression in HT1080 Cell Lines.***

Cells were grown in media with and without dexamethosone (DEX) (4 µM). LAMP-1 in conditioned cells and media (3-6 days) were immunoquantified using the BB6 2-tier assay (section 2.2.11.3). Panel A, LAMP-1 expression with and without DEX in LAMP-1 (+Tail) expressing HT1080 cells; Panel B, LAMP-1 expression with and without DEX in LAMP-1 (-Tail) expressing HT1080 cell media. LAMP-1 levels expressed by HT1080 cells grown without DEX (■); LAMP-1 levels expressed by HT1080 cells grown in the presence of DEX (■).

cell media of HT1080 cells were monitored for approximately 12 months and was found to remain at the induced level (data not shown). Cell media from LAMP-1 (+Tail) expressing HT1080 cells were assayed for LAMP-1 in order to determine whether the cause of the decrease in expression of LAMP-1 (+Tail) in HT1080 cells was because the recombinant protein was not being retained within the cell but was secreted into the media. Recombinant protein was not secreted into the cell media, indicating that after long periods of exposure to DEX these cells reverted back to their uninduced state of expression.

### *3.1.2.3 Amplification of Clonal CHO-K1 and HT1080 Cell Lines*

In an attempt to increase expression levels of LAMP-1 in both HT1080 and CHO-K1 expression cell lines, MTX was added into the cell media. MTX was used to amplify the MTX-resistant DHFR gene in the pCI-neo.N-dhfr expression vector, this in turn would theoretically increase the copy number of the LAMP-1 gene and expression of the protein would therefore increase. MTX concentration was increased from 200 nM to 25 μM, doubling the concentration approximately every 4 days. Cells were resistant to MTX levels up to 6.4 μM thus amplification was either not evident or not significant, however when 12.8 μM and 25 μM MTX was added, cells with higher MTX resistance were selected and amplification became notable (Figure 3.3). There was a 3-fold increase in the level of LAMP-1 (+Tail) and LAMP-1 (-Tail) expressed by transfected CHO-K1 cells as compared to the unamplified parental clone. A greater amplification was expected, however because the original CHO-K1 clone was highly resistant to MTX, the potential for amplification was limited. Above 25 μM MTX, transport of MTX into the cell becomes the limiting factor preventing further gene amplification (Ioannou *et al.*, 1992; Van Hove *et al.*, 1996). When MTX selection pressure was

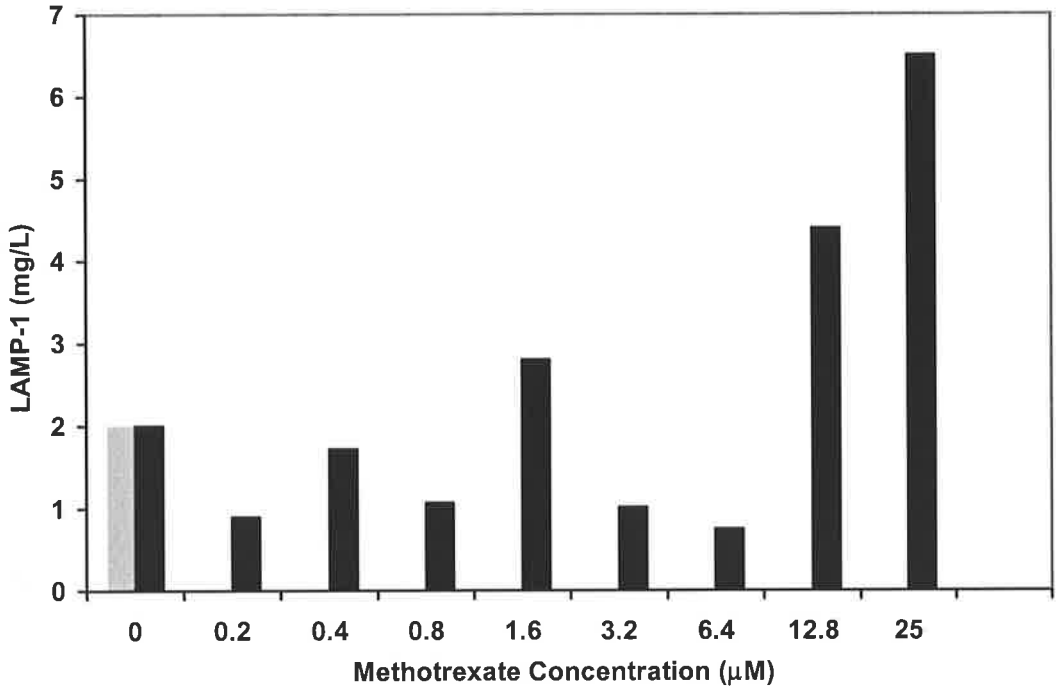



***Figure 3.3 Effects of Methotrexate Amplification of LAMP-1 (-Tail) CHO-K1 Expression Cells Lines.***

Methotrexate (200 nM) was added into the media of CHO-K1 cells expressing LAMP-1 (-Tail) and concentrations of MTX were doubled every 4 days up to 25 μM. LAMP-1 (-Tail) levels expressed by CHO-K1 cells grown without MTX (■); LAMP-1 (-Tail) levels expressed by CHO-K1 cells grown in the presence of MTX (■).

removed, expression of LAMP-1 (+Tail) and LAMP-1 (-Tail) remained stable at approximately 18.9 μg/mg and 6 mg/L, respectively. Amplification was attempted on HT1080 clones, however, the addition of MTX had no effect on expression levels.

## 3.2 Purification of Recombinant LAMP-1

To obtain large quantities of recombinant LAMP-1, cell factories and roller bottles of expressing cells were set up. HT1080 F+T1 cells were grown in 6 x 1700 $cm^2$ roller bottles in Iscoves media containing 4 μM DEX. CHO L1+T7 cells were grown in 6 x 1700 $cm^2$ roller bottles in α-MEM minus nucleosides media containing 25 μM MTX. HT1080 F+T1 and CHO L1+T7 cells were grown to confluence and harvested. HT1080 F-T7 cells were grown in 1264 $cm^2$ two-tiered cell factories in MEM media containing 4 μM DEX. CHO L1-T40 cells were grown in 1264 $cm^2$ two-tiered cell factories in α-MEM minus nucleosides media containing 25 μM MTX. HT1080 F-T7 and CHO L1-T40 cells were grown to confluence and condition media was collected.

### 3.2.1 Purification of LAMP-1 (+Tail)

Recombinant LAMP-1 (+Tail) was purified from HT1080 and CHO expression cells. Cells were lysed by forcing through a 26 gauge needle, LAMP-1 (+Tail) were then solubilised from the cell membranes with 1% (w/v) CHAPS (section 2.2.5.1). Expanding HT1080 F+T1 and CHO L1+T7 into roller bottles decreased expression to approximately 1 μg/mg and 1.8 μg/mg of total cell protein, respectively. From six roller bottles a total of about 80 μg of LAMP-1 (+Tail) was collected from the HT1080 F+T1 cells. Table 3.1 outlines the approximate recovery of each step of purification of HT1080 LAMP-1 (+Tail). CHO LAMP-1 (+Tail) recoveries were similar. Recovery of

LAMP-1 (+Tail) was very low with approximately 40% loss when membranes were lysed by 6 freeze/thaw cycles and a further 25% was lost when membranes were dialysed prior to affinity purification. Only 23% of HT1080 LAMP-1 (+Tail) was affinity purified from the 4F5 anti-LAMP-1 monoclonal antibody column. We were unable to obtain any CHO LAMP-1 (+Tail) that was sufficiently pure. In an attempt to improve purification yields of LAMP-1 (+Tail), a column with a different anti-LAMP-1 antibody was used. The BB6 anti-LAMP-1 monoclonal antibody was thought to have greater affinity for LAMP-1 as it works well in the immunoquantification assays, however purification yields was not improved. Other reports have used wheat germ agglutinin-agarose columns to purify LAMP-1 (Sawada *et al.*, 1993; Carlsson *et al.*, 1993), however in this study, no improvements to the purification yield was seen. Due to the difficulty in purifying LAMP-1 (+Tail), only HT1080 LAMP-1 (+Tail) was used in subsequent experiments.

**Table 3.1 Recovery of LAMP-1 (+Tail) from HT1080.**

| Purification Step | Total HT1080 LAMP-1 (+Tail) (μg) | % Recovery in Each Step | % Recovery of Total LAMP-1 |
|---|---|---|---|
| Lysed cells | 86 | - | 100 |
| Freeze Thawed Membranes | 54 | 63 | 63 |
| Post Dialysis/Pre Affinity Sample | 41 | 76 | 48 |
| Affinity Purified | 9.5 | 23 | 11 |

### 3.2.2 Purification of LAMP-1 (-Tail)

To obtain pure forms of recombinant LAMP-1 (-Tail) from the condition media of CHO-K1 and HT1080, cells were required to be grown in serum free media. However,

CHO-K1 and HT1080 cells were not viable for more than 6 days in serum free media, therefore, cells were cycled between 1% and 0% FCS. Cells were conditioned (4-6 days) with 250 ml of serum free media and the media collected for purification (section 2.2.5.2). When placed into a cell factory, expression of LAMP-1 (-Tail) by HT1080 and CHO-K1 cells dropped to approximately 1.5 mg/L and 2.5 mg/L, respectively. Table 3.2 outlines the average HT1080 LAMP-1 (-Tail) protein recoveries for each of the purification steps. CHO LAMP-1 (-Tail) recoveries were similar. Total recovery was obtained when cell media was concentrated in a hollow fibre concentrator. When the concentrated cell media was subjected to octanoic acid precipitation, 70% LAMP-1 (-Tail) was recovered, with no further loss during dialysis. Thus from the collection of the condition media to the pre-affinity purification stage of LAMP-1 (-Tail) approximately 30% of the recombinant protein was lost. Affinity purification was carried out with either the 4F5 anti-LAMP-1 monoclonal antibody column or the BB6 anti-LAMP-1 monoclonal antibody column. The capacities of the columns were limited and approximately 60% of LAMP-1 (-Tail) were recovered.

**Table 3.2 Recovery of LAMP-1 (-Tail) from HT1080.**

| Purification Step | Total HT1080 LAMP-1 (-Tail) (μg) | % Recovery in Each Step | % Recovery of Total LAMP-1 |
|---|---|---|---|
| 6 Day Conditioned Media | 362 | - | 100 |
| Hollow Fibre Concentrated Media | 362 | 100 | 100 |
| Post Octanoic Precipitation | 261 | 72 | 72 |
| Post Dialysis/Pre Affinity Sample | 261 | 100 | 72 |
| Affinity Purified | 150 | 57 | 41 |

## 3.3 Characterisation of the HT1080 Expression Cell Lines and Recombinant LAMP-1

Affinity purified recombinant LAMP-1 produced from the HT1080 expression cell lines were run on 1D SDS polyacrylamide gels to determine the purity of the proteins. Western analysis was performed to ensure that the protein purified was indeed LAMP-1 and also to determine the difference in molecular weights of LAMP-1 (+Tail) and LAMP-1 (-Tail). To further characterise the LAMP-1 HT1080 expression cell lines, immunoflourescence and flow cytometry were performed on these cell lines using anti LAMP-1 monoclonal antibodies and antibodies specific for the sialyl-Lewis X antigen.

### 3.3.1 SDS-PAGE of LAMP-1

One dimensional gel electrophoresis (section 2.2.13) was carried out on 50 ng of affinity purified HT1080 LAMP-1 (±Tail) and silver stained (section 2.2.13.2). Figure 3.4 shows that the affinity purified LAMP-1 (+Tail) was approximately 90% pure and LAMP-1 (-Tail) was 100% pure. HT1080 LAMP-1 (+Tail) was distinctly larger than HT1080 LAMP-1 (-Tail) at 120 kDa compared to 100 kDa, respectively. Bands at 60 and 70 kDa of HT1080 LAMP-1 (+Tail) are possibly cell proteins that have been co-purified with the LAMP-1 (+Tail). There is evidence suggesting that LAMP-1 (+Tail) interacts strongly with various proteins (see Chapter 5) and this may also be a reason for the difficulty in obtaining pure LAMP-1 (+Tail). LAMP-1 (-Tail) produced by HT1080 was considerably larger (100 kDa) than LAMP-1 (-Tail) produced by CHO cells (90 kDa) (Figure 3.5). This is thought to be due to differences in glycosylation in the recombinant proteins.

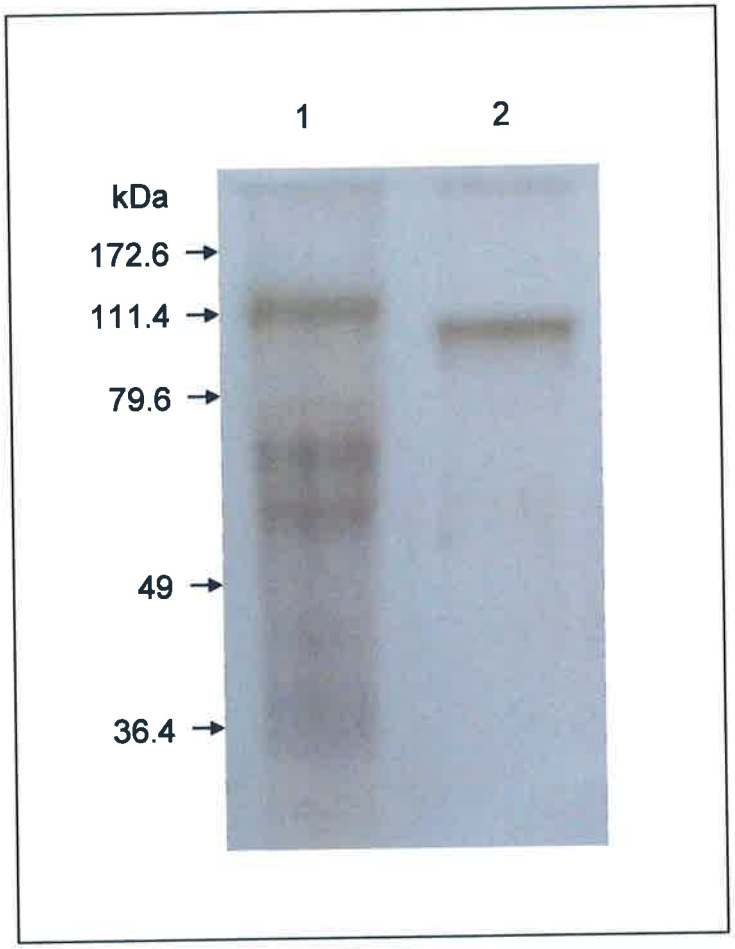



***Figure 3.4 SDS-PAGE of Affinity Purified HT1080 LAMP-1 (±Tail)***

Affinity purified HT1080 LAMP-1 (+Tail) and HT1080 LAMP-1 (-Tail) (50 ng) were run on 12.5% polyacrylamide gels at a constant voltage (180 V) (section 2.2.13) and then silver stained (section 2.2.13.2). Lane 1 = HT1080 LAMP-1 (+Tail) (120 kDa); lane 2 = HT1080 LAMP-1 (-Tail) (100 kDa).

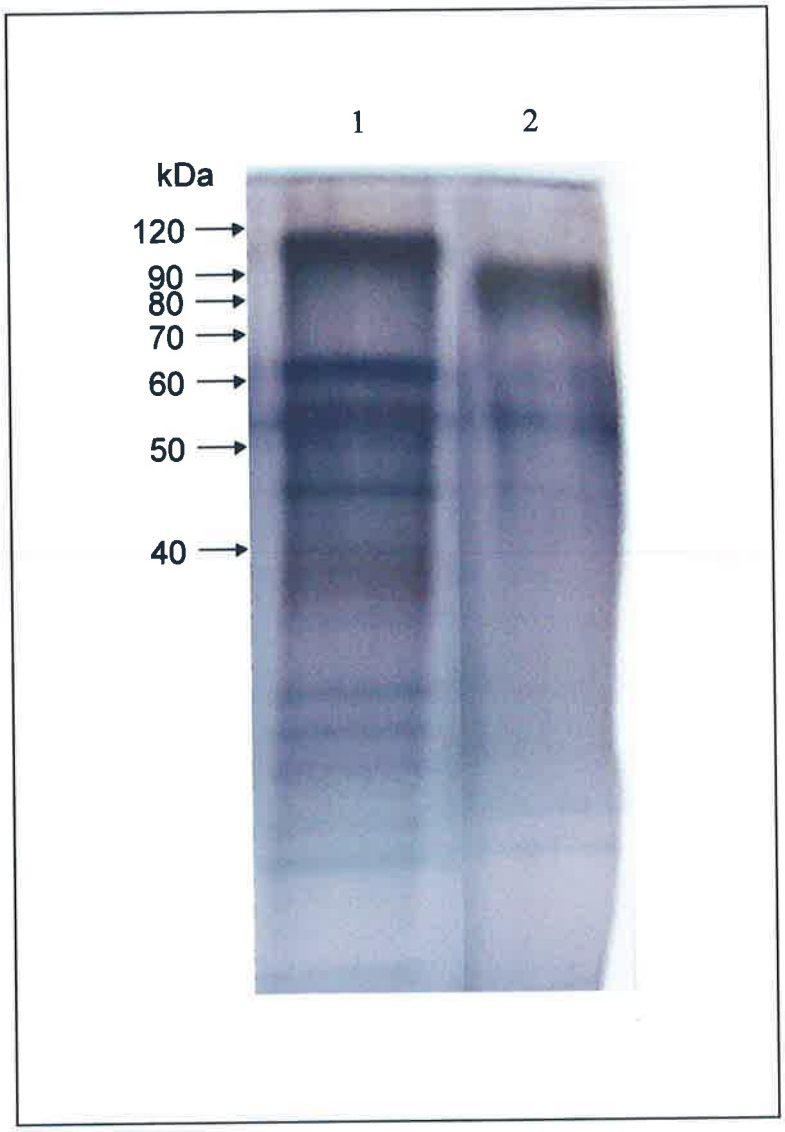



***Figure 3.5 SDS-PAGE of HT1080 LAMP-1 (-Tail) and CHO LAMP-1 (-Tail)***

HT1080 LAMP-1 (-Tail) and CHO LAMP-1 (-Tail) (100 ng) were run on 12.5% polyacrylamide gels at a constant voltage (180 V) (section 2.2.13) and then silver stained (section 2.2.13.2). Lane 1 = HT1080 LAMP-1 (-Tail) (100 kDa); lane 2 = CHO LAMP-1 (-Tail) (90 kDa).

### 3.3.2 Western Analysis of LAMP-1

Western analysis was performed on 15 ng of affinity purified HT1080 LAMP-1 (±Tail), probing with rabbit anti-LAMP-1 polyclonal antibody (Figure 3.6). A single band at 120 kDa representing LAMP-1 (+Tail) and a band at 100 kDa representing LAMP-1 (-Tail) were detected. However a second broad band at approximately 75 kDa was also detected in the LAMP-1 (-Tail) sample. This is believed to be a break down product of LAMP-1 (-Tail) or a product of incomplete glycosylation due to the absence of a membrane anchor as LAMP-1 (-Tail) is trafficked through the ER and Golgi. This was confirmed in the size exclusion chromatography elution profile of HT1080 LAMP-1 (-Tail) (section 4.5.2).

To determine whether the sialyl-Lewis X antigen is being expressed on the recombinant LAMP-1 proteins, Western analysis was carried out probing with CD15s anti-sialyl-Lewis X monoclonal antibody. A negative result was obtained, indicating that sialyl-Lewis X antigen may not be expressed on our recombinant LAMP-1 proteins. However, it is possible that either the concentration of sialyl-Lewis X expressed on the protein was too low to be detected or the affinity of the anti-sialyl-Lewis X antibody was insufficient to detect low amounts of the antigen.

### 3.3.3 Immunofluorescence of HT1080 LAMP-1 Cell Lines

HT1080 wild type and LAMP-1 expression cell lines were probed with BB6 anti-LAMP-1 monoclonal antibody to characterise the expression of LAMP-1 in these cell lines. Cells were also probed with CD15s anti-sialyl Lewis X monoclonal antibody to determine whether the LAMP-1 cell lines were expressing the sialyl-Lewis X antigen. Immunofluorescence staining of HT1080 wild type cells with BB6 resulted in

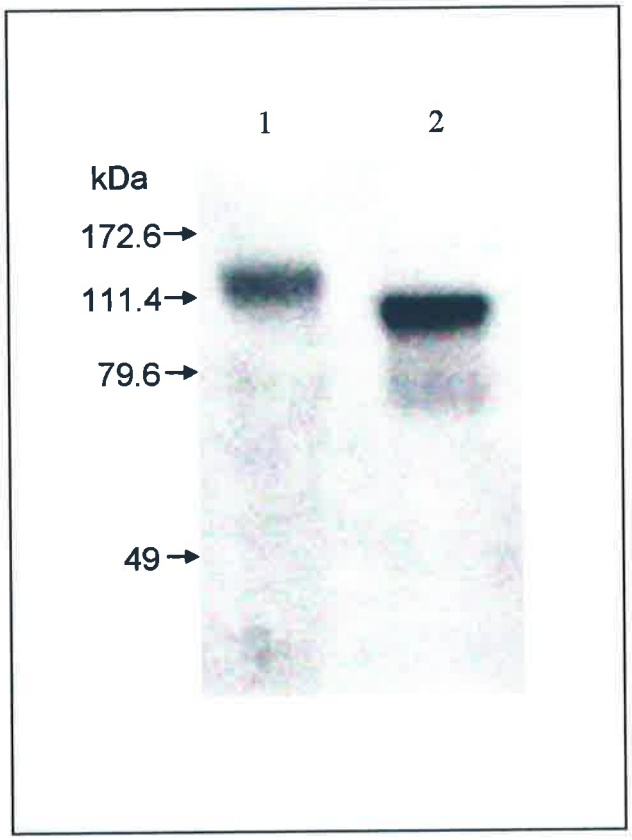



***Figure 3.6 Western Analysis of Affinity Purified HT1080 LAMP-1 (±Tail)***

Affinity purified HT1080 LAMP-1 (±Tail) (15 ng) were electrophoresed on a 12.5% polyacrylamide gel at a constant voltage (180 V) (section 2.2.13) and then transferred to a PVDF membrane at a constant current (250 mA). The membrane was probed with rabbit anti-LAMP-1 polyclonal antibody (1 μg/mL) and developed by chemiluminescence (section 2.2.14). Lane 1 = HT1080 LAMP-1 (+Tail) (120 kDa); lane 2 = HT1080 LAMP-1 (-Tail) (100 kDa).

perinuclear staining characteristic of lysosomes (Figure 3.7A). HT1080 cells expressing LAMP-1 (+Tail) showed similar but a more intense staining pattern (Figure 3.7B), indicative of endosomes and lysosomes. HT1080 cells expressing LAMP-1 (-Tail) displayed an increased staining concentrated around the nucleus characteristic of the ER and Golgi, showing LAMP-1 (-Tail) being processed (Figure 3.7C). These results indicated that over expression of LAMP-1 (±Tail) was achieved within the cell organelles of HT1080 expression cells.

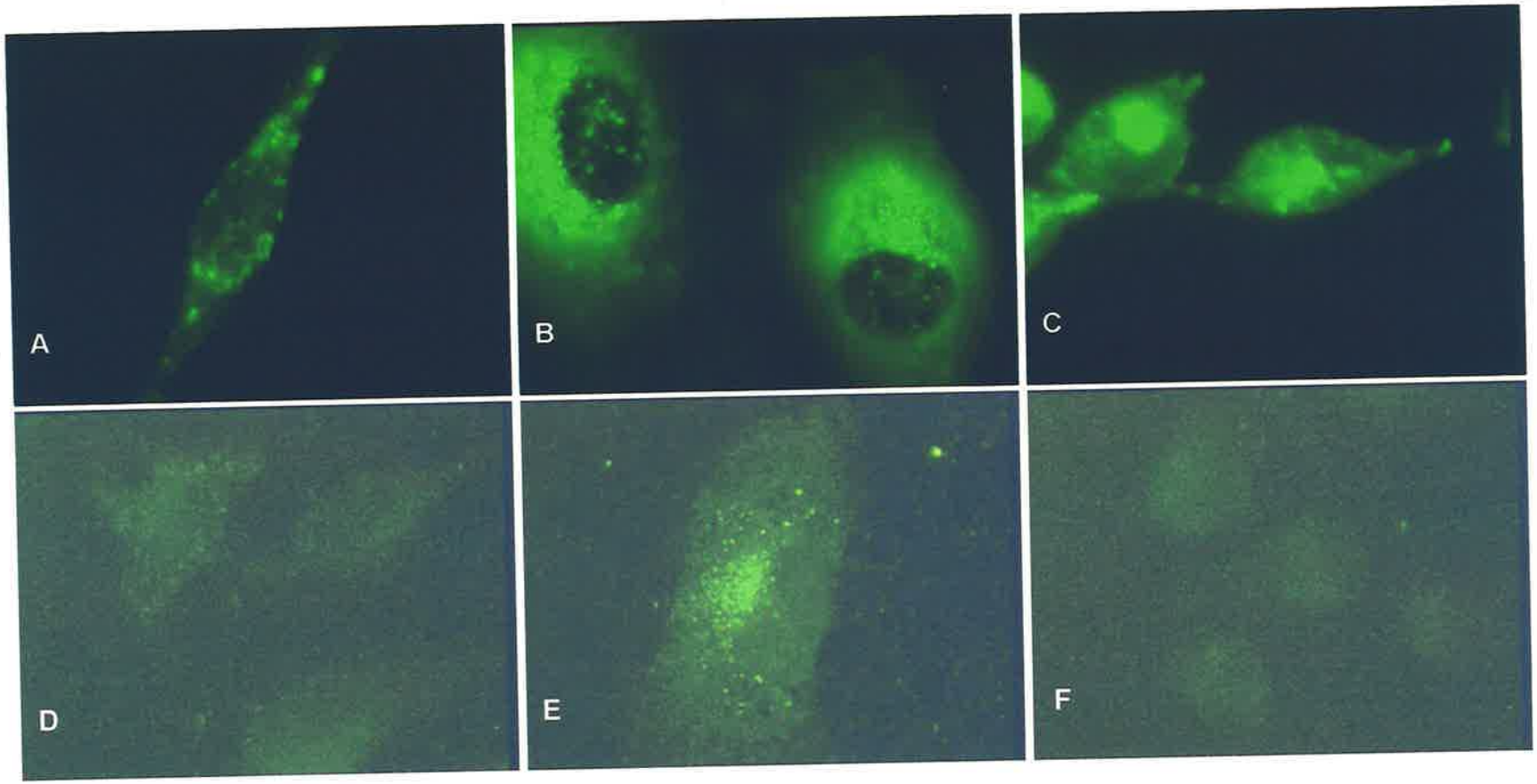
HT1080 wild type cells labelled with CD15s showed slight surface staining (Figure 3.7D) while HT1080 LAMP-1 (+Tail) cells showed the same level of surface staining in addition to intracellular staining (Figure 3.7E). HT1080 LAMP-1 (-Tail) cells resulted in negative staining (Figure 3.7F). The expression of sialyl-Lewis X antigen in the cell lines was not as we had expected. Expression in the wild type was very low and only slightly increased in HT1080 cells expressing LAMP-1 (+Tail). This would suggest minimal expression of the glycosyl transferases required to produce the sialyl-Lewis X antigen in this cell line. It is also possible that the over expression of LAMP-1 saturated the processing and/or trafficking pathways for the production of the sialyl-Lewis X antigen.

### 3.3.4 Flow Cytometry of HT1080 LAMP-1 Cell Lines

Flow cytometry cell staining with BB6 and CD15s of HT1080 wild type and expression cell lines were carried out to specifically examine cell surface expression of LAMP-1 (±Tail) and sialyl-Lewis X antigen.

***Figure 3.7 Immunofluorescence Staining of HT1080 Cells Expressing LAMP-1 (±Tail) for LAMP-1 and the Sialyl-Lewis X Antigen***

HT1080 cells expressing LAMP-1 (+Tail) and LAMP-1 (-Tail) were grown in 2 chambered slides (37°C, 24 hr). Cells were fixed on the slides with 1% (w/w) formaldehyde, permeabilised with digitonin and probed with either BB6 anti-LAMP-1 monoclonal antibody (panels A-C) or CD15s anti-sialyl Lewis X monoclonal antibody (panels D-F), and detecting with FITC-conjugated sheep anti-mouse IgG antibody (section 2.2.8.1). Panels A and D, HT1080 Wild Type cells; panels B and E, HT1080 LAMP-1 (+Tail) cells; panels C and F, HT1080 LAMP-1 (-Tail) cells. Magnification at 100x.


A
B
C
D
E
F

HT1080 wild type cells labelled with BB6 resulted in little surface staining while HT1080 LAMP-1 (+Tail) and HT1080 LAMP-1 (-Tail) showed a significant increase in surface staining for LAMP-1 (Figure 3.8). Staining with CD15s gave negative results.

These results confirmed that LAMP-1 (+Tail) was being over expressed on the cell surface of HT1080 expression cells. However, the presence of LAMP-1 (-Tail) on the cell surface of the LAMP-1 (-Tail) expression cell line was unexpected as the membrane spanning domain of this form of LAMP-1 had been removed. A possible explanation is that some cells may have been permeated in the labelling process and the results shown may be the consequence of some internal labelling of LAMP-1. Another possibility is that we have labelled LAMP-1 at the cell surface as it is in the process of being secreted from the HT1080 cells. Attachment of LAMP-1 (-Tail) may have resulted from the interaction of this protein with other surface proteins on the HT1080 cells.

## 3.4 Discussion

Human LAMP-1 was previously expressed in CHO-K1 cells (Meikle *et al.*, 1999b). As this is a non-human cell line, it is likely that the LAMP-1 produced would be somewhat different, in terms of post-translational modifications, to that found *in vivo*, thus we aimed to express LAMP-1 in a human cell line. Human fibrosarcoma (HT1080) cells and human pancreatic (PANC) cells were investigated as potential LAMP-1 expression systems. HT1080 was chosen to express human LAMP-1 proteins over PANC cells as it was found to express higher levels of LAMP-1 and grow better, thus reducing the time required to produce LAMP-1 proteins.

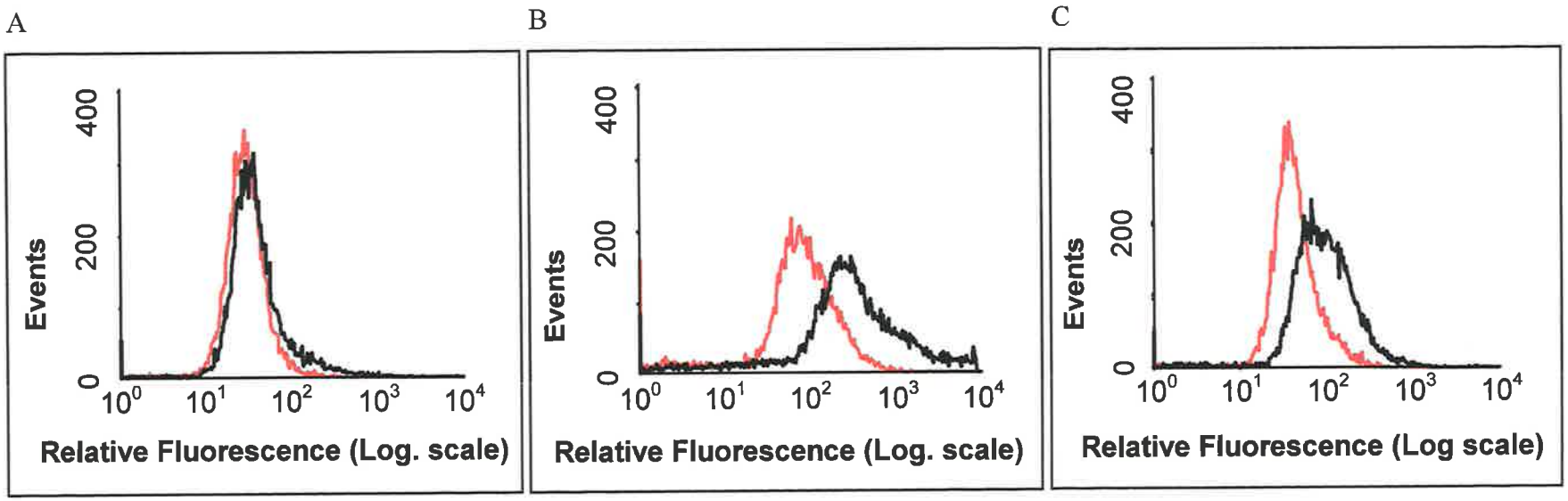



***Figure 3.8 Flow Cytometry Analysis of Cell Surface LAMP-1 on HT1080 Cells Expressing LAMP-1 (±Tail).***

HT1080 cells expressing LAMP-1 (+Tail) and LAMP-1 (-Tail) were grown to confluence in T75 flasks, harvested and labelled with BB6 anti-LAMP-1 monoclonal antibody and detected with a PE-conjugated rabbit anti-mouse IgG antibody (section 2.2.8.2). The x-axis represents fluorescence intensity; the y-axis represents cell number. Red curves represent cells stained with negative control antibody (IgG1); black curves represents cells stained with BB6 anti-LAMP-1 monoclonal antibody. The shift in each histogram (black curves on the right) correlates with cell surface expression of LAMP-1 above background fluorescence (red curves on the left). Panel A, HT1080 Wild Type; panel B, HT1080 (+Tail); panel C, HT1080 (-Tail).

Induction of LAMP-1 (±Tail) production by transfected HT1080 cells was evident when DEX was introduced into the culture media. The expression of LAMP-1 (-Tail) by HT1080 cells remained stable after inducing with DEX, however, LAMP-1 (+Tail) expression decreased considerably after cells had been growing in DEX for greater than 28 days (Figure 3.2). When fresh cells (that which had never been exposed to DEX) were placed into media containing DEX, expression of LAMP-1 (+Tail) increased transiently and dropped to levels reminiscent of non-induced cells, after approximately 6 weeks. It is not known why this occurred, however it is possible that HT1080 F+T1 cells somehow lost its expression vector over time. Moreover, it has been found that high levels of protein expression often lead to unstable cell lines that degenerate (in terms of expression levels) (Ioannou *et al.*, 1992; Van Hove *et al.*, 1996; Bielicki *et al.*, 1998). Production of sufficient amounts of LAMP-1 (+Tail) was therefore difficult, particularly when HT1080 cells were expanded into roller bottles.

The use of MTX to amplify the LAMP-1 gene, resulted in a 3-fold increase in the level of LAMP-1 (+Tail) and LAMP-1 (-Tail) produced by the CHO expression cell lines, approximately 18.9 μg/mg and 6 mg/L, respectively (section 3.1.2.3). CHO-K1 is a DHFR negative cell line thus amplification can be achieved in clones that contain the expression vector, pFRNEE.2 (Figure 3.1). The HT1080 expression cell lines, on the other hand, are able to produce endogenous DHFR in addition to the expressed DHFR encoded in the pGRE-neo.N-dhfr expression vector. HT1080 cells were found to be highly resistant to MTX and amplification of this gene in HT1080 cells required concentrations of MTX above 25 μM. Previous reports have found that above 25 μM, the transport of MTX into the cell is inhibited (Ioannou *et al.*, 1992; Van Hove *et al.*,

1996), and so the attempted amplification was not successful in the HT1080 expression cell lines.

The purification of HT1080 LAMP-1 (+Tail) proved to be difficult with only very small amounts being purified (section 3.2.1). We suspect LAMP-1 (+Tail) may be binding tightly with cell proteins such that even with the addition of 1% (w/v) CHAPS (section 2.2.5.1) this interaction was not disrupted. Other studies (Sawada *et al.*, 1993; Carlsson *et al.*, 1993) have used wheat germ agglutinin-agarose columns to purify LAMP-1, however when this column was used to purify our LAMP-1 (+Tail) protein, purification yields did not improve. A large proportion (about 30%) of LAMP-1 (+Tail) was also lost after CHAPS detergent was dialysed out of the LAMP-1 (+Tail) supernatant (Table 3.1). The elimination of CHAPS may have caused LAMP-1 (+Tail) aggregation leading to the protein becoming insoluble and precipitating out of solution. Due to the difficulties in producing and obtaining pure LAMP-1 (+Tail), affinity purified HT1080 LAMP-1 (+Tail) was used sparingly and only in size exclusion chromatography studies (Chapter 4) to investigate aggregation.

The recombinant proteins produced were 120 kDa for HT1080 LAMP-1 (+Tail), 100 kDa for HT1080 LAMP-1 (-Tail) and 90 kDa for CHO LAMP-1 (-Tail) (Figures 3.4 and 3.5). These molecular weight ranges for LAMP-1 corresponds to other reports (Carlsson *et al.*, 1988; Lin *et al.*, 1997; Kundra and Kornfeld, 1999). The difference in molecular weights observed in several studies, particularly between different species, is presumably the result of differential post-translational modification, in this case HT1080 LAMP-1 proteins are more glycosylated than CHO LAMP-1 proteins. The polypeptide core of LAMP-1 is approximately 42 kDa, thus more than 50% of the protein is made up

of oligosaccharide side chains. This was confirmed by the removal (deglycosylation) of Asn-linked oligosaccharides from mature LAMP-1 proteins with endoglycosidase H, which showed the molecular weight of the protein decreasing progressively as treatment time with endoglycosidase H increased (Kundra and Kornfeld, 1999). The 20 kDa size difference between LAMP-1 (+Tail) and LAMP-1 (-Tail) cannot be accounted for by the cytoplasmic tail in LAMP-1 (+Tail). Presumably, this difference in size is also due to differential post-translational modifications. The presence of the cytoplasmic tail in LAMP-1 (+Tail) may result in different translocation through the ER and Golgi apparatus (section 1.1.1), compared to LAMP-1 (-Tail), such that LAMP-1 (+Tail) is retained within the organelles for longer periods resulting in greater glycosylations. Different processing times resulting in different sizes of LAMP-1 have been reported. Carlsson and Fukuda (Carlsson and Fukuda, 1992) reported a $t_{1/2}$ of 35 min for transport of a 125 kDa LAMP-1 from the ER to the Golgi apparatus in human HL-60 cells, whereas Meikle *et al.* (Meikle *et al.*, 1999b) reported a $t_{1/2}$ of 17 min for transport of a smaller LAMP-1 protein (82-110 kDa) in human skin fibroblasts.

Immunofluorescence and flow cytometry of our LAMP-1 expression cell lines were performed to examine the expression of LAMP-1 and the presence of the sialyl-Lewis X antigen. LAMP-1 was shown to be over expressed intracellularly as well as on the cell surface of the HT1080 expression cell lines (Figures 3.7 and 3.8). The cytoplasmic tail is missing in the LAMP-1 (-Tail) form of the protein, therefore the signalling motif that trafficks this protein to the lysosome (section 1.4.1.1) is also missing. Immunofluorescence staining with BB6 anti-LAMP-1 monoclonal antibody confirmed that the LAMP-1 (-Tail) form was specifically being over expressed in the HT1080 cell line (Figure 3.7E), as it was detected in the ER and Golgi rather than in endosomes and

lysosomes, such as that shown for LAMP-1 (+Tail) (Figure 3.7D). The results also showed that expression of the sialyl-Lewis X antigen was minimal. The slight surface staining observed on both wild type (Figure 3.7D) and LAMP-1 (+Tail) expressing (Figure 3.7E) HT1080 cells was probably endogenous sialyl-Lewis X antigen. Tumour and cancer cells have been reported to have an increased expression of sialyl-Lewis X on the cell surface (Saitoh *et al.*, 1992; Sawada *et al.*, 1993). Immunofluorescence staining with CD15s anti-sialyl-Lewis X monoclonal antibody showed that there was an increase in intracellular sialyl-Lewis X (Figure 3.7D). However, sialyl-Lewis X antigens were not detected on the LAMP-1 expressed on the cell surface, indicating that the sialyl-Lewis X antigens were probably proteolytically cleaved from LAMP-1 within the lysosomes. It is possible that the synthesis and the placement of sialyl-Lewis X onto the carbohydrate chains of LAMP-1 by glycosyl transferases was inhibited or retarded by the over expression of LAMP-1 in these cells. The adhesion molecule, selectin E, is known to bind to LAMP-1 molecules that display the sialyl-Lewis X antigen (Sawada *et al.*, 1993). Thus the near absence of sialyl-Lewis X on our recombinant LAMP-1 was an advantage in this study as we were seeking novel proteins that interact with LAMP-1 in circulation (Chapter 5). Sawada *et al.* (Sawada *et al.*, 1993) have shown that fucosyltransferase is the limiting factor in sialyl-Lewis X antigen expression. Thus to over express LAMP-1 displaying the sialyl-Lewis X antigen, cells would have to be co-transfected with the LAMP-1 and the fucosyltransferase cDNA.

# CHAPTER FOUR:

# Development of LAMP-1 Immunoassays and Determination of LAMP-1 In Circulation

## 4 Introduction and Aims

In a study of the trafficking and turnover of LAMP-1, Meikle *et al.* (Meikle *et al.*, 1999b) found that approximately 25% of LAMP-1 within the lysosome of skin fibroblasts from Pompe and control cells was in a soluble form. The soluble LAMP-1 that was purified from within human skin fibroblasts contained little or no cytoplasmic tail {LAMP-1 (-Tail)}. In contrast, soluble LAMP-1 that was secreted from the cells did contain the cytoplasmic tail {LAMP-1 (+Tail)}. The secreted LAMP-1 represented 5.6% and 8.5% of newly synthesised LAMP-1 in Pompe and control cells, respectively. It is not known which form predominates in the LAMP-1 that is secreted into circulation of healthy individuals and LSD affected individuals. In addition, LAMP-1 is postulated to form aggregates, following reports on the aggregation properties of soluble membrane-associated proteins, particularly of LAMP-2 which is a close relative of LAMP-1 (Colomer *et al.*, 1996; Jadot *et al.*, 1997). However it is unknown whether aggregation occurs in both LAMP-1 (-Tail) and LAMP-1 (+Tail) or in only one form.

The BB6 anti-LAMP-1 monoclonal antibody and a polyclonal antibody that was raised against the cytoplasmic tail of LAMP-1, the anti-LAMP-1 (Tail) polyclonal antibody, were used to develop two immunosassays in order to quantify and differentiate the two forms of LAMP-1 in biological samples. LAMP-1 forms in the plasma from 12

unaffected and 48 LSD affected individuals representing 8 LSD was investigated by using the two immunoassays. The aggregation properties of LAMP-1 were also investigated using these immunoassays together with size exclusion chromatography.

## 4.1 Preparation of Immunoquantification Reagents

Monoclonal and polyclonal antibodies against LAMP-1 were purified over a 5 mL Protein G column. Polyclonal antibodies were further affinity purified over LAMP-1 affinity columns. The purified antibodies were used to develop LAMP-1 immunoassays to capture LAMP-1 proteins in different samples. Affinity purified LAMP-1 (-Tail) from the media of HT1080 expression cells (Chapter 3) was used as calibration standards in these assays. Due to the difficulties of purifying LAMP-1 (+Tail) from HT1080 cells, LAMP-1 (+Tail) calibration standards were obtained from membrane preparations of skin fibroblast cells.

### 4.1.1 Production of Polyclonal Antibody Against LAMP-1 Cytoplasmic Tail

A peptide was made by Chiron Mimotopes representing the cytoplasmic tail of LAMP-1 (H-CLUGRKRSHAGYQTI-OH). This peptide was coupled to diptheria toxoid at a ratio of 3.9 mg peptide to 14.4 mg of toxoid (Mimotopes, Melbourne, Australia). The conjugate was resuspended in 1x PBS to 3 mg/mL. The conjugate had a tendency to precipitate, therefore sonification (2 min) of the antigen and dounce homogenisation was required to obtain an even suspension. The conjugate was diluted in PBS (1 mg/mL) and aliquoted (200 μL). This conjugate was used to inoculate a semilop rabbit at four to six subcutaneous sites along the neck and back, using the protocol outlined in Meikle *et al.* (Meikle *et al.*, 1999b). Immunisation of the rabbit with the peptide antigen was carried out by the Institute of Medical and Veterinary Science (IMVS, Adelaide, Australia) with booster shots given at 14 day intervals. Test bleeds were taken from the ear vein and serum (20 mL) prepared. Test bleeds were titrated against the peptide conjugate and against an unrelated peptide, galactosamine-6-sulphatase (FGSPNCHFGPY), conjugated to diptheria toxoid (Mimotopes, Melbourne, Australia).

This was done by coating a 96 well microtitre plate with the peptide tail conjugate (2 μg/mL, 4°C, overnight). To differentiate between the polyclonal antibody raised against the LAMP-1 peptide tail and that raised against the diptheria toxoid or the linker between the peptide and the toxoid, a negative control plate was set up, coating with a non-specific peptide coupled to diptheria toxoid (2 μg/mL, 4°C, overnight). The plates were washed (x1) with 20 mM Tris/HCl, 0.25 M NaCl, pH 7.0. Serum samples were diluted (1:4000) in 1% (w/v) BSA, 20 mM Tris/HCl, 0.25 M NaCl, pH 7.0, and then serially diluted 1:2 across the plate (100 μL per well). The plates were shaken (10 min), incubated (37°C, 4 hr) and then washed with DELFIA® wash buffer (x6). $Eu^{3+}$-labelled sheep anti-rabbit-IgG polyclonal antibody was diluted in DELFIA® assay buffer (100 ng/mL), added to the wells (100 μL/well) and incubated (37°C, 4 hrs). Plates were washed (x6) with DELFIA® wash buffer and DELFIA® enhancement solution (200 μL) was added to each well. Plates were shaken (20°C, 10 min) and the fluorescence read on a WALLAC 1234 DELFIA® Research Fluorometer (Wallac, North Ryde, Australia). Figure 4.1 shows the titration curve of the rabbit serum against the peptide antigen after the final antigen boost. The polyclonal antibody reacted strongly against the LAMP-1 peptide tail conjugate and was approximately two folds greater as compared to the reaction against the negative control peptide conjugate, indicating that the antibody had a strong and specific reaction against the LAMP-1 tail.

### 4.1.2 Purification of Anti-LAMP-1 (Tail) Polyclonal Antibody

Rabbit anti-LAMP-1 (Tail) polyclonal antibody from rabbit serum (8 mL) immunised against LAMP-1 tail peptide was purified over a 5 mL Protein G column (section 2.2.6.1). The yield of immunoglobulin was 38 mg. To affinity purify antibodies that

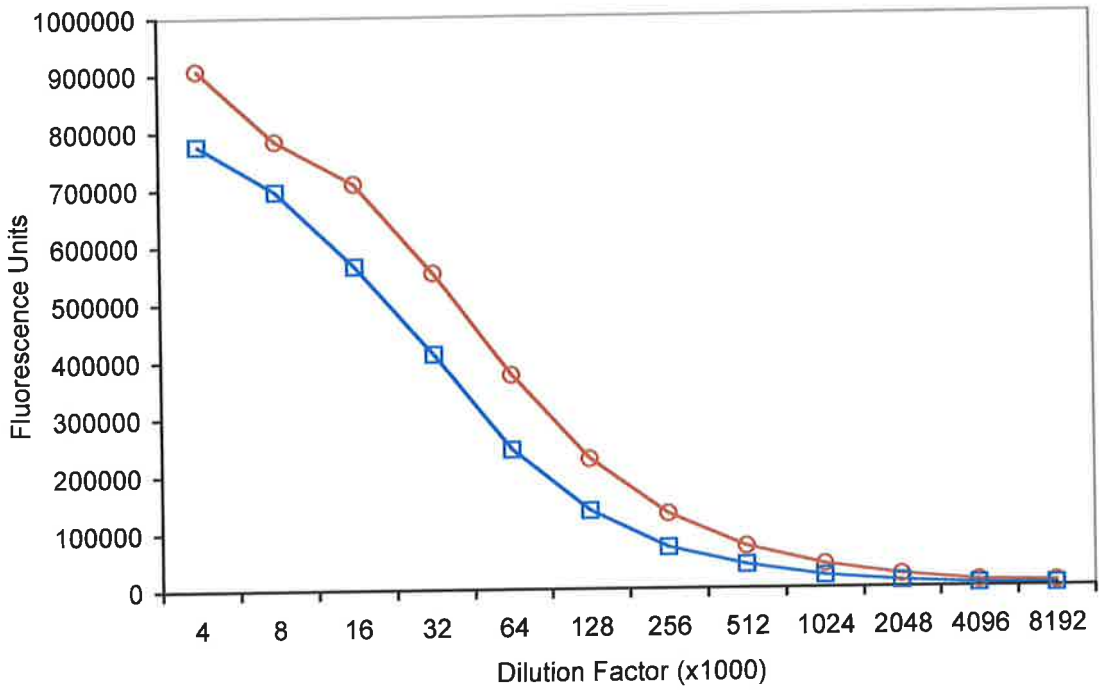



***Figure 4.1 Titration of Rabbit anti-LAMP-1 (Tail) Polyclonal Antibody***

A rabbit was immunised with LAMP-1 tail peptide conjugated to diptheria toxoid (section 4.1.1). Serum from the final bleed was diluted (1:4000) in 1% (w/v) BSA, 20 mM Tris/HCl, 0.25 M NaCl, pH 7.0 and titrated against the LAMP-1 tail conjugate (o) as well as a negative control (galactosamine-6-sulphatase) peptide conjugate (□) in an immunoassay (section 4.1.1).

were specifically against the tail domain of LAMP-1, the antibodies eluted from the Protein G column were passed over the LAMP-1 peptide tail column (section 2.2.6.1) (Figure 4.2) and the anti-LAMP-1 (Tail) specific antibody (peak B) was collected and pooled. The yield of purified anti-LAMP-1 (Tail) polyclonal antibody was 0.35 mg. When the LAMP-1 peptide tail column flow through was re-loaded onto the column, a similar amount of anti-LAMP-1 (Tail) polyclonal antibody was purified, indicating that the capacity of the LAMP-1 peptide column was limited.

### 4.1.3 Purification of BB6 Anti-LAMP-1 Monoclonal Antibody

Diacults were used to produce the BB6 anti-LAMP-1 monoclonal antibodies as they are able to generate concentrated antibodies in sufficient amounts, required for these studies. BB6 diacults (50 mL) were harvested and clarified by centrifugation (2,000 x g, 10 min) (section 2.2.6.2). The resulting supernatant was filtered through a 0.2 μm filter and passed through a Protein G column (section 2.2.6.1). The yield of monoclonal antibodies was 7 mg. The protein G elution profile is shown in Figure 4.3.

### 4.1.4 Calibration of Skin Fibroblast LAMP-1 (+Tail)

While the purification of HT1080 LAMP-1 (+Tail) protein (section 3.2.1) was being carried out, skin fibroblast (SF) membrane preparations (section 2.2.11.2) were used as the LAMP-1 (+Tail) standard in the LAMP-1 immunoassays. However, over time the difficulty of obtaining sufficient quantities of HT1080 LAMP-1 (+Tail) became evident. As we had already established the use of SF LAMP-1 (+Tail) as a standard in the LAMP-1 immunassays, we continued to use this rather than replacing it with HT1080 membrane preparations.

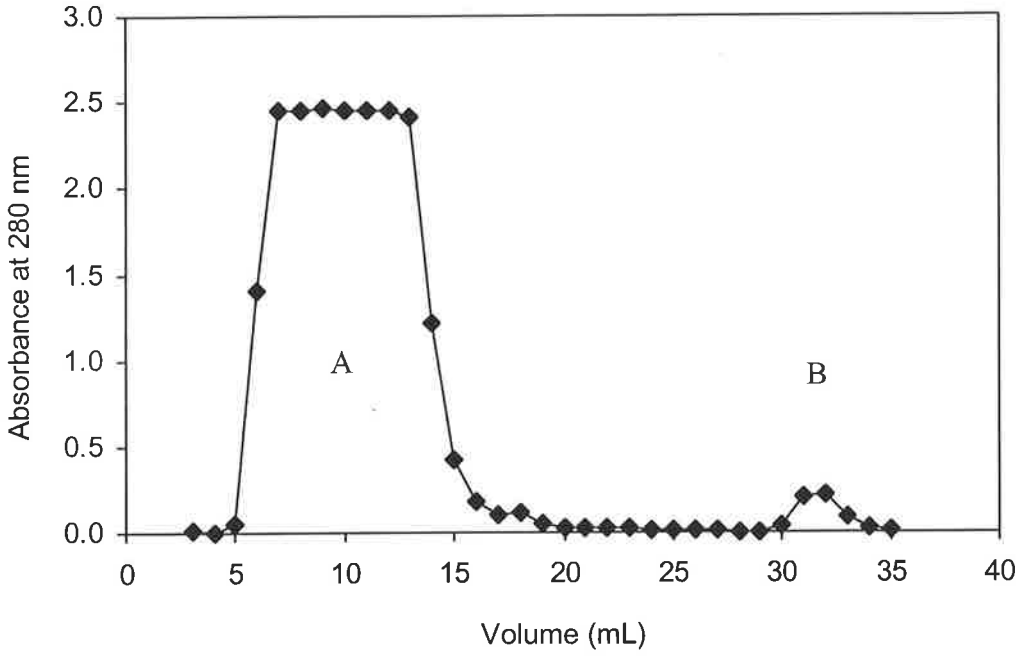



***Figure 4.2 Elution Profile of Rabbit Anti-LAMP-1 (Tail) Polyclonal Antibody from LAMP-1 Peptide (Tail) Affinity Column***

Rabbit serum from LAMP-1 peptide tail immunised rabbit was first purified over a Protein G column and then affinity purified over a LAMP-1 Peptide Tail affinity column at a flow rate of 0.5 ml/min (sections 2.2.6.1 and 2.2.6.2). The column was washed with PBS (18 mL) and the bound antibody eluted with 0.1 M $H_3PO_4$/NaOH, pH 2.5 (13 mL). Peak A represents the unbound proteins. Peak B represents the eluted rabbit anti-LAMP-1 (Tail) polyclonal antibody.

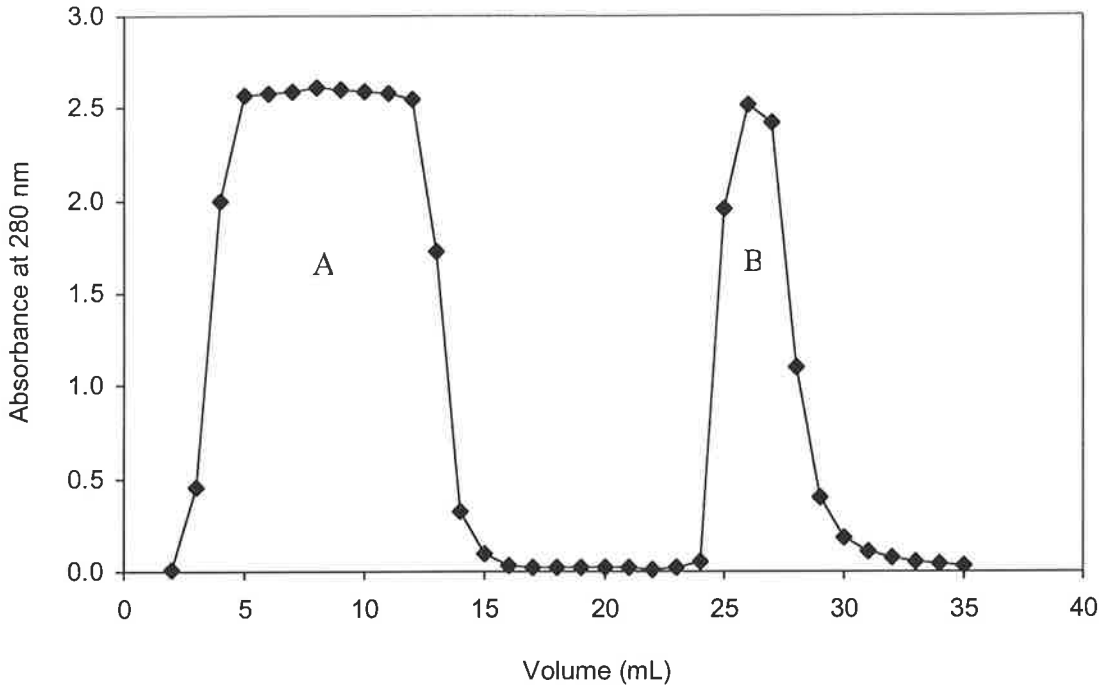



***Figure 4.3 Elution Profile of BB6 anti-LAMP-1 monoclonal antibody from Protein G Column***

BB6 diacult cells grown for 3 weeks in dialysis tubing suspended in RPMI/10% (v/v) FCS media were harvested by centrifugation (2,000 x g, 10 min). The resulting supernatant containing the BB6 anti-LAMP-1 monoclonal antibody was retained (50 mL) and loaded onto a 5 mL Protein G column at a flow rate of 0.5 mL/min (sections 2.2.6.1 and 2.2.6.2). The column was washed with PBS (7 mL) and the bound antibody eluted with 0.1 M $H_3PO_4$/NaOH, pH 2.5 (16 mL). Peak A represents the unbound proteins. Peak B represents the eluted BB6 anti-LAMP-1 monoclonal antibody.

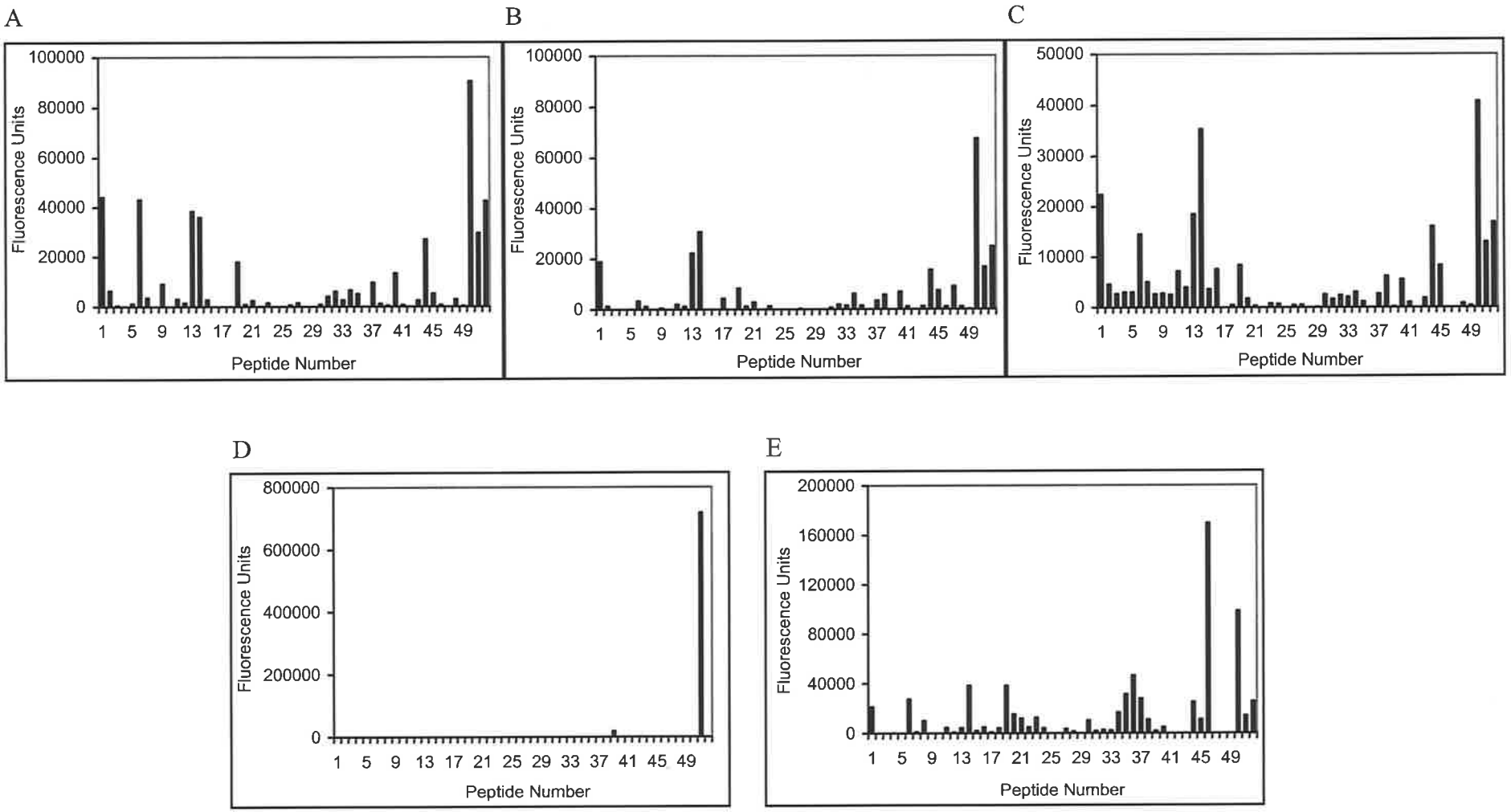
The concentration of LAMP-1 (+Tail) in the SF membrane preparations was quantified using the BB6 LAMP-1 immunoquantification assay (section 2.2.11.3) with purified HT1080 LAMP-1 (-Tail) as the standard. A calibration curve of SF LAMP-1 (+Tail) was then prepared in DELFIA assay buffer at 0, 0.0625, 0.125, 0.25, 0.5, 1 ng/mL. The SF calibration curve was then aligned with the HT1080 LAMP-1 (-Tail) calibration curve. Western analysis was performed on HT1080 LAMP-1 (-Tail) and SF LAMP-1 (+Tail) at a range of concentrations (1, 2, 4 and 8 ng) to confirm that the estimated SF LAMP-1 (+Tail) concentration matched that of the HT1080 LAMP-1 (-Tail) standard (data not shown).

## 4.2 Epitope Mapping of Anti-LAMP-1 Monoclonal and Polyclonal Antibodies

Throughout these studies several monoclonal and polyclonal antibodies against LAMP-1 were used. In order to understand their reactivity and performance in the different immunoassays, epitope mapping of the antibodies was performed (section 2.2.7). Figure 4.4 shows the epitope maps of the LAMP-1 antibodies. The results showed that epitope number 50, representing the membrane domain of LAMP-1 (Table 4.1), is a particularly reactive epitope with all but the anti-LAMP-1 (Tail) polyclonal antibody recognising this peptide (Figure 4.4). The membrane domain of LAMP-1 is a hydrophobic region, explaining the non-specific binding of the antibodies to this part of the protein. Similarly, non-specific binding of the antibodies to peptide number 1 (H-MAPRSARRPLLLLLP-GSG), representing a portion of the signal peptide, can be explained by the hydrophobicity of this region. Moreover, the LAMP-1 antigens used to produce all the anti-LAMP-1 antibodies did not contain the signal peptide as this part of the protein is cleaved off in mature LAMP-1, therefore none of the antibodies could have been specific for this epitope.

***Figure 4.4 Epitope Maps of Anti-LAMP-1 Monoclonal and Polyclonal Antibodies***

Microtitre plates were coated with streptavidin (5 μg/mL) and 52 peptides coupled to biotin, representing the total LAMP-1 sequence, were bound to the streptavidin coated plate and used to capture antibodies against LAMP-1 (section 2.2.7). Monoclonal antibodies were detected with $Eu^{3+}$-labelled anti-mouse IgG and polyclonal antibodies were detected with $Eu^{3+}$-labelled anti-rabbit IgG. Epitope maps of: panel A, BB6 monoclonal antibody; panel B, 1B1 monoclonal antibody; panel C, 4F5 monoclonal antibody; panel D, rabbit anti-LAMP-1 (Tail) polyclonal antibody; panel E, rabbit-anti-LAMP-1 polyclonal antibody.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Fluorescence Units
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50000
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800000
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400000
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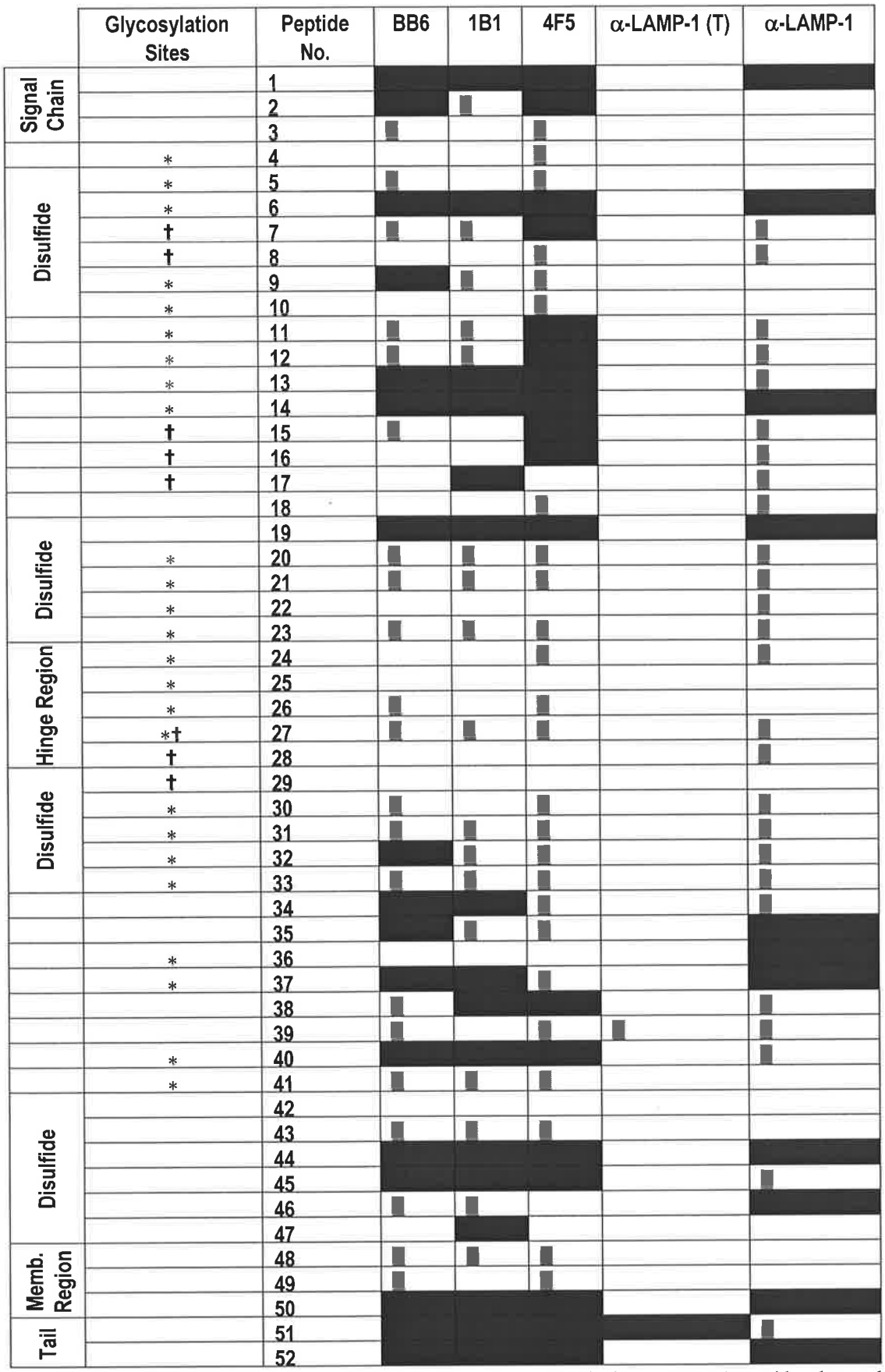
160000
120000
1 5 9 13 17 21 25 29 33 37 41 45 49

**Table 4.1 Summary of Epitope Mapping of Anti-LAMP-1 Antibodies.**




* = N or O-linked carbohydrates; † = polyacetyllactosamines; solid blocks = LAMP-1 peptides detected by the antibodies; short blocks = fluorescence units ≤10% of max. fluorescence unit readings.

There appears to be multiple epitopes for BB6, 1B1 and 4F5 anti-LAMP-1 monoclonal antibodies, not typical of monoclonal antibodies (Figures 4.4A-C). The possibility that these antibodies may recognise protein conformation rather than a linear sequence was unlikely, as we were able to use them to detect LAMP-1 in Western analyses. All 3 monoclonal antibodies showed affinity for common epitopes (peptides 13, 14 and 19), however, these epitopes lie at or adjacent to a site of glycosylation (Table 4.1). The generated peptides do not have the carbohydrate side chains of LAMP-1, thus binding of antibodies to epitopes that, in the native form, are glycosylated is probably not specific as these epitopes would not be exposed in the native protein.

The recognition of the monoclonal antibodies to peptides 51 and 52, the LAMP-1 tail domain, was also non-specific as it was shown that all 3 antibodies recognised LAMP-1 (-Tail) in the immunoassays (section 4.3.1), which does not have the cytoplasmic tail. BB6, 1B1 and 4F5 monoclonal antibodies are believed to recognise the same epitope as they cannot be used together in a sandwich immunoassay. Therefore, the most probable epitopes for these anti-LAMP-1 monoclonal antibodies are at peptides 44 and 45 and peptide 47 for 1B1 (Figures 4.4A, B, C).

Rabbit-anti-LAMP-1 polyclonal antibody recognises several epitopes (Figure 4.4E), using the same rationale as above, the majority of these epitopes can be ruled out. Thus the most probable epitopes for rabbit-anti-LAMP-1 polyclonal antibody is at peptides 44 and 46. The epitope map of the anti-LAMP-1 (Tail) polyclonal antibody shows that it only recognises peptide 51, the tail region of LAMP-1 (Figure 4.4D), which was the peptide used to generate this antibody.

## 4.3 LAMP-1 Immunoquantification Assays

In order to detect LAMP-1 in various samples and to distinguish between LAMP-1 (+Tail) and LAMP–1 (-Tail), three LAMP-1 immunoassays were developed and then optimised; the BB6 assay, the LAMP-1 (Tail) assay and the 1B1 assay.

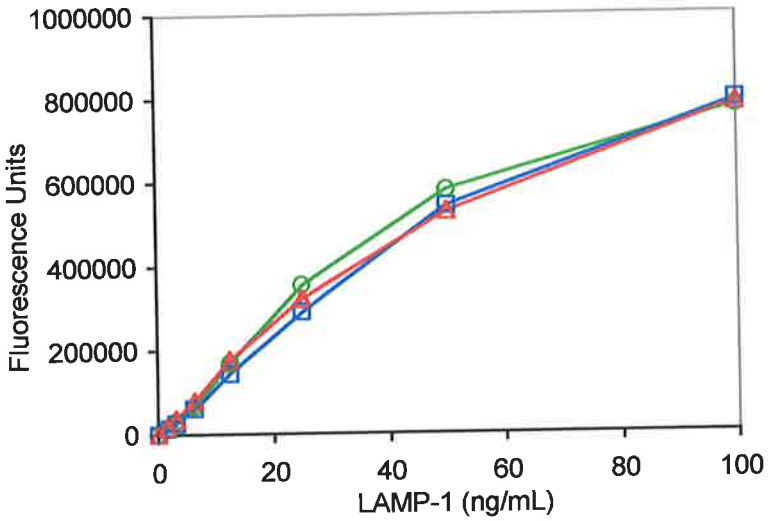
### 4.3.1 Optimisation of LAMP-1 Immunossays

Figure 4.5 shows the final calibration curves of the three LAMP-1 immunoassays generated using optimised parameters. The BB6 assay was optimised as a 2-step assay (section 2.2.11.3) in which 96 well plates are coated with BB6 anti-LAMP-1 monoclonal antibody (5 μg/mL, 4°C, overnight) and used to capture LAMP-1 standards or samples (4°C, overnight). Captured LAMP-1 were detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 37°C, 4 hr). This assay detects both LAMP-1 (+Tail) and LAMP-1 (-Tail) with equal sensitivity (Figure 4.5A). The LAMP-1 (Tail) assay is a 1-step assay (section 2.2.11.4) that only detects LAMP-1 (+Tail) (Figure 4.5B). Plates are coated with rabbit-anti-LAMP-1 (Tail) polyclonal antibody (5 μg/mL, 4°C, overnight), which only has affinity to the cytoplasmic tail of LAMP-1, as shown on the epitope map (Figure 4.4D). LAMP-1 (+Tail) standards or samples are captured and detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight). Both the BB6 and the LAMP-1 (Tail) assays gave linear responses up to 25 ng/mL of LAMP-1, all assays were therefore carried out within this range. The 1B1 assay is a 1-step assay in which plates are coated with 1B1 anti-LAMP-1 monoclonal antibody (5 μg/mL, 4°C, overnight) and LAMP-1 standards or samples are captured and detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight). This assay detects both LAMP-1 (+Tail) and LAMP-1 (-Tail) however it has higher affinity for the -Tail form (Figure 4.5C). The epitope map of 1B1 shows that this

***Figure 4.5 LAMP-1 Immunoassays***

Optimum immunoquantification conditions were used to generate the final BB6 2-step (panel A), 1B1 1-step (panel B) and LAMP-1 (Tail) 1-step (panel C) calibration curves for use in the immunoquantification of LAMP-1 (+Tail) and LAMP-1 (-Tail). Microtitre plates were coated with primary BB6 anti-LAMP-1 monoclonal antibody (panel A), anti-LAMP-1 (Tail) polyclonal antibody (panel B) and 1B1 anti-LAMP-1 monoclonal antibody (panel C) (5 μg/mL, 4°C, overnight). In the BB6 2-step assay, standards were incubated in duplicate (4°C, overnight) and detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 37°C, 4 hr). In the 1-step assays, standards were incubated in duplicate with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight). The standards were HT1080 LAMP-1 (-Tail) (○), CHO LAMP-1 (-Tail) (□) and SF LAMP-1 (+Tail) (△).

A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80
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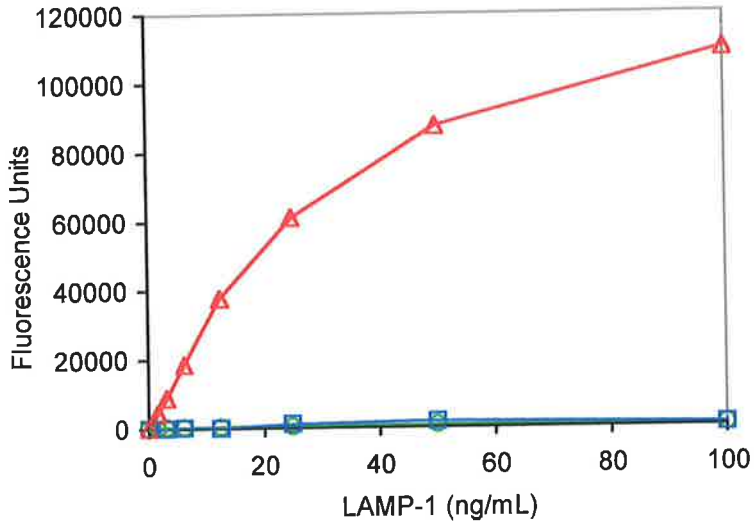
LAMP-1 (ng/mL)

B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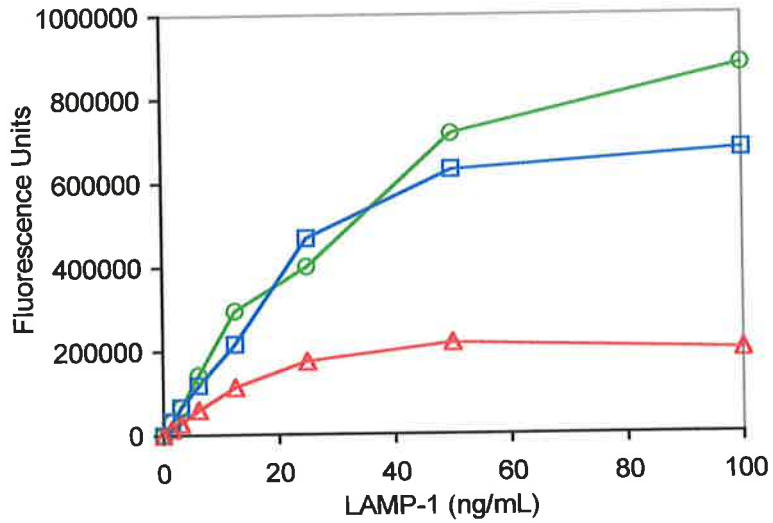
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Fluorescence Units
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80
100
LAMP-1 (ng/mL)

antibody, unlike BB6 or 4F5, has affinity to peptide 47, which is adjacent to the transmembrane region (Figure 4.4B and Table 4.1). This region may be somewhat masked in LAMP-1 (+Tail), which may explain it's higher affinity for LAMP-1 (-Tail).

As the 1B1 assay does not have equal affinity to both forms and cannot absolutely distinguish one form of LAMP-1 from the other in any given sample, no further work was done on this assay. The BB6 and 4F5 antibodies recognise the same epitopes, however as BB6 gave stronger signals in the epitope maps compared to the 4F5 antibody (Figures 4.4A and C), indicating greater affinity for LAMP-1, we did not evaluate 4F5 in these immunoassays.

All standards and samples were assayed in duplicate, the coefficient of variance were always less than 10%. In all figures the average of the duplicates assayed are shown. The following section describes the optimisation steps of the BB6 and LAMP-1 (Tail) assays.

#### *4.3.1.1 Optimisation of Concentrations of Coating and Detecting Antibodies*

The primary coating antibodies {BB6 and anti-Lamp-1 (Tail)} and the detecting $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody concentrations of the LAMP-1 immunoassays were optimised previously by C. Hua (Women's and Children's Hospital, SA, Australia). This was achieved by keeping all parameters constant while testing different antibody concentrations. It was determined that a coating concentration of 5 μg/mL and a final concentration of 200 ng/mL per well of detecting antibody were optimum for the assays as a compromise between assay sensitivity, consumption of reagents and consistency in the two assays.

#### *4.3.1.2 Determination of One or Two-Step Incubation of BB6 and LAMP-1 (Tail) Immunoassays*

To determine whether a 1 or 2-step assay worked best for the BB6 assay, calibration curves of HT1080 LAMP-1 (-Tail) and SF LAMP-1 (+Tail) standards were compared in the different assay conditions. In the 1-step assay, calibration curves were generated by incubating the standards with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 detection antibody (200 ng/mL, 4°C, overnight). In the 2-step assay, calibration curves were generated by incubating the standards first (4°C, overnight) followed by incubation with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 (200 ng/mL, 37°C, 4 hrs). Figure 4.6 shows the resulting calibration curves. The 1-step immunoassay was more sensitive for the HT1080 LAMP-1 (-Tail) standard, while the 2-step assay detected both HT1080 LAMP-1 (-Tail) and SF LAMP-1 (+Tail) standards equally.

The percentage of LAMP-1 (+Tail) and LAMP-1 (-Tail) standards bound in the 1 and 2-step assays were determined by transferring the standards after incubation and re-assaying the unbound standards. It was found that in the 1-step BB6 assay (Figures 4.7A, B), approximately 92% of HT1080 LAMP-1 (-Tail) bound while approximately 83% of SF LAMP-1 (+Tail) bound. In the 2-step assay (Figures 4.7C, D), approximately 70% of both standards bound. Although similar amounts of SF LAMP-1 (+Tail) bound as compared to HT1080 LAMP-1 (-Tail) in the 1 and 2-step assays, the response given by SF LAMP-1 (+Tail) and HT1080 LAMP-1 (-Tail) in the 1-step assay was different. As HT1080 LAMP-1 (-Tail) and SF LAMP-1 (+Tail) gave the same response in the 2-step assay, the 2-step BB6 assay was chosen as the optimum and used in subsequent assays. It is thought that in the 1-step assay, LAMP-1 (+Tail) forms aggregates that are

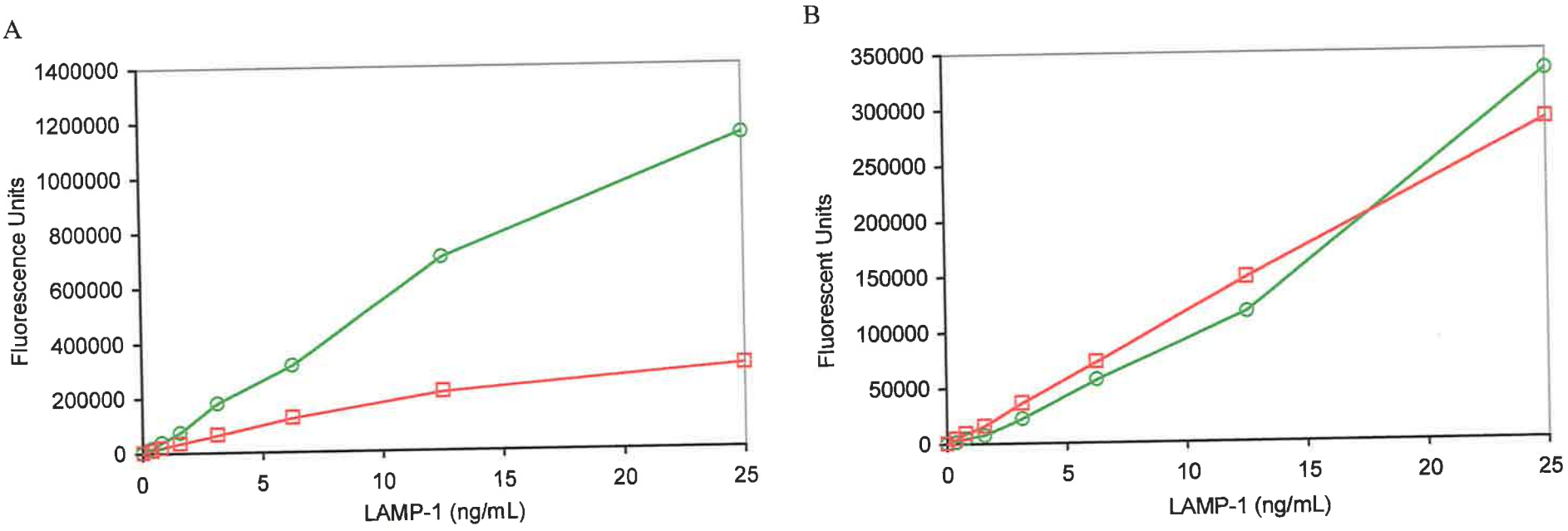


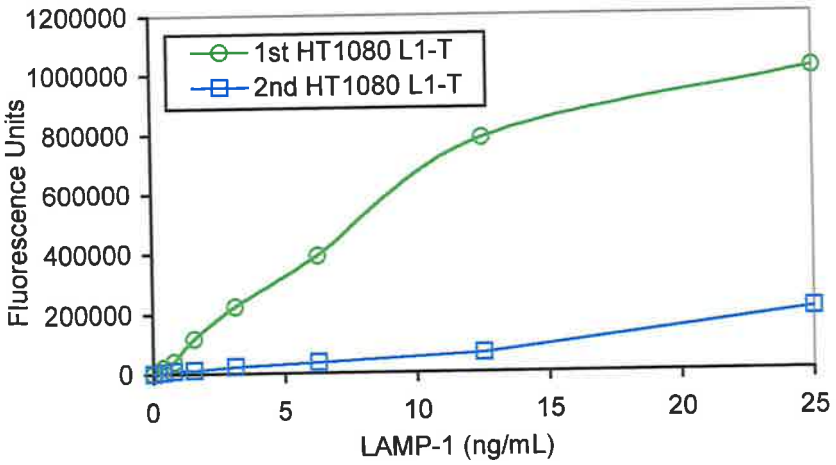

***Figure 4.6 BB6 1 and 2-Step Assays***

Microtitre plates were coated with BB6 anti-LAMP-1 monoclonal antibody (5 µg/mL, 4°C, overnight) and HT1080 LAMP-1 (-Tail) (○) and SF LAMP-1 (+Tail) (□) standards were assayed. In the BB6 1-step assay (panel A), standards were incubated with the detecting $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight). In the BB6 2-step assay (panel B), standards were incubated separately (4°C, overnight) followed by $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 37°C, 4 hr).

***Figure 4.7 Determination of Binding Efficiency of LAMP-1 Standards in BB6 1 and 2-Step Assays***

Microtitre plates were coated with BB6 anti-LAMP-1 monoclonal antibody (5 μg/mL, 4°C, overnight). In the BB6 1-step assay (panel A, B), HT1080 LAMP-1 (-Tail) (○, □) and SF LAMP-1 (+Tail) (△, +) standards were incubated with the detecting $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight) ($1^{st}$ binding), then transferred and assayed in a fresh BB6 coated plate ($2^{nd}$ binding). In the BB6 2-step assay (panel C, D), standards were incubated (4°C, overnight) ($1^{st}$ binding), transferred to a fresh BB6 coated plate for a further overnight incubation ($2^{nd}$ binding) and both plates were detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 37°C, 4 hr). (○) = HT1080 LAMP-1 (-Tail) $1^{st}$ binding; (□) = HT1080 LAMP-1 (-Tail) $2^{nd}$ binding; (△) = SF LAMP-1 (+Tail) $1^{st}$ binding; (+) = SF LAMP-1 (+Tail) $2^{nd}$ binding.

A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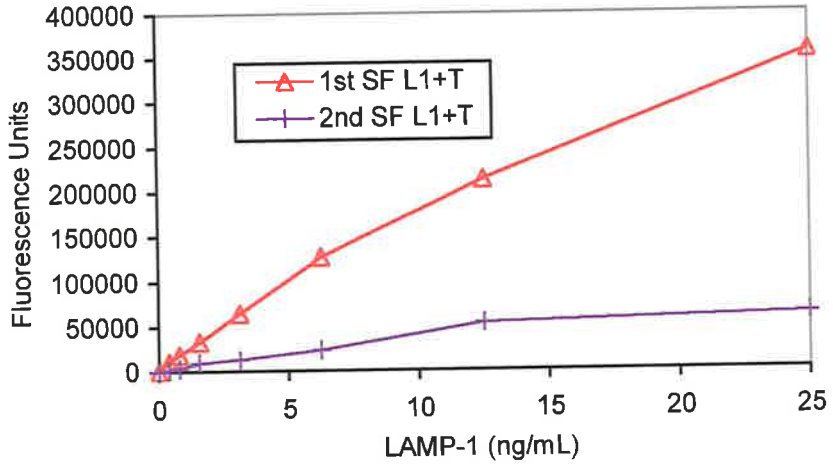
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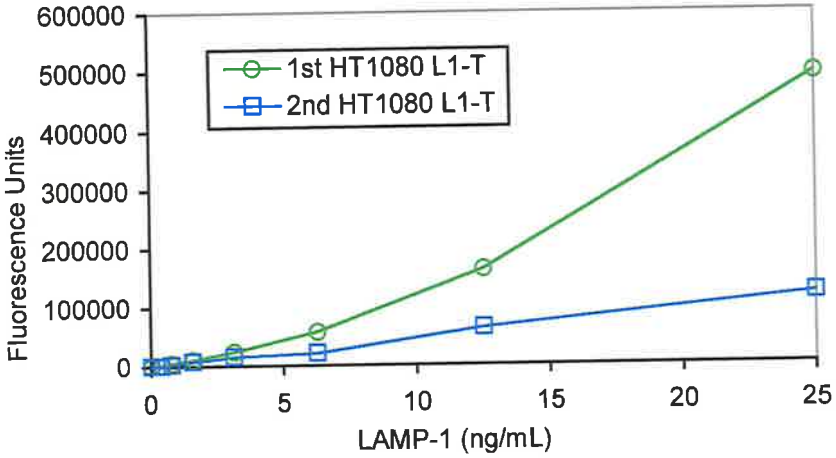
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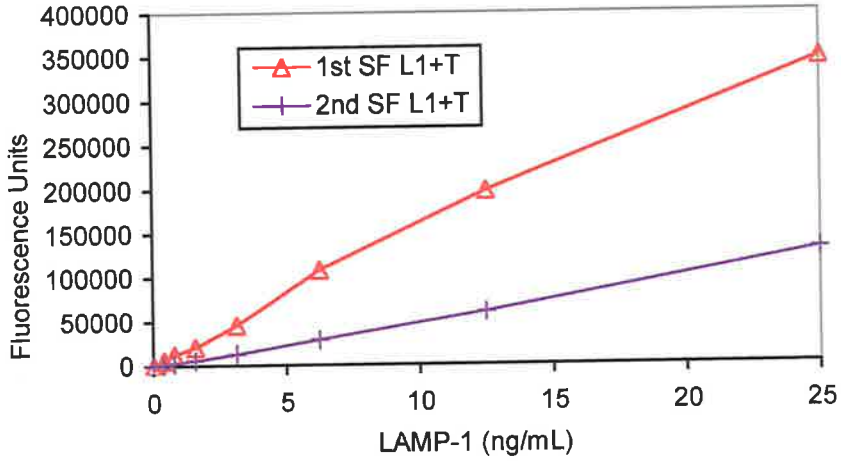
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stabilised by the $Eu^{3+}$-labelled rabbit anti-LAMP-1 polyclonal antibody, thus providing less antibody binding sites and resulting in decreased fluorescence intensity compared to the LAMP-1 (-Tail) standard. In the 2-step assay, the LAMP-1 (+Tail) aggregates are not stabilised by the $Eu^{3+}$-labelled polyclonal antibody but are disrupted as they bind to the immobilised BB6 monoclonal antibody, thus giving a signal from the LAMP-1 (+Tail) that is equal to the signal from the LAMP-1 (-Tail) (Figure 4.6). The aggregation properties of LAMP-1 (±Tail) are discussed in section 4.5.

One or 2-step assays were also tested for the LAMP-1 (Tail) assay. Calibration curves of HT1080 LAMP-1 (-Tail) and SF LAMP-1 (+Tail) standards were compared in the different assay conditions. In the 1-step assay, calibration curves were generated by incubating the standards with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 detection antibody (200 ng/mL, 4°C, overnight). In the 2-step assay, calibration curves were generated by incubating the standards first (4°C, overnight) followed by incubation with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 (200 ng/mL, 37°C, 4 hrs) in a second step. Figure 4.8 shows the resulting calibration curves. Both the 1 and 2-step assays detected only the SF LAMP-1 (+Tail) standard, however, the 1-step assay (Figure 4.8A) was approximately 20-fold more sensitive as compared to the 2-step assay (Figure 4.8B), giving fluorescent unit counts of up to 70,000. This was probably partly due to the difference in the incubation time of the $Eu^{3+}$-labelled rabbit-anti-LAMP-1 detecting antibody. Moreover, the signals obtained from the LAMP-1 (+Tail) standard in the LAMP-1 (Tail) 1 and 2-step assays (Figure 4.8) was approximately 5-fold and 70-fold lower compared to the signals obtained from the LAMP-1 (+Tail) standard in the BB6 1 and 2-step assays (Figure 4.6), respectively. This suggests that the rabbit anti-LAMP-1 (Tail) polyclonal antibody has a relatively lower affinity for LAMP-1 than the BB6 monoclonal antibody. Thus in

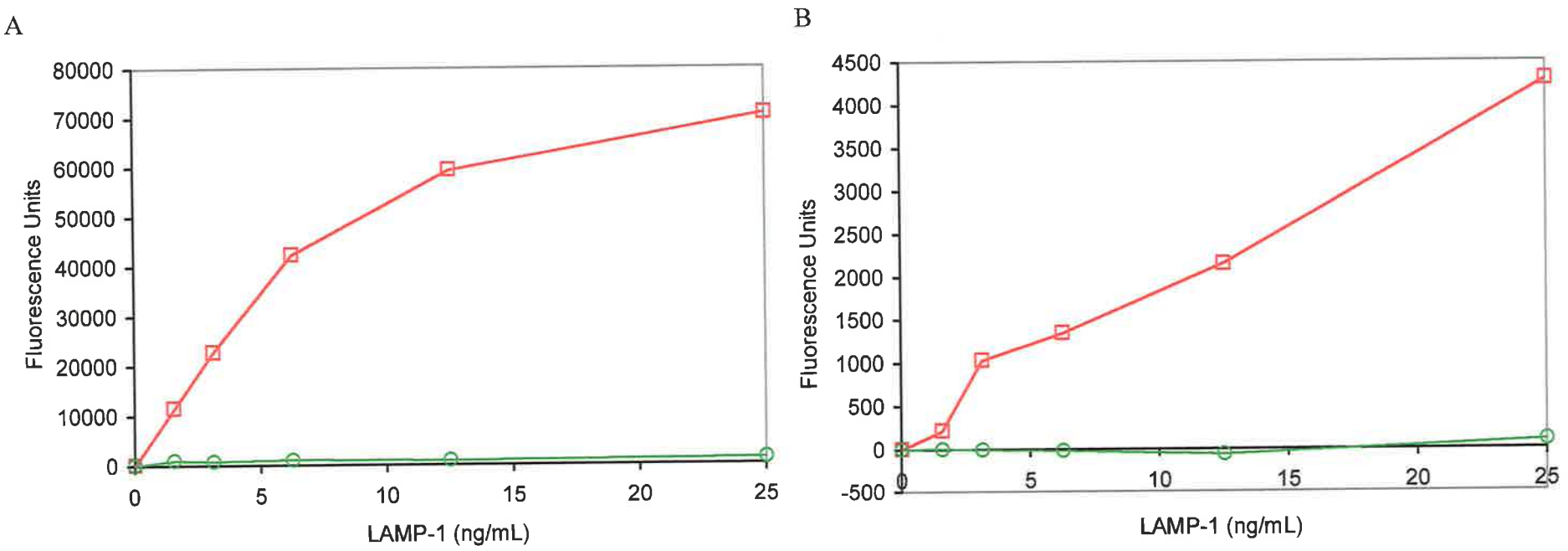



***Figure 4.8 LAMP-1 (Tail) 1 and 2-Step Assays***

Microtitre plates were coated with anti-LAMP-1 (Tail) polyclonal antibody (5 µg/mL, 4°C, overnight) and HT1080 LAMP-1 (-Tail) (○) and SF LAMP-1 (+Tail) (□) standards were assayed. In the LAMP-1 (Tail) 1-step assay (panel A), standards were incubated with the detecting $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight). In the LAMP-1 (Tail) 2-step assay (panel B), standards were incubated separately (4°C, overnight) followed by $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 37°C, 4 hr).

the LAMP-1 (Tail) 1-step assay, it is thought that the LAMP-1 (+Tail) forms aggregates that are stabilised by the $Eu^{3+}$-labelled rabbit anti-LAMP-1 polyclonal antibody that then bind to the coated plate multivalently. In the LAMP-1 (Tail) 2-step assay, the LAMP-1 (+Tail) aggregates are disrupted as in the BB6 2-step assay, however, as the rabbit anti-LAMP-1 (Tail) polyclonal antibody has a lower affinity for LAMP-1, the LAMP-1 (+Tail) does not bind efficiently therefore giving the reduced fluorescence intensity observed.

The 1-step LAMP-1 (Tail) assay was the optimum and used for subsequent assays.

#### *4.3.1.3 Optimisation of Incubation Times*

Incubation time of coating was optimised previously by C. Hua (Women's and Children's Hospital, SA, Australia). It was determined that an overnight incubation at 4°C was required to ensure that maximum binding of the primary antibody was achieved. This was also true for sampling time, to ensure LAMP-1 binding had reached equilibrium.

To examine the effect of incubation time of the detecting secondary antibody on the BB6 2-step assay, calibration curves were generated by varying the incubation time for the $Eu^{3+}$-labelled rabbit-anti-LAMP-1 secondary antibody (4 hr at 37°C compared to overnight at 4°C). All other parameters were kept constant. The resulting calibration curves from both incubation times were similar, indicating that binding of the $Eu^{3+}$-labelled rabbit-anti-LAMP-1 is a rapid process (data not shown). Thus incubation times of 4 hr at 37°C and overnight at 4°C are interchangeable. In order to minimise the assay time, the detection incubation time of 4 hr at 37°C was used in the BB6 2-step assay. In

the LAMP-1 (Tail) 1-step assay, sampling and detection is carried together, thus the incubation time of overnight at 4°C was used to allow for the assay to reach equilibrium.

### 4.3.2 Effect of Plasma on the LAMP-1 Immunoquantification Assays

Plasma samples of de-identified individuals were obtained from the National Referral Laboratory for Lysosomal Peroxisomal and Related Genetic Diseases (Women's and Children's Hospital, SA, Australia). To determine the effect of plasma on the BB6 and LAMP-1 (Tail) assays, plasma from a normal individual was spiked into the LAMP-1 standards, 4 μL for the BB6 assay and 1, 2, and 4 μL for the LAMP-1 (Tail) assay. To determine whether plasma from LSD-affected individuals had any effects on the BB6 and LAMP-1 (Tail) assays, 5 μL and 2 μL of different LSD-affected plasma was added to the LAMP-1 standards, respectively. Prior to using plasma in the immunoassays, the plasma was dialysed against PBS (4°C, overnight), as some plasma samples contained EDTA, which interferes with the detection $Eu^{3+}$-labelled rabbit anti-LAMP-1 polyclonal antibody. Plasma was also clarified of cellular debris by ultracentrifugation (100,000 x g, 15 min).

There were no significant effects on the BB6 assay with the addition of 4 μL of plasma (Figure 4.9A). Figure 4.9B shows slight inhibition when 5 μL of normal and LSD plasma was added to the SF LAMP-1 (+Tail) standard and there were no difference in the extent of inhibition between control and LSD-affected plasma. However the inhibition observed was minimal at the lower end of the calibration curve where the working area is.

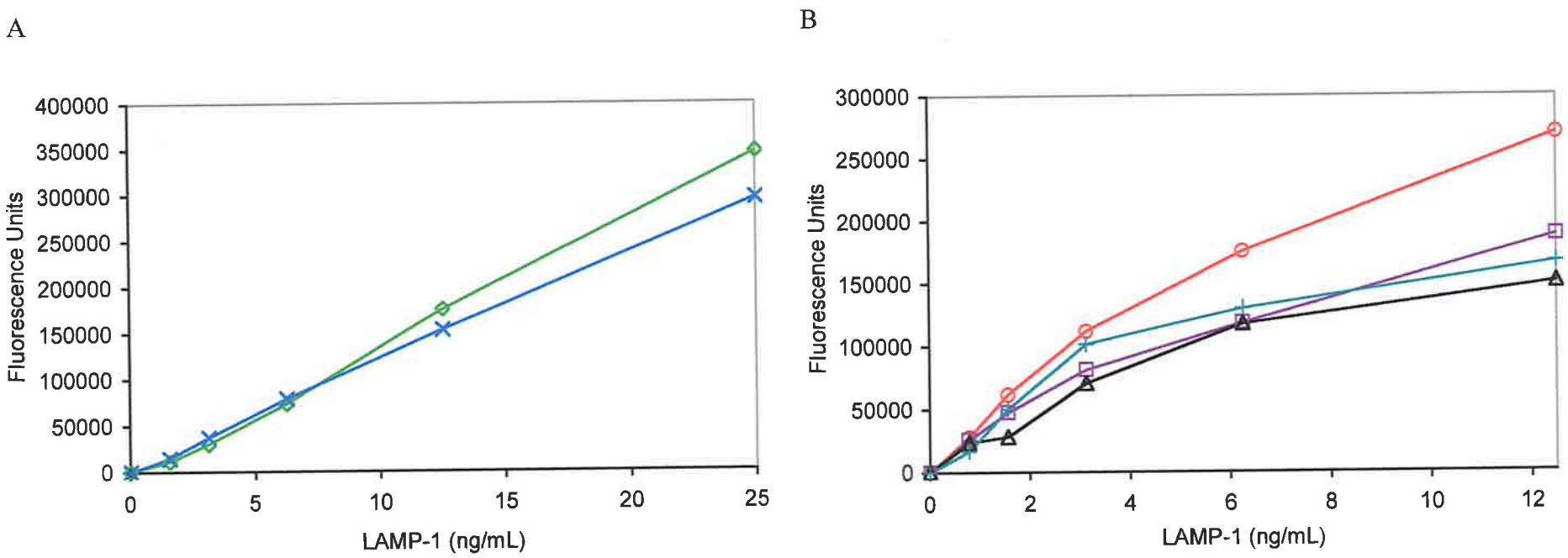



***Figure 4.9 Effects of Plasma on BB6 2-Step Assay***

Microtitre plates were coated with BB6 monoclonal antibody (5 µg/mL, 4°C, overnight). HT1080 LAMP-1 (-Tail) standard (panel A) without (◇) and with 4 µL (×) control plasma were detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight). SF LAMP-1 (+Tail) standard (panel B) without (○) and with 5 µL of control (□), Gaucher (△) and Niemann-Pick A/B (+) LSD plasma were detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 37°C, 4 hr).

A marked inhibition of the LAMP-1 (Tail) assay was observed with 1 μL of control plasma giving a reduction of approximately 87% and almost total inhibition when 4 μL of plasma was added, when compared to the signals obtained for the normal standard curve (Figure 4.10A). The extent of inhibition differed with plasma sources from different individuals. Figure 4.10B shows 2 μL of Gaucher LSD-affected plasma had the greatest inhibitory effect, followed by MPS II LSD-affected plasma then control plasma. The inhibition observed is thought to be due to proteins present in plasma that may bind to LAMP-1 (+Tail), thereby inhibiting the assay. With the addition of smaller volumes of plasma the inhibitory effect was alleviated, suggesting that the inhibitory protein(s) may have been diluted out.

As each plasma sample imnhibited the LAMP-1 (Tail) assay to a different degree, a separate calibration curve was generated for each plasma sample assayed. This was done by spiking the SF LAMP-1 (+Tail) standard curve with the plasma at an equal volume to that being assayed.

## 4.4 Determination of LAMP-1 Forms in Circulation

Using the BB6 and the LAMP-1 (Tail) assays, 12 plasma samples from control individuals, and 6 plasma samples each from MPS I, MPS II, MPS IIIA, MPS IVA, MPS VI, Fabry, Gaucher, Niemann-Pick A/B and 5 samples from Pompe affected individuals were assayed. All plasma samples were dialysed against PBS (4°C, overnight) and clarified of cellular debris by ultracentrifugation (100,000 x g, 15 min) such that LAMP-1 quantification is solely on soluble LAMP-1 in circulation.

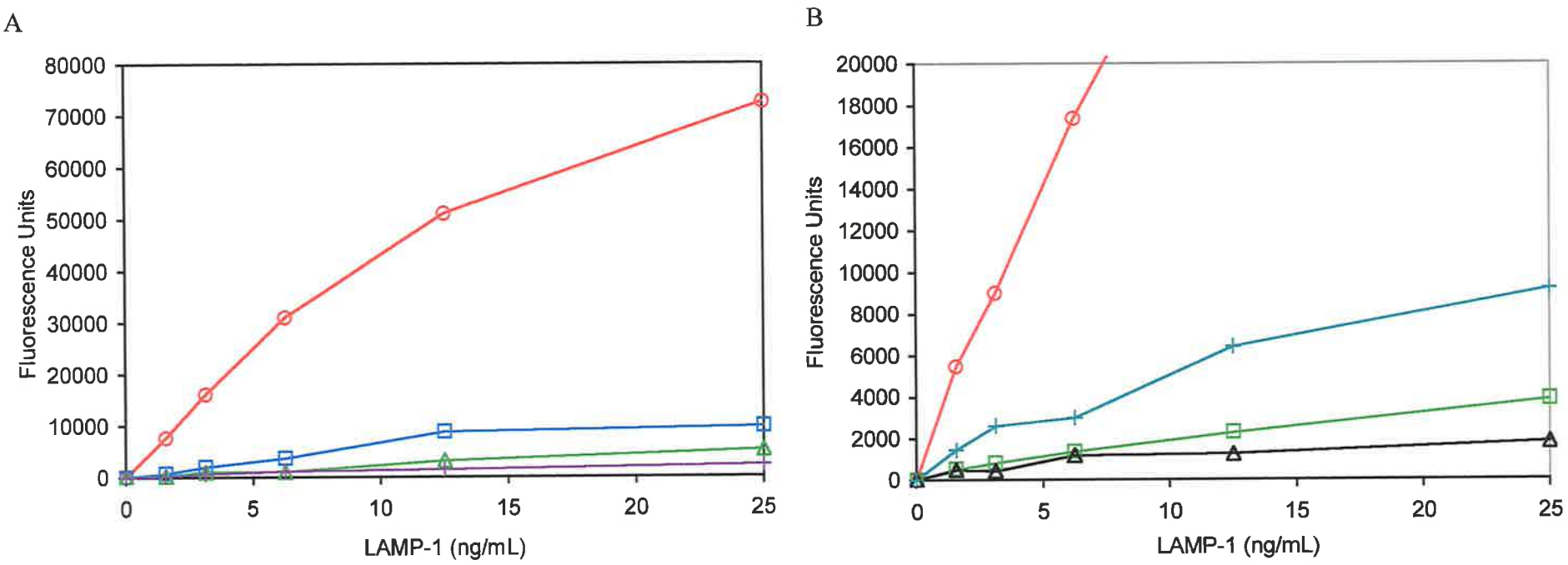



***Figure 4.10 Effects of Plasma on LAMP-1 (Tail) 1-Step Assay***

Microtitre plates were coated with anti-LAMP-1 (Tail) polyclonal antibody (5 µg/mL, 4°C, overnight). In panel a), SF LAMP-1 (+Tail) standards without (○) and with 1 µL (□), 2 µL (△) and 4 µL (+) control plasma were detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight). In panel b), SF LAMP-1 (+Tail) standard without (○) and with 2 µL of control (□), Gaucher (△) and MPS II (+) LSD plasma were detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 37°C, 4 hr).

### 4.4.1 Determination of LAMP-1 Forms in the Plasma of Control and LSD Affected Individuals

Volumes of 2, 5 or 10 μL of control and LSD-affected plasma were used to give an adequate signal in the BB6 and LAMP-1 (Tail) assays. The working range of the BB6 assay was 0-25 ng/mL, and for the LAMP-1 (Tail) was 0-6.25 ng/mL of LAMP-1.

LAMP-1 concentrations as determined by the BB6 2-step assay were calculated with the MultiCalc Advanced program (Wallac, NSW, Australia) using a spline fit of the calibration curve. Due to the plasma inhibition on the LAMP-1 (Tail) assay, calculation of LAMP-1 (+Tail) concentrations was carried out by calculating the LAMP-1 (+Tail) concentration in the 0 point of the calibration curve (plasma only) from the calibration curve corrected for the high plasma background (Figure 4.11). The LAMP-1 (Tail) calibration curve for a control plasma sample is shown in Figure 4.11. The calibration curve was generated by spiking the SF LAMP-1 (+Tail) calibration curve with 2 μL of plasma from a healthy individual. Calculation of the LAMP-1 (+Tail) in the plasma using the corrected calibration curve gave a concentration of 3 ng/mL in a 100 μL assay volume. However, because 2 μL of plasma was assayed, the actual LAMP-1 (+Tail) concentration in the plasma is 150 ng/mL of plasma.

The BB6 assay gave the plasma concentration of total LAMP-1, that is LAMP-1 (+Tail) and LAMP-1 (-Tail), whereas the LAMP-1 (Tail) assay gave the plasma concentration of LAMP-1 (+Tail) only. In theory, subtracting the LAMP-1 (+Tail) concentration from the total LAMP-1 concentration will give the concentration of the LAMP-1 (-Tail) in the plasma sample, however there are some limitations with this system, particularly with the LAMP-1 (Tail) assay. Firstly, the BB6 assay may detect break down products of

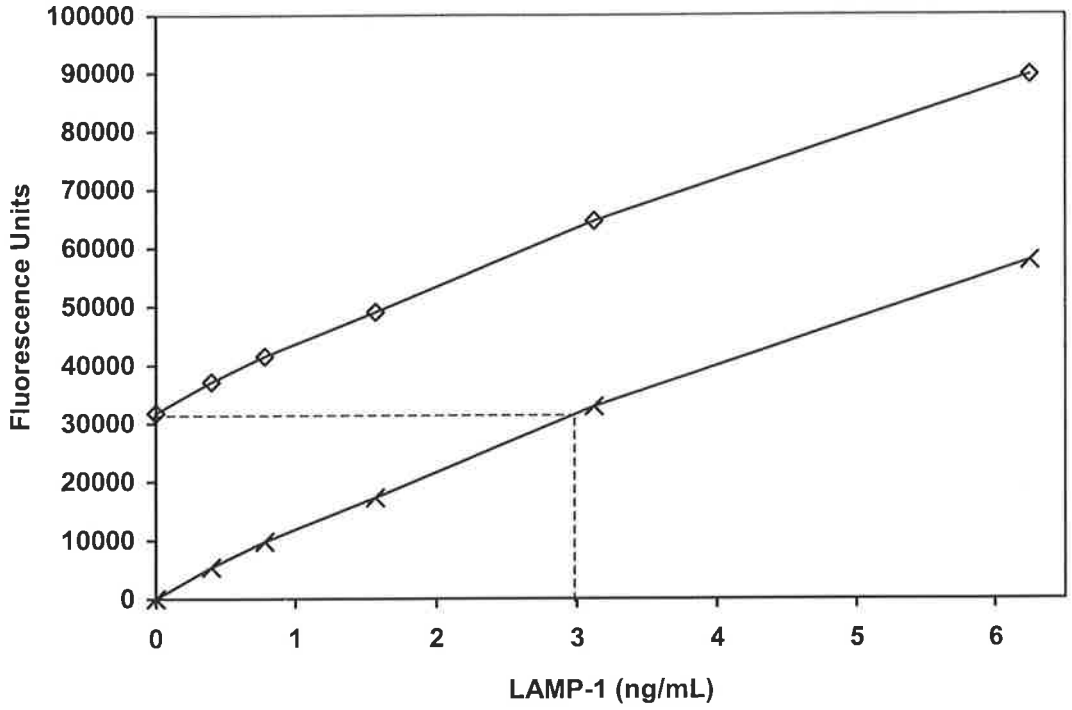



*Figure 4.11 Determination of LAMP-1 (+Tail) Concentration in Plasma Samples with the LAMP-1 (Tail) 1-Step Assay*

Microtitre plates were coated with anti-LAMP-1 (Tail) polyclonal antibody (5 μg/mL, 4°C, overnight). SF LAMP-1 (+Tail) standards were spiked with 2 μL of control plasma and detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight). The concentration of LAMP-1 (+Tail) in the assayed plasma was calculated by calculation of the 0 point of the calibration curve (◇) on the plasma corrected calibration curve (×).

LAMP-1 thus over estimating the actual LAMP-1 concentration. Secondly, if the plasma inhibition effect on the LAMP-1 (Tail) assay is very severe then an accurate concentration is difficult to obtain. However, the primary aim of this experiment was to determine which form predominates in the different plasma samples and this was achieved. Table 4.2 shows the total amount of LAMP-1 in each sample, based on the BB6 assay. Also shown are the predominant form of LAMP-1 and the approximate percentage of LAMP-1 (+Tail) in the plasma samples assayed. In cases where the LAMP-1 (+Tail) concentration was greater than the total LAMP-1 concentration the percentage of LAMP-1 (+Tail) was taken to be 100%.

There does not appear to be any correlations between control and LSD plasma and the form of LAMP-1. The majority (67%) of the plasma samples assayed contained predominantly the LAMP-1 (+Tail) form. Thus measuring the levels of LAMP-1 (+Tail) in plasma cannot distinguish between unaffected and LSD-affected individuals. However, LAMP-1 in 5 of the Gaucher and all of the Niemann-Pick A/B affected plasma samples were predominantly LAMP-1 (+Tail). Altered trafficking of LAMP-1 have been reported to occur in cells taken from LSD-affected patients, including Gaucher patients, where an increased level of LAMP-1 was seen on the cell surface (Zimmer *et al.*, 1999). It is not known whether this altered trafficking of LAMP-1 also causes an increase in the secretion of LAMP-1 (+Tail) into circulation, as observed in these patients.

## 4.5 Aggregation Properties of LAMP-1 (+Tail) and LAMP-1 (-Tail)

The aggregation properties of LAMP-1 and its interactions in the immunassays were investigated using the LAMP-1 immunoassays and size exclusion chromatography.

**Table 4.2 LAMP-1 Form in Plasma Samples**

| Plasma Sample | Number | Total LAMP-1 (ng/mL) | LAMP-1 (+Tail) (%) |
|---|---|---|---|
| Control | 1 | 19 | 50 |
| | 2 | 19 | 64 |
| | 3 | 40 | 8 |
| | 4 | 46 | 10 |
| | 5 | 32 | 100 |
| | 6 | 35 | 59 |
| | 7 | 33 | 25 |
| | 8 | 50 | 25 |
| | 9 | 12 | 100 |
| | 10 | 8 | 100 |
| | 11 | 30 | 65 |
| | 12 | 26 | 55 |
| MPS I | 1 | 175 | 36 |
| | 2 | 73 | 31 |
| | 3 | 50 | 53 |
| | 4 | 65 | 58 |
| | 5 | 66 | 31 |
| | 6 | 37 | 100 |
| MPS II | 1 | 35 | 9 |
| | 2 | 113 | 6 |
| | 3 | 111 | 28 |
| | 4 | 28 | 100 |
| | 5 | 37 | 100 |
| | 6 | 43 | 100 |
| MPS III | 1 | 74 | 46 |
| | 2 | 108 | 8 |
| | 3 | 82 | 30 |
| | 4 | 28 | 100 |
| | 5 | 58 | 82 |
| | 6 | 35 | 100 |
| MPS IV | 1 | 46 | 12 |
| | 2 | 50 | 40 |
| | 3 | 104 | 43 |
| | 4 | 24 | 83 |
| | 5 | 26 | 54 |
| | 6 | 23 | 100 |
| MPS VI | 1 | 97 | 56 |
| | 2 | 107 | 11 |
| | 3 | 86 | 17 |
| | 4 | 57 | 44 |
| | 5 | 59 | 69 |
| | 6 | 43 | 69 |
| Fabry | 1 | 49 | 57 |
| | 2 | 63 | 45 |
| | 3 | 37 | 37 |
| | 4 | 24 | 100 |
| | 5 | 28 | 98 |
| | 6 | 31 | 100 |
| Gaucher | 1 | 99 | 33 |
| | 2 | 323 | 100 |
| | 3 | 161 | 51 |
| | 4 | 14 | 100 |
| | 5 | 28 | 100 |
| | 6 | 48 | 100 |
| Niemann-Pick A/B | 1 | 28 | 100 |
| | 2 | 31 | 84 |
| | 3 | 39 | 69 |
| | 4 | 24 | 100 |
| | 5 | 38 | 100 |
| | 6 | 51 | 100 |
| Pompe | 1 | 65 | 31 |
| | 2 | 50 | 26 |
| | 3 | 65 | 100 |
| | 4 | 44 | 100 |
| | 5 | 51 | 100 |

LAMP-1 (+Tail) was found to have significantly different properties to LAMP-1 (-Tail) in that LAMP-1 (+Tail) formed aggregates while LAMP-1 (-Tail) did not.

### 4.5.1 Effect of CHAPS on the LAMP-1 Immunoquantification Assays

To determine whether LAMP-1 formed aggregates based on hydrophobic interactions, CHAPS was used to dissociate any interactions that may exist in the HT1080 LAMP-1 (-Tail) and SF LAMP-1 (+Tail) calibration standards. CHAPS was added to the calibration standards at 0.1, 1 and 2% and assayed with the BB6 2-step assay (Figure 4.12) and LAMP-1 (Tail) 1-step assay (Figure 4.13). Figure 4.12A shows the effects of CHAPS on the HT1080 LAMP-1 (-Tail) calibration standard in the BB6 assay. The addition of CHAPS inhibited the detection of HT1080 LAMP-1 (-Tail) in the BB6 assay. At the addition of 0.1% of CHAPS, the assay was inhibited more than 50% and at 1 and 2% the assay was inhibited 85%. Figure 4.12B shows the effects of CHAPS on the SF LAMP-1 (+Tail) calibration standard in the BB6 assay. The addition of CHAPS to SF LAMP-1 (+Tail) also inhibited the BB6 assay however the inhibition was not as great as the effect on HT1080 LAMP-1 (-Tail). At the addition of 0.1% CHAPS, inhibition was not evident. When 1% CHAPS was added, the assay was inhibited approximately 60% and at 2% the inhibition observed was 70%. Figure 4.13A shows the effects of CHAPS on the SF LAMP-1 (+Tail) calibration curve in the LAMP-1 (Tail) assay. In this assay, the addition of CHAPS enhanced the LAMP-1 (Tail) signal. When 0.1% CHAPS was added, the assay was enhanced nearly 5-fold. When 1 and 2% CHAPS was added, the signal obtained was lower than the signal given from the addition of 0.1% CHAPS, however the signals were still 2-fold greater than when no CHAPS was added.

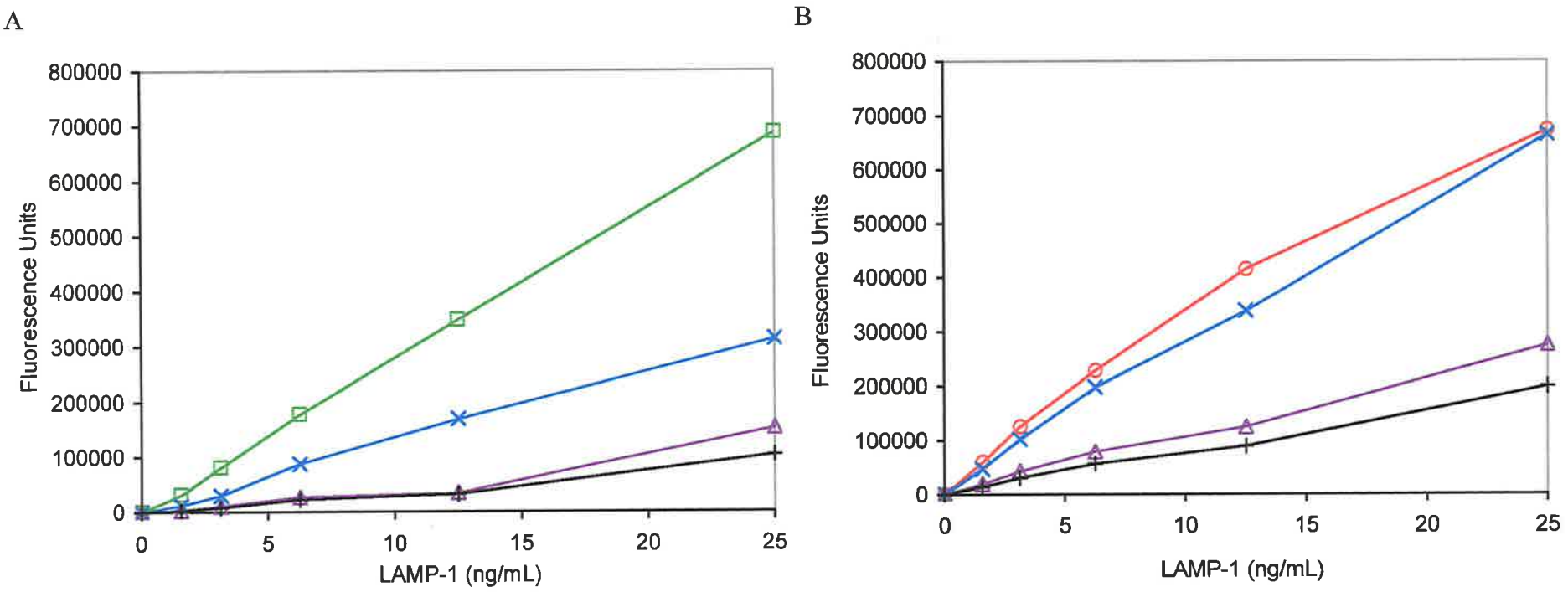



***Figure 4.12 Effects of CHAPS on the Immunoquantification of LAMP-1 in the BB6 2-Step Assay***

Microtitre plates were coated with BB6 monoclonal antibody (5 µg/mL, 4°C, overnight). In panel a), HT1080 LAMP-1 (-Tail) standards without (□) and with 0.1% (×), 1% (△) and 2% (+) CHAPS were detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight). In panel b), SF LAMP-1 (+Tail) standards without (○) and with 0.1% (×), 1% (△) and 2% (+) CHAPS were detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight).

The results indicate that CHAPS inhibits the BB6 2-step assay by disrupting the binding of LAMP-1 to the BB6 capture antibody. LAMP-1 (-Tail) was increasingly inhibited with the addition of increasing CHAPS concentration in the BB6 2-step assay (Figure 4.12A). The binding of SF LAMP-1 (+Tail) to BB6 antibody was not disrupted at the addition of 0.1% CHAPS (Figure 4.12B), possibly due to the increased avidity of aggregated LAMP-1 (+Tail) to the immobilised antibody on the plate. These results suggest that LAMP-1 (-Tail) does not form aggregates but LAMP-1 (+Tail) does. The inhibition on SF LAMP-1 (+Tail) was not as great at the higher CHAPS concentrations as compared to the inhibition on HT1080 LAMP-1 (-Tail), with the effect of disrupting BB6 binding of LAMP-1 (+Tail) offset by the continued partial aggregation of the LAMP-1 (+Tail).

Aggregation of SF LAMP-1 (+Tail) was also indicated with the effects of CHAPS in the LAMP-1 (Tail) assay (Figure 4.13A). A dual CHAPS effect was also observed for the LAMP-1 (Tail) assay, whereby CHAPS causes the dissociation of LAMP-1 aggregations as well as the disruption of LAMP-1 binding. In this case, CHAPS may disrupt either the binding of LAMP-1 (+Tail) to the anti-LAMP-1 (Tail) capture antibody or the binding of $Eu^{3+}$-labelled rabbit-anti-LAMP-1 detection antibody to LAMP-1 (+Tail). With 0.1% CHAPS disruption of SF LAMP-1 (+Tail) aggregation was seen to enhance the assay, this enhancement was off set, however, when CHAPS concentration was increased to 1-2% and disruption of LAMP-1 binding to the capture or detection antibody was observed as a decrease in the signal (Figure 4.13A). As the SF LAMP-1 (+Tail) calibration standard was not an affinity pure protein but a solubilised membrane preparation (section 2.2.11.2), it is possible that the aggregation observed may not be self aggregation but interaction with other membrane proteins.

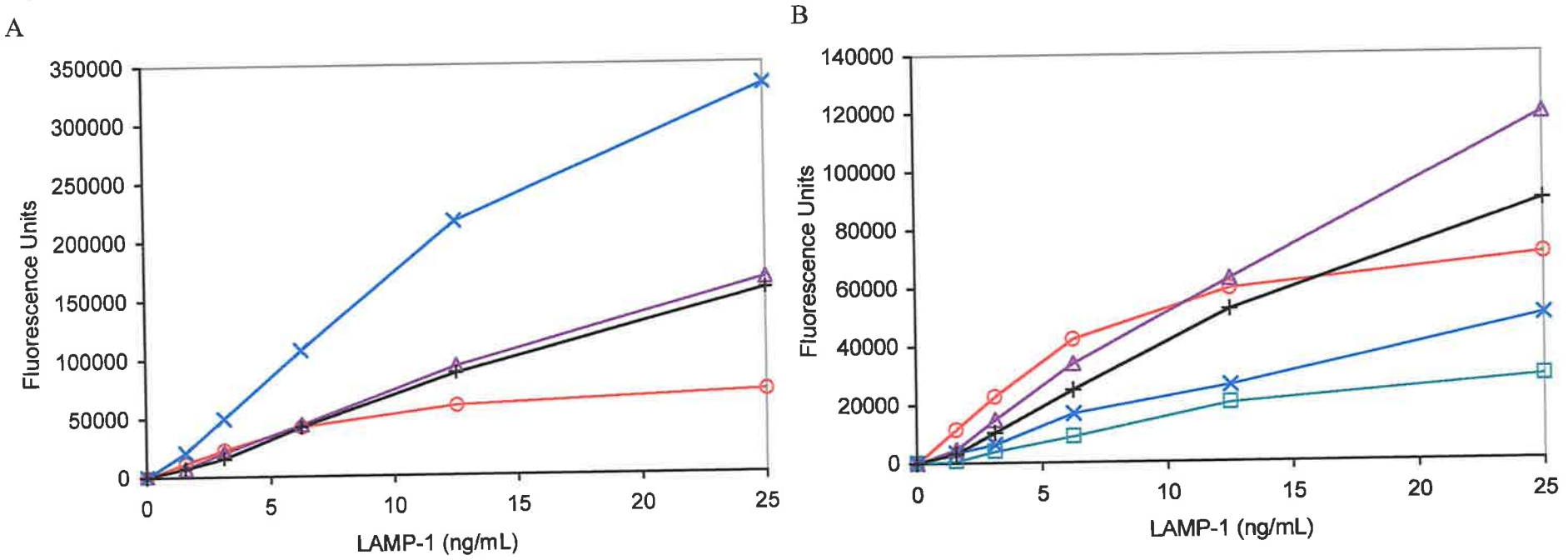



***Figure 4.13 Effects of CHAPS on the Immunoquantification of LAMP-1 in the LAMP-1 (Tail) 1-Step Assay***

Microtitre plates were coated with anti-LAMP-1 (Tail) polyclonal antibody (5 µg/mL, 4°C, overnight). In panel a), SF LAMP-1 (+Tail) standards without (○) and with 0.1% (×), 1% (△) and 2% (+) CHAPS were detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 4°C, overnight). In panel b), SF LAMP-1 (+Tail) standard without (○), with 2 µL of control plasma (□) and with 2 µL of plasma plus 0.1% (×), 1% (△) and 2% (+) CHAPS were detected with $Eu^{3+}$-labelled rabbit-anti-LAMP-1 polyclonal antibody (200 ng/mL, 37°C, 4 hr).

Investigation into the interaction of SF LAMP-1 (+Tail) with plasma in the LAMP-1 (Tail) assay was carried out by adding CHAPS at 0.1, 1 and 2% to SF LAMP-1 (+Tail) calibration standards spiked with 2 μL of control plasma (Figure 4.13B). The addition of CHAPS alleviated the inhibition caused by the plasma, however 0.1% CHAPS provided only a slight increase in signal and 1-2% CHAPS was required to give signals equivalent to the assay with CHAPS but without plasma (compare Figure 4.13B to Figure 4.13A). This suggests that the inhibition caused by plasma proteins interacting with LAMP-1 is stronger than the inhibition present in the SF LAMP-1 (+Tail) standard alone that only requires 0.1% CHAPS to provide maximum increase in the signal. Moreover, the SF LAMP-1 (+Tail) interaction with plasma proteins appears to be concentration dependent. The calibration curve begins to plateau at a LAMP-1 (+Tail) concentration of 6.25 ng/mL, suggesting that either the LAMP-1 (Tail) assay had been saturated or that at higher concentrations there may be an increase in SF LAMP-1 (+Tail) interaction (Figure 4.13B). The latter is most likely the case as when CHAPS was added the calibration curve no longer plateaus, indicating that SF LAMP-1 (+Tail) interaction with plasma proteins had been disrupted.

### 4.5.2 Size Exclusion Chromatography of LAMP-1

To further investigate the aggregation properties of LAMP-1, size exclusion chromatography was carried out on affinity purified HT1080 LAMP-1 (±Tail) (section 2.2.9). The Superose 12 column was calibrated with the following molecular weight standards; Catalase (232 kDa), Aldolase (158 kDa), Bovine Serum Albumin (67 kDa), Chrymotrypsinogen A (25 kDa), and Periodic Acid (214 Da) (Pharmacia Biotech., Uppsala, Sweden). These were used to determine the weight range of each eluted fraction. Each standard was dissolved in PBS (10 mg/mL). A solution mix containing

100 μg of each was prepared and made up to a total of 200 μL with PBS. From the elution profile of the calibration standards (Figure 4.14), we expected that non-aggregated LAMP-1 (-Tail) and LAMP-1 (+Tail) to elute between volumes of 11 and 13 mL as their molecular weights are 100 and 120 kDa, respectively. The Superose 12 column has a separation range of 1-300 kDa, therefore any proteins eluting at volumes of less than 8 mL would have molecular weights greater than 300 kDa. Aggregated LAMP-1 was therefore expected to elute at a volume of about 8 or 9 mL.

Affinity purified full length HT1080 LAMP-1 (100 ng) and HT1080 LAMP-1 (-Tail) (5 μg) were each made up to 200 μL with PBS and applied onto a Superose 12 size exclusion chromatography column (Figure 4.15A) (section 2.2.9). BB6 anti-LAMP-1 monoclonal antibody (100 μg) was mixed with HT1080 LAMP-1 (±Tail) to form aggregates in order to determine the volume in which aggregated LAMP-1 elutes from the column (Figure 4.15B). Eluted fractions were assayed for LAMP-1 using the BB6 2-step assay.

Aggregated LAMP-1 was found to elute from the size exclusion column at a volume of 9 mL, as expected (Figure 4.15B). Figure 4.15A shows that the size exclusion chromatography of HT1080 LAMP-1 (-Tail) resulted in two elution peaks. LAMP-1 (-Tail) does not appear to form any aggregates, the majority of the protein eluted at a volume of 12 mL that represented non-aggregated LAMP-1 (-Tail), and a smaller fraction eluted at a volume ranging from 20-25 mL, possibly proteolytic fragments. The size exclusion chromatography of HT1080 LAMP-1 (+Tail) resulted in three elution peaks, the first at a volume of 9 mL, representing aggregated LAMP-1 (+Tail). A

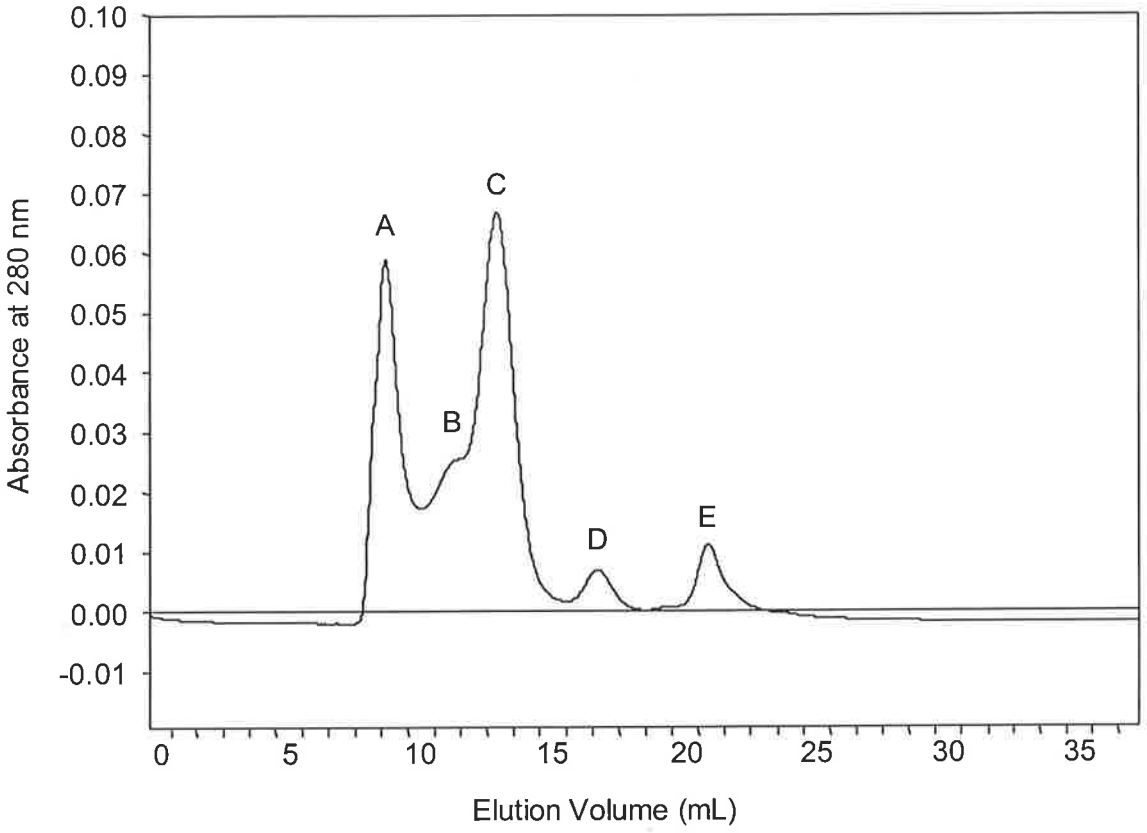



***Figure 4.14 Elution Profile of Molecular Weight Standards from a Superose 12 Chromatography Column***

Samples were applied to a Superose 12 size exclusion chromatography column (1 x 30 cm) at a flow rate of 0.5 mL/min and eluted with PBS (40 mL) (section 2.2.9). Samples applied onto the Superose column were A) Catalase (232 kDa); B) Aldolase (158 kDa); C) Bovine Serum Albumin (67 kDa); D) Chrymotrypsinogen A (25 kDa); E) Periodic Acid (<25 kDa).

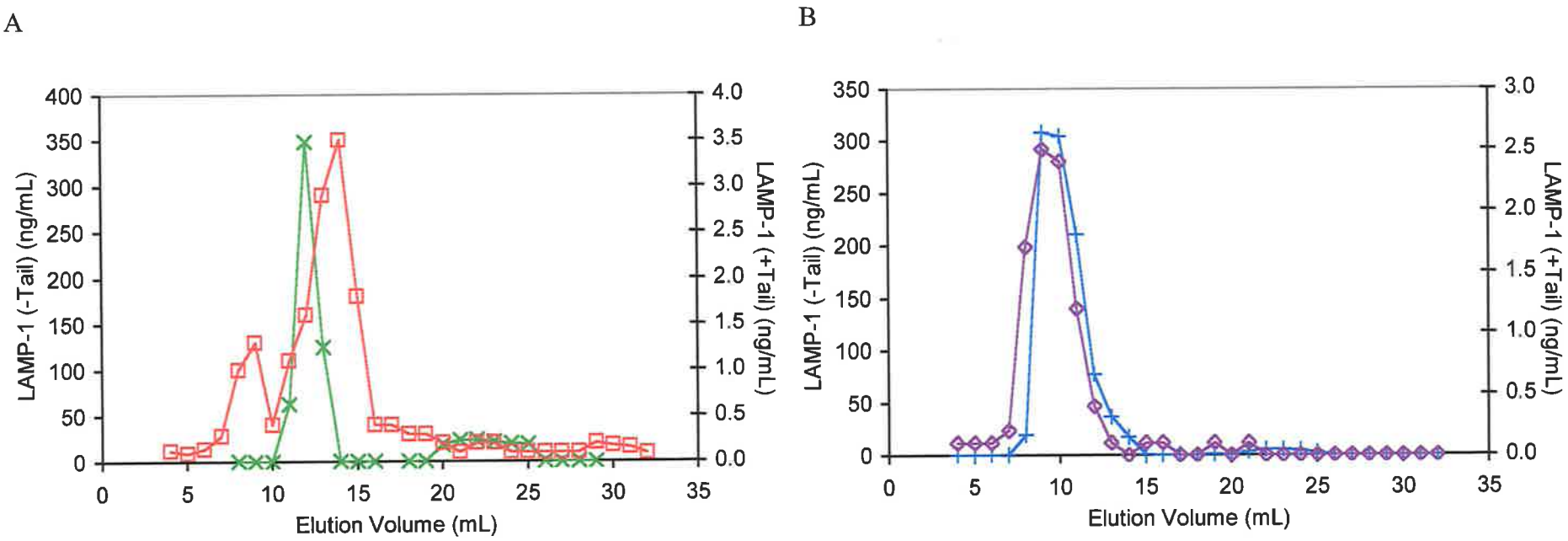



***Figure 4.15 Elution Profile of HT1080 LAMP-1 (±Tail) from a Superose 12 Chromatography Column***

Samples were applied to a Superose 12 size exclusion chromatography column (1 x 30 cm) at a flow rate of 0.5 mL/min and eluted with PBS (40 mL) (section 2.2.9). Panel A, elution profiles of HT1080 LAMP-1 (-Tail) (5 µg) (×) and HT1080 LAMP-1 (+Tail) (100 ng) (□). Panel B, elution profiles of HT1080 LAMP-1 (-Tail) (5 µg) with BB6 monclonal antibody (100 µg) (+) and HT1080 LAMP-1 (+Tail) (100 ng) with BB6 monclonal antibody (100 µg) (◇).

shoulder was seen to elute at a volume of 12 mL and the third peak eluted at a volume of 14 mL, both are thought to represent non-aggregated LAMP-1 (+Tail). Why LAMP-1 (+Tail) should elute at an apparently lower molecular weight than LAMP-1 (-Tail) is unclear, this could reflect a conformational change in the LAMP-1 (+Tail) or some interaction between the LAMP-1 (+Tail) and the column. These results show that only LAMP-1 (+Tail) is able to self aggregate, supporting the interpretation of the immunoassay results (section 4.5.1), and that the majority of LAMP-1 (+Tail) is in a non-aggregated form.

Size exclusion chromatography was also carried out on control plasma (200 μL) from two healthy individuals (1 and 2 from Table 4.2) and plasma from MPS II 1(100 μL), Niemann-Pick A/B 4 (100 μL) and Gaucher 2 (75 μL) affected individuals (see Table 4.2). Control plasma and LSD affected plasma were made up to 500 μL with PBS. These plasma samples were chosen according to their LAMP-1 (+Tail) content, the control plasma contained approximately equal amounts of both LAMP-1 (±Tail), MPS II 1 contained predominantly LAMP-1 (-Tail), Niemann-Pick A/B 4 and Gaucher 2 contained predominantly LAMP-1 (+Tail).

Both control plasma samples (Figure 4.16) appear to be predominantly made up of non-aggregated LAMP-1, eluting at a volume of 12 mL. As non-aggregated LAMP-1 (-Tail) eluted at volume 12 mL, as did a fraction of LAMP-1 (+Tail) (Figure 4.13), we are unable to determine whether the peak eluted from the control samples is LAMP-1 (-Tail) or LAMP-1 (+Tail). However, from the immunoassays, controls 1 and 2 had LAMP-1 (+Tail) levels of approximately 50 and 64%, respectively (Table 4.2),

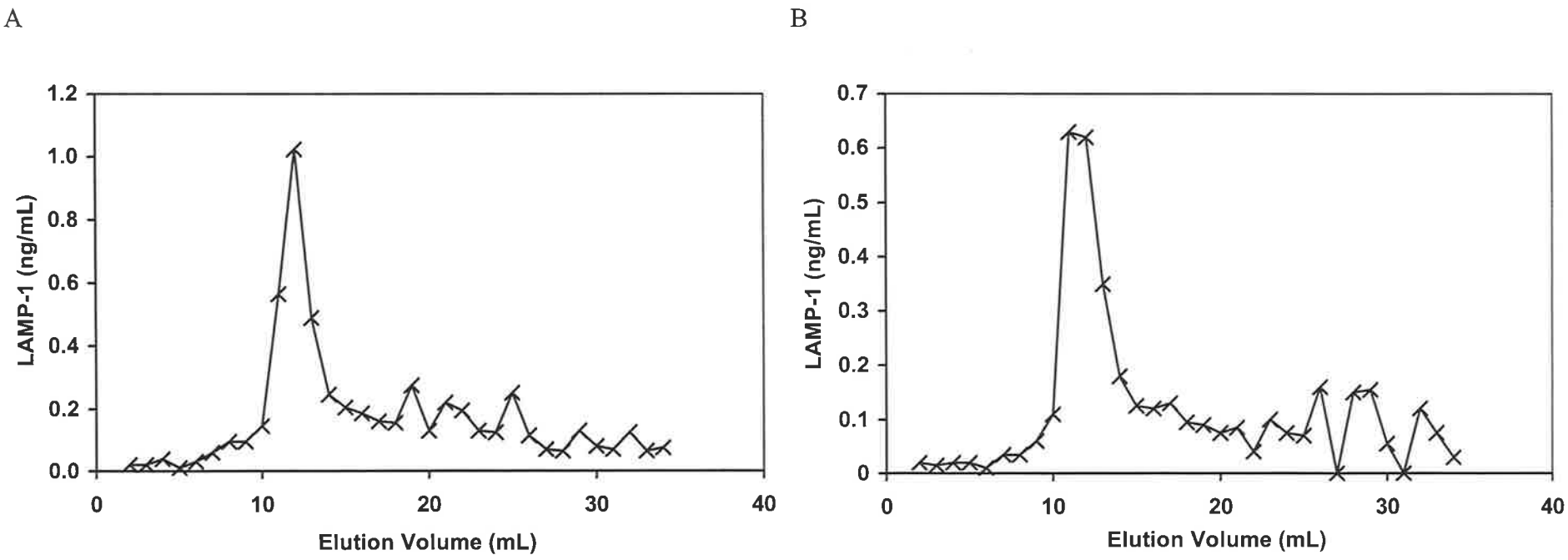


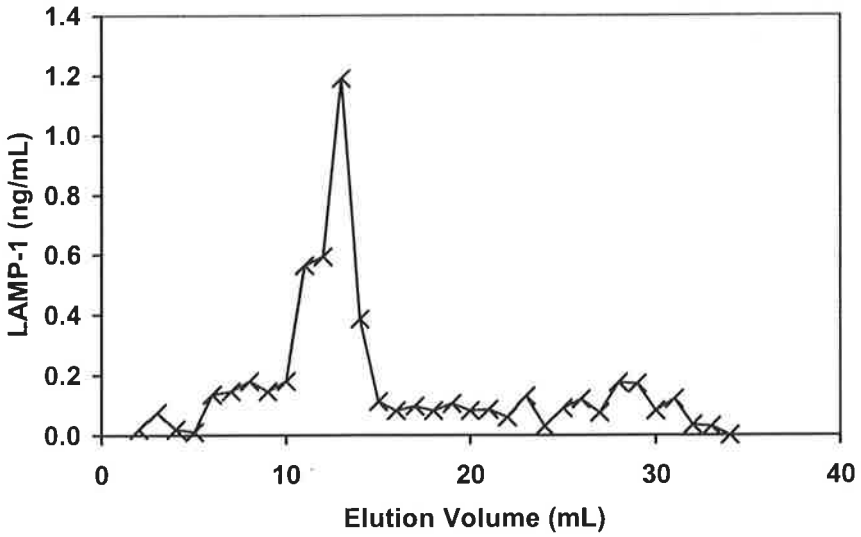

***Figure 4.16 Elution Profile of Control Plasma Samples from a Superose 12 Chromatography Column***

Control plasma samples (200 µL) were applied to a Superose 12 size exclusion chromatography column (1 x 30 cm) at a flow rate of 0.5 mL/min and eluted with PBS (section 2.2.9). Fractions were assayed for LAMP-1 using the BB6 2-step assay (section 2.2.11.3). Panel A, control plasma 1; panel B, control plasma 2.

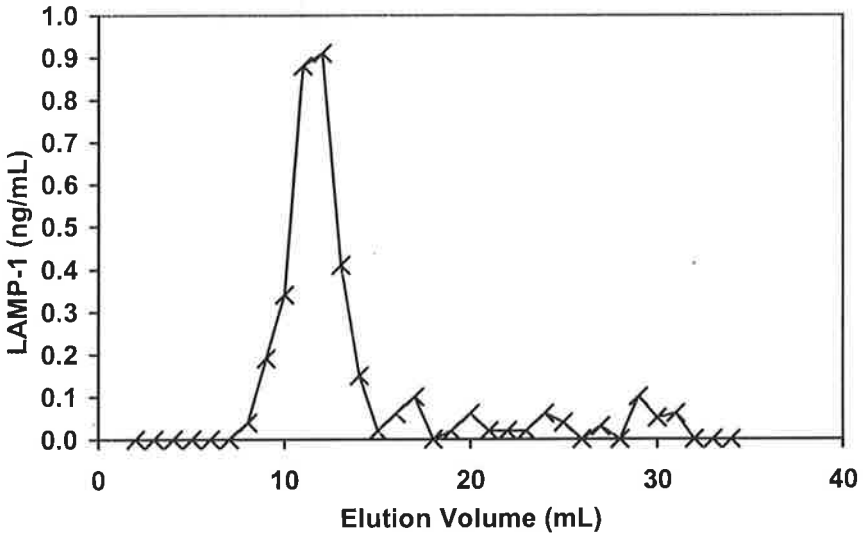
***Figure 4.17 Elution Profile of LSD-Affected Plasma from a Superose 12 Chromatography Column***

LSD affected plasma samples were applied to a Superose 12 size exclusion chromatography column (1 x 30 cm) at a flow rate of 0.5 mL/min and eluted with PBS (section 2.2.9). Fractions were assayed for LAMP-1 using the BB6 2-step assay (section 2.2.11.3). Panel A, MPS II plasma (100 μL); panel B, Niemann-Pick A/B plasma (100 μL); panel C, Gaucher plasma (75 μL).

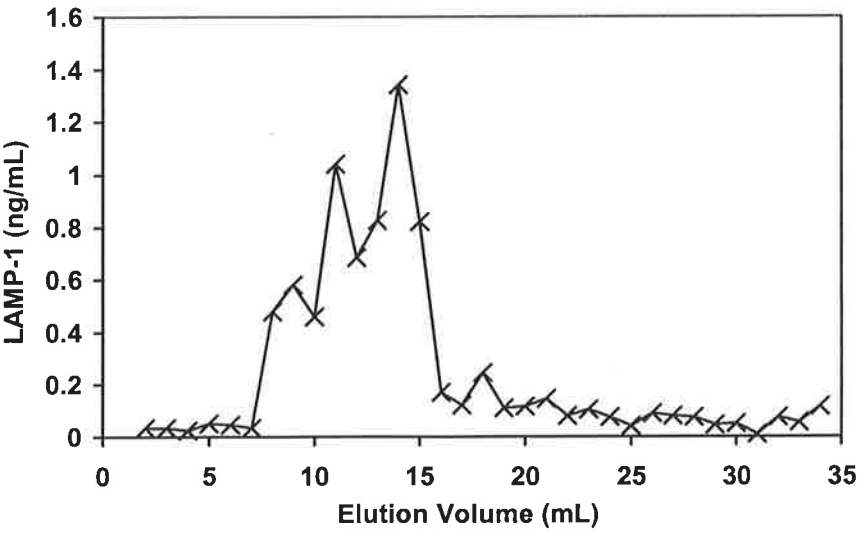
A


LAMP-1 (ng/mL)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
Elution Volume (mL)

B


LAMP-1 (ng/mL)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
Elution Volume (mL)

C


LAMP-1 (ng/mL)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
Elution Volume (mL)

therefore, both non-aggregated LAMP-1 (-Tail) and LAMP-1 (+Tail) are probably represented in the eluted peak of these plasma samples. Size exclusion chromatography of MPS II 1 plasma (Figure 4.17A) resulted in three elution peaks, similar to the elution profile of HT1080 LAMP-1 (+Tail) (Figure 4.15A). As indicated by the immunoassays (Table 4.2), MPS II 1 plasma contained predominantly non-aggregated LAMP-1 (-Tail). Size exclusion chromatography of Niemann-Pick A/B 4 plasma (Figure 4.17B) resulted in a main peak at an elution volume of 11-12 mL with a slight shoulder at 9-10 mL, representing aggregated LAMP-1 (+Tail). Niemann-Pick A/B 4 plasma was assayed to contain 100% of LAMP-1 (+Tail) (Table 4.2), thus the eluted peak at 11-12 mL probably represents non-aggregated LAMP-1 (+Tail). Size exclusion chromatography of Gaucher 2 plasma (Figure 4.17C) had a similar elution profile to HT1080 LAMP-1 (+Tail) (Figure 4.15A), correlating to the LAMP-1 immunoassay of this Gaucher plasma whereby 100% was found to be LAMP-1 (+Tail). The eluted peaks at 11 mL and 14 mL must therefore represent non-aggregated LAMP-1 (+Tail). For plasma samples with predominantly LAMP-1 (+Tail), aggregated LAMP-1 (+Tail) was expected to be observed, however, this was not the case. It is unclear why a more obvious peak at 9 mL was not seen in both control 1 and 2 plasma samples (Figure 4.16) or the Niemann-Pick A/B 4 plasma sample. It is possible that some plasma proteins may be interacting with the LAMP-1 (+Tail) in these plasma samples such as to prevent self aggregation. It was also noted that the aggregated LAMP-1 (+Tail) was only detected in the MPS II 1 and Gaucher 2 plasma samples, which had a higher LAMP-1 concentration (Table 4.2), compared to the other plasma samples. Thus, there may be a critical LAMP-1 concentration required to achieve LAMP-1 (+Tail) aggregation and the control and Niemann-Pick A/B plasma samples were below this concentration.

There are two possible causes for aggregation of LAMP-1 in plasma. One is that the aggregation is formed by LAMP-1 (+Tail) self aggregating, another is that LAMP-1 (-Tail) or LAMP-1 (+Tail) may be interacting with plasma proteins to form aggregates. However, as plasma does not effect the HT1080 LAMP-1 (-Tail) calibration curve but has a slight inhibition effect on the SF LAMP-1 (+Tail) calibration curve in the BB6 2-step assay (Figure 4.9 and section 4.3.2), aggregation in plasma is at least partially due to LAMP-1 (+Tail) interacting with plasma proteins. Therefore, the aggregated form of LAMP-1 seen in the size exclusion chromatography of the plasma samples is thought to be only LAMP-1 (+Tail). The amount of aggregated LAMP-1 in a plasma sample seemed to correlate to the degree of inhibition the plasma sample placed upon the LAMP-1 (Tail) assay (data not shown). When the Gaucher plasma was assayed, it exhibited a marked inhibition (approximately 70%) on the LAMP-1 (Tail) assay and it was this plasma sample that contained the greatest level of aggregated LAMP-1 form. Control plasma samples 1 and 2 that showed the lowest level of inhibition (approximately 10% and 1%, respectively) in the LAMP-1 (Tail) assay, also contained the least level of aggregated LAMP-1 form. This may result from the LAMP-1 (+Tail) in plasma interacting with the SF LAMP-1 (+Tail) calibration standard to form aggregates.

## 4.6 Discussion

The discovery of two forms of soluble LAMP-1 (Meikle *et al.*, 1999b) and reports on the aggregation of LAMP-2 (Jadot *et al.*, 1997) prompted this investigation into the form and aggregation properties of LAMP-1 in circulation.

Anti-LAMP-1 antibodies; BB6 and 1B1 monoclonal antibodies and the rabbit anti-LAMP-1 (Tail) and rabbit anti-LAMP-1 polyclonal antibodies, were used to develop 3 LAMP-1 immunoassays (Figure 4.5) (section 4.3). The first 3 antibodies were used to capture LAMP-1 and $Eu^{3+}$-labelled rabbit anti-LAMP-1 polyclonal antibody was used to detect bound LAMP-1. Initially it was thought that 1B1 monoclonal antibody would recognise only the LAMP-1 (-Tail) form, however, after optimisation of the immunoassays it was found that 1B1 recognised both LAMP-1 (±Tail) although gave a higher response for LAMP-1 (-Tail) (Figure 4.5C). The BB6 assay recognised both forms equally (Figure 4.5A) and the LAMP-1 (Tail) assay recognised only LAMP-1 (+Tail) (Figure 4.5B). The epitope maps of the anti-LAMP-1 monoclonal and polyclonal antibodies (Figure 4.4) showed that BB6, 4F5 and 1B1 monoclonal antibodies recognised similar epitopes (section 4.2), explaining the similar results obtained from the 1B1 and BB6 assays (section 4.3.1). Anti-LAMP-1 (Tail) polyclonal antibody only recognised the cytoplasmic tail and rabbit anti-LAMP-1 polyclonal antibody recognised peptide 46 strongly and epitopes either side of the hinge region (peptides 19, 35 and 44) (Table 4.1).

Affinity purified HT1080 LAMP-1 (-Tail) (section 3.2.2) was used as the calibration standard in the LAMP-1 immunoassays. Due to the difficulties in obtaining affinity purified HT1080 LAMP-1 (+Tail) (section 3.2.1), we were unable to use this as a calibration standard. Therefore, SF LAMP-1 (+Tail) membrane preparations (section 2.2.11.2) were used (section 4.1.4). The assays of control and LSD affected plasma showed that the majority of the plasma samples contained predominantly LAMP-1 (+Tail) and that there was no difference between the form of LAMP-1 in control plasma and the form of LAMP-1 in LSD plasma (section 4.4). Although the LAMP-1 (Tail)

assay distinguished between LAMP-1 (-Tail) and (+Tail), it was limited by the inhibition resulting from LAMP-1 (+Tail) self aggregating or by LAMP-1 (+Tail) interacting with plasma proteins (section 4.3.2 and section 4.5). Moreover, the degree of inhibition exhibited by plasma in the LAMP-1 (Tail) assay correlated to the amount of aggregated LAMP-1 in the plasma sample as determined by size exclusion chromatography.

The ability of CHAPS to alleviate the inhibition of the assay (Figure 4.13) was not realised until after all the plasma samples were assayed. Due to the limited reagents and samples available it was not possible to repeat these studies. In future, when using the LAMP-1 (Tail) assay, CHAPS will be added to the samples to ensure a more accurate quantification of LAMP-1 (+Tail).

The aggregation properties of LAMP-1 (±Tail) were investigated through the LAMP-1 immunoassays and size exclusion chromatography of affinity purified HT1080 LAMP-1 (±Tail) (section 4.5). The results showed that self aggregation of LAMP-1 was only observed in LAMP-1 (+Tail) (Figure 4.15). Furthermore, LAMP-1 interaction with plasma proteins was seen only for LAMP-1 (+Tail) (Figures 4.9 and 4.10). Taken together, these observations indicate that self aggregation and interaction with plasma proteins, are via the cytoplasmic tail of LAMP-1. This would explain the inhibition effect of plasma on the SF LAMP-1 (+Tail) calibration standard in the BB6 and LAMP-1 (Tail) assays. In addition, plasma samples with a greater proportion of aggregated LAMP-1 (+Tail) content (Figure 4.17C) would be able to aggregate with the SF LAMP-1 (+Tail) calibration standard and exert an inhibitory effect in the LAMP-1 (+Tail) assay (section 4.5.2). The BB6 anti-LAMP-1 monoclonal antibody recognises an epitope close

to the membrane spanning domain which may be slightly masked when LAMP-1 self aggregates or interacts with plasma proteins, thus preventing or limiting BB6 from binding LAMP-1 (+Tail) (Figure 4.9). The anti-LAMP-1 (Tail) polyclonal antibody recognises only the cytoplasmic tail of LAMP-1, thus when LAMP-1 self aggregates or interacts with plasma proteins via its tail the binding of anti-LAMP-1 (Tail) polyclonal antibody to LAMP-1 (+Tail) is greatly inhibited (Figure 4.10).

The aggregation of LAMP-1 in plasma is a complex process and is probably a composite of self aggregation and interaction with plasma proteins, both actions through the cytoplasmic tail.

The results of this study suggest that LAMP-1 (-Tail) does not interact with plasma proteins. However, it may be that, compared to LAMP-1 (+Tail), LAMP-1 (-Tail) interacts with less plasma proteins or that the interaction is less specific so that the binding is weaker than the interaction with the antibodies. Some of the potential binding plasma proteins are identified in Chapter 5.

# CHAPTER FIVE:

# Identifying Proteins that Interact with LAMP-1 in Circulation

## 5 Introduction and Aims

On the cell surface, LAMP-1 has been found to interact with two adhesion molecules, galectin-3 and E-selectin. Galectin-3, a mammalian lectin, is found in a wide range of tissues and cells and their expression is developmentally regulated (Inohara *et al.*, 1999). Galectin-3 has affinity for β-galactoside-containing glycoconjugates and preferentially binds to poly-N-acetyl-lactosaminoglycans (Barondes *et al.*, 1994). E-selectin is expressed on activated endothelial cells and recognises the sialyl-Lewis X antigen, expressed on neutrophils, monocytes and certain T lymphocytes (Fukuda *et al.*, 1984; Mizoguchi *et al.*, 1984).

Sialyl-Lewis X is formed at the termini of N- and O-glycan chains, in particular, poly-N-acetyllactosamines are often modified to express sialyl-Lewis X (Lee *et al.*, 1990; Saitoh *et al.*, 1992; Wilkins *et al.*, 1996). LAMP-1 and LAMP-2 were shown to be the major carriers for poly-N-acetyllactosamines (Laferte and Dennis, 1989). Moreover, LAMP-1 was shown to be the cellular ligand for galectin-3 in human fibrosarcoma and human colon-adenocarcinoma cells (Sarafian *et al.*, 1998) and was found to be the main E-selectin ligand on human colon tumour cells (Tomlinson *et al.*, 2000). Cell surface LAMP-1 has therefore been proposed to assist in cell-cell interactions. However, the role of soluble LAMP-1 in circulation is not known.

In this chapter the functional role of LAMP-1 in circulation is investigated by using affinity chromatography and proteomic methods to identify plasma proteins that interact with LAMP-1 in circulation.

## 5.1 Determination of Proteins Interacting with LAMP-1 in Circulation

Recombinant LAMP-1 (-Tail) produced from CHO-K1 and HT1080 cells and the peptide sequence of the cytoplasmic tail of LAMP-1 were coupled to affinity columns (section 2.2.4). Plasma was run over these LAMP-1 columns and plasma proteins that bound were isolated by SDS-PAGE and 2D gel electrophoresis (section 2.2.13). All plasma proteins of interest were excised from gels stained with Coomassie blue and peptide mass fingerprinting was then performed by APAF (Australian Proteome Analysis Facility, North Ryde, Australia) and proteins identified by searching EMBL and Swiss-Prot databases.

### 5.1.1 Preparation of LAMP-1 Affinity Columns

Affinity purified recombinant CHO LAMP-1 (-Tail) and HT1080 LAMP-1 (-Tail) (4 mg) (section 2.2.5) were coupled to 2 mL of Affi-Gel 15 (section 2.2.4). The binding efficiency of CHO LAMP-1 (-Tail) was 25 % with 0.5 mg/mL LAMP-1 bound to the Affi-Gel. The binding efficiency of HT1080 LAMP-1 (-Tail) was 50% with 1 mg/mL LAMP-1 bound. LAMP-1 cytoplasmic tail peptide (H-CLUGRKRSHAGYQTI-OH) was bound to Sepharose at 0.5 mg/mL by Chiron Mimotopes (Mimotopes, Melbourne, Australia).

Two control columns were made to pre-clear non-specific plasma proteins before purification over the LAMP-1 columns. The first control column that was used was a Blank column containing only Affi-Gel 15 (2 mL), while the second column was prepared by coupling BSA (1 mg/mL) to Affi-Gel 15 (2 mL).

### 5.1.2 Optimisation of Affinity Chromatography of Plasma Proteins on LAMP-1 Columns

Plasma (400 mL) was collected from approximately 10 healthy de-identified volunteers by the Department of Immunology (Women's and Children's Hospital, South Australia, Australia) over a period of 2 weeks and pooled. Preliminary experiments were carried out with this plasma to determine the volume required to purify sufficient amounts of plasma proteins. Plasma collected from a single healthy individual was then used for preparative experiments. While HT1080 LAMP-1 (-Tail) protein was still being purified (section 3.2.2), preliminary experiments were carried out on the CHO LAMP-1 (-Tail) and LAMP-1 Peptide Tail columns.

#### *5.1.2.1 Preliminary Analysis of Plasma Proteins of Interest*

Plasma (20 mL) was clarified by centrifugation (13,000xg) and applied to the Blank Affi-Gel 15 column and the CHO LAMP-1 and LAMP-1 Peptide Tail columns separately at a flow rate of 0.5 mL/min. Each column was washed with PBS until all unbound protein was removed as determined by absorbance at 280 nm and the bound plasma proteins eluted with elution buffers of increasing strength, 1 M NaCl/PBS then 0.1 M $H_3PO_4$/NaOH (pH 2.5). These were followed by pre-elution buffer (10 mM $NaH_2PO_4$/NaOH, pH 6.5) then 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5). Fractions under the NaCl, low pH and $MgCl_2$ elution peaks were collected and assayed to determine the amount of plasma proteins eluted (section 2.2.3). Table 5.1 shows the amount of plasma proteins recovered from each of the columns. There was substantial variation in the protein recovered from the 1 M NaCl wash of each column and between the first and second run of each column type. This may be the result of inefficient washing of the columns despite monitoring the eluate for the absorbance at 280 nm.

Plasma proteins and washes may have been channeled through the column preventing efficient washing and giving an impression that a high amount of proteins had bound and were eluted with the 1 M NaCl. However, the level of protein eluted with the low pH was much more consistent between first and second runs, suggesting that chanelling was not a problem.

**Table 5.1 Total Amount of Plasma Proteins Recovered From Blank and LAMP-1 Columns**

| Elution Buffer | [a]Blank Affi-Gel 15 | [b]Blank Affi-Gel 15 | [a]CHO LAMP-1 (-Tail) | [b]CHO LAMP-1 (-Tail) | [a]Peptide Tail | [b]Peptide Tail |
|---|---|---|---|---|---|---|
| [c]Total Proteins 1 M NaCl (mg) | 0.77 | 0.13 | 0.58 | 0.22 | 0.07 | 0.32 |
| [c]Total Proteins pH 2.5 (mg) | 0.046 | 0.055 | 0.16 | 0.23 | 0.22 | 0.17 |
| [c]Total Proteins 3 M $MgCl_2$ (mg) | 0.082 | 0.0058 | 0.16 | 0.039 | 0.011 | 0.051 |

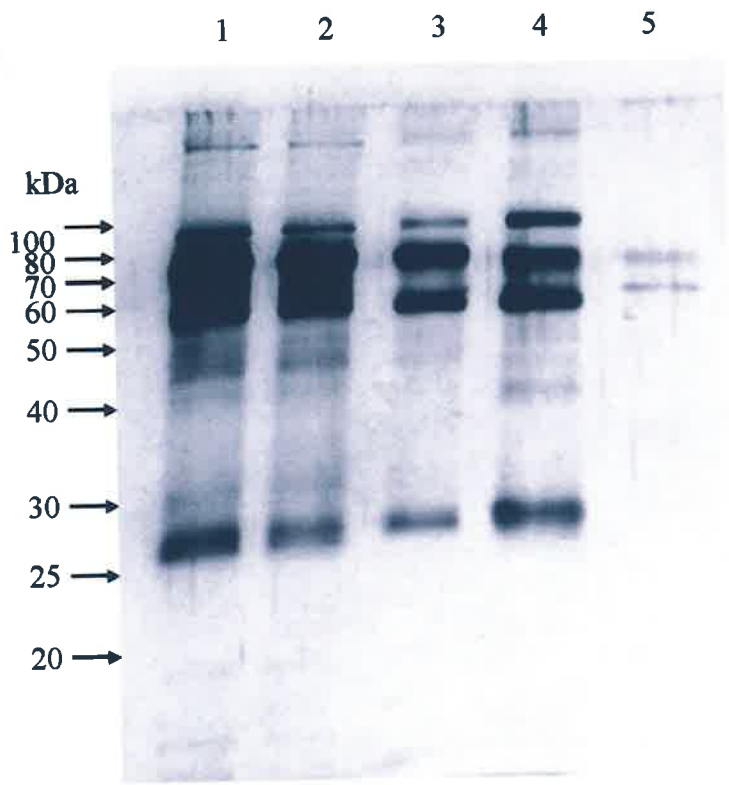
[a] Recoveries of plasma proteins from first run; [b] Recoveries of plasma proteins from re-applying first run column flow through onto columns; [c] Total amount of plasma proteins eluted with 1 M NaCl/PBS (pH 7.4), 0.1 M $H_3PO_4$/NaOH(pH 2.5) and 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5) was calculated as an average of 2 runs where 20 mL of plasma loading was used.

Eluted plasma proteins were analysed on SDS-PAGE (section 2.2.13). Figure 5.1 shows the plasma proteins that were eluted from the Blank column (Figure 5.1A), and also CHO LAMP-1 (-Tail) and LAMP-1 Peptide Tail columns (Figure 5.1B). By comparing the plasma proteins eluted from the LAMP-1 columns to that eluted from the Blank column, proteins that bound specifically to LAMP-1 were identified. Several plasma proteins of interest, indicated by the arrows, were found, particularly from the 0.1 M $H_3PO_4$/NaOH (pH 2.5) elutions. Very little protein was eluted using 3 M $MgCl_2$, 10 mM

***Figure 5.1 SDS-PAGE of Plasma Proteins Eluted from LAMP-1 Affinity Columns***

Plasma samples (20 mL) were applied onto the Blank Affi-Gel 15, CHO LAMP-1 (-Tail) and LAMP-1 Peptide Tail columns at a flow rate of 0.5 mL/min. The columns were washed with PBS and plasma proteins were eluted from the columns successively with 1 M NaCl/PBS, 0.1 M $H_3PO_4$/NaOH (pH 2.5) and 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5). Eluted plasma proteins (250 ng) were run on 12.5% polyacrylamide gels at a constant voltage (180 V) (section 2.2.13) then silver stained (section 2.2.13.2). Panel A, proteins eluted from the Blank column; panel B, proteins eluted from CHO LAMP-1 (-Tail) (lanes 1-5) and LAMP-1 Peptide Tail columns (lanes 6-9). Lanes A1, B1 and B6 = plasma load; lanes A2, B2 and B7 = column flow through; lanes A3, B3 and B8 = plasma protein eluted with 1 M NaCl; lanes A4, B4 and B9 = plasma protein eluted with 0.1 M $H_3PO_4$/NaOH (pH 2.5); lanes A5 and B5 = plasma protein eluted with 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5). Arrows indicates plasma proteins of interest.

A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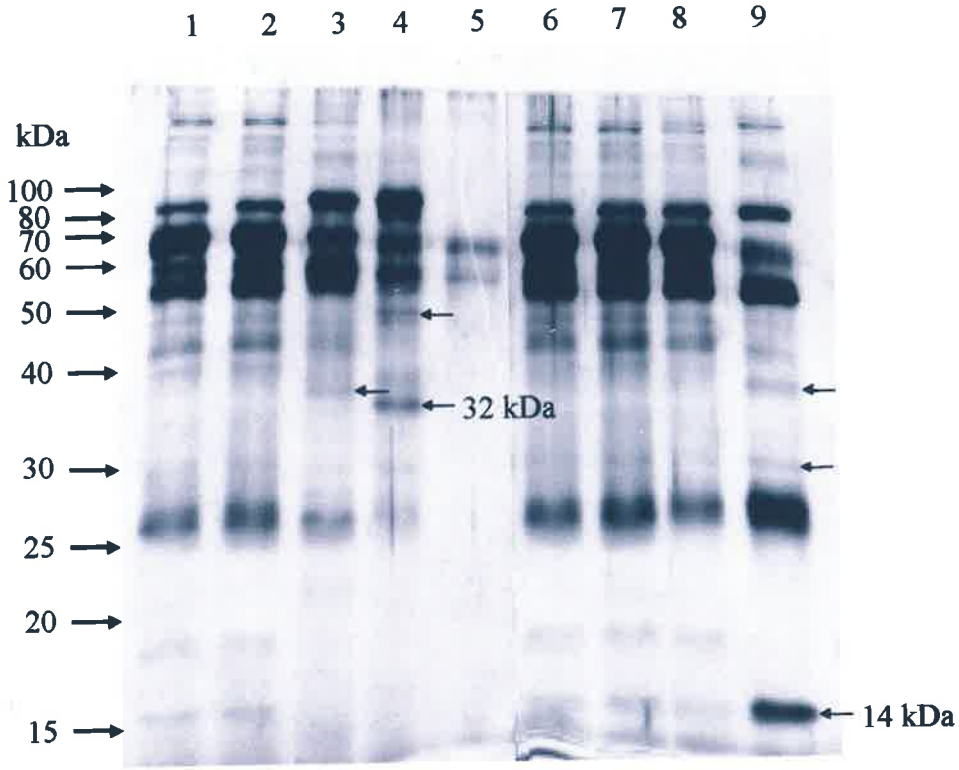
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$NaH_2PO_4$/NaOH (pH 6.5), thus this buffer was used to clear the columns of remnant plasma proteins after each run.

To determine whether all proteins of interest were bound with one run through of the LAMP-1 columns, the flow throughs from each column were re-applied to the separate columns and the plasma proteins that were subsequently eluted were analysed on SDS-PAGE. Figure 5.2 shows proteins eluted with 0.1 M $H_3PO_4$/NaOH (pH 2.5) in the first and second run through. There was an enhancement of the proteins of interest in the second run of the 20 mL plasma load as compared to the first run. The protein recovery is shown in Table 5.1. It is thought that non-specific plasma proteins were substantially removed during the first run, leaving the proteins that interact with LAMP-1 to bind more efficiently during the second run.

#### *5.1.2.2 Determination of Plasma Volume Required for Affinity Chromatography*

In order to determine the minimum volume of plasma needed to purify the plasma proteins of interest and to attempt to reduce non-specific binding to the affinity columns, 1 mL of plasma was diluted 1:10 in PBS. This was applied onto the Blank, CHO LAMP-1 (-Tail) and LAMP-1 Peptide Tail columns and plasma proteins eluted as described (section 5.1.2.1). Diluting the plasma reduced the level of non-specific proteins (data not shown). Re-applying the flow through showed that most of the proteins were removed in the first run for CHO LAMP-1 (-Tail) and LAMP-1 Peptide Tail columns (Table 5.2) and the plasma proteins of interest were enhanced in the second run as in Figure 5.2. However, the amount of plasma proteins eluted from the 1 mL plasma load was not sufficient to carry out peptide mass fingerprinting. A plasma volume of 10 mL was therefore used to purify sufficient amounts of plasma proteins.

**Table 5.2 Total Amount of Plasma Proteins Recovered From 1 mL of Plasma Applied onto the Blank and LAMP-1 Columns**

| Elution Buffer | [a]Blank Affi-Gel 15 | [b]Blank Affi-Gel 15 | [a]CHO LAMP-1 (-Tail) | [b]CHO LAMP-1 (-Tail) | [a]Peptide Tail | [b]Peptide Tail |
|---|---|---|---|---|---|---|
| Total Proteins 1 M NaCl (mg) | 0.016 | 0.023 | 0.015 | 0.0071 | 0.062 | 0.0081 |
| Total Proteins pH 2.5 (mg) | 0.0038 | 0.0046 | 0.0091 | 0.0042 | 0.022 | 0.0092 |
| Total Proteins 3 M $MgCl_2$ (mg) | 0.0053 | 0.0049 | 0.0079 | 0.018 | 0.079 | 0.0086 |

[a] Recoveries of plasma proteins from first run; [b] Recoveries of plasma proteins from re-applying first run column flow through onto columns.

In an attempt to reduce non-specific plasma proteins, the Blank Affi-Gel 15 column was connected to a LAMP-1 column such that the flow through from the Blank column was run onto one of the three LAMP-1 columns (Figure 2.1). To ensure that the plasma proteins bound to the column the plasma was recirculated through the Blank column and the LAMP-1 column (5x) at a flow rate of 0.5 mL/min. The Blank column was first used to reduce non-specific binding plasma proteins, but was replaced by a BSA column coupled to Affi-Gel 15 in an attempt to further reduce non-specific binding proteins. To ensure conformity, plasma from one healthy individual, rather than pooled plasma, was used in the subsequent experiments.

Using the optimal conditions outlined above, the CHO LAMP-1 (-Tail) and HT1080 LAMP-1 (-Tail) columns were shown to have a maximum binding capacity of 1.4 and 1.5 mg plasma protein, respectively, while the LAMP-1 Peptide Tail column had a binding capacity of 1 mg. The total amount of plasma proteins eluted from the columns

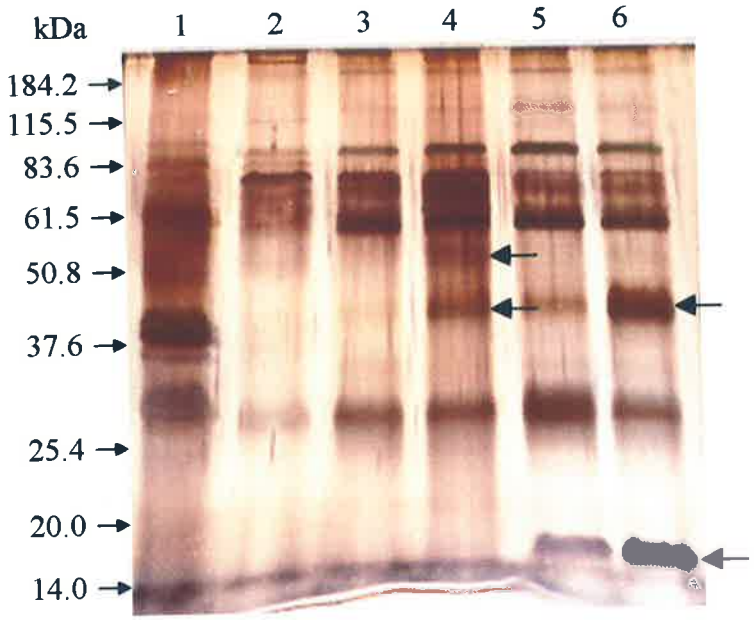



***Figure 5.2 SDS-PAGE of Plasma Proteins Eluted with 0.1 M $H_3PO_4$/NaOH (pH 2.5) from LAMP-1 Affinity Columns After Re-Application of Column Flow Through***

Plasma samples (20 mL) were applied onto the Blank Affi-Gel 15 (lanes 1 and 2), CHO LAMP-1 (-Tail) (lanes 3 and 4) and LAMP-1 Peptide Tail columns (lanes 5 and 6) at a flow rate of 0.5 mL/min. The columns were washed with PBS and plasma proteins were eluted from the columns with 1 M NaCl, 0.1 M $H_3PO_4$/NaOH (pH 2.5) and 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5). The plasma flow through from the columns were re-applied onto each column and eluted as before. Low pH eluted plasma proteins (250 ng) were run on 12.5% polyacrylamide gels at a constant voltage (180 V) (section 2.2.13) then silver stained (section 2.2.13.2). Lanes 1, 3 and 5 = plasma proteins eluted with 0.1 M $H_3PO_4$/NaOH, pH 2.5, on the first run through; lanes 2, 4 and 6 = plasma proteins eluted with 0.1 M $H_3PO_4$/NaOH (pH 2.5), on second run through. Arrows indicate plasma proteins of interest that were enhanced by re-applying column flow through in the second run through.

with 1 M NaCl and 0.1 M $H_3PO_4$/NaOH, pH 2.5, are shown in Table 5.3. The average amount of plasma proteins in the 1 M NaCl eluate of the CHO LAMP-1 (-Tail) column is approximately 1.1 mg, and 0.02 mg in the pH 2.5 eluate. The average amount of plasma proteins in the 1 M NaCl eluate of the HT1080 LAMP-1 (-Tail) column is approximately 0.89 mg, and 0.045 mg in the pH 2.5 eluate. The average amount of plasma proteins in the 1 M NaCl eluate of the LAMP-1 Peptide Tail column is approximately 0.5 mg, and 0.15 mg in the pH 2.5 eluate. The pre-column (Blank and BSA) connected to the LAMP-1 columns, used to pre-clear non-specific binding proteins, had very low recoveries (Table 5.3). This may be the result of recirculating the plasma over the columns. It is thought that as the plasma first flows through the columns plasma proteins bind to the pre-column in a non-specific manner with low affinity, therefore on the second and subsequent flow throughs these proteins are pulled off, perhaps due to their interaction with other plasma proteins. However, by comparing the SDS-PAGE of plasma proteins eluted from the pre-column and each of the 3 LAMP-1 columns, proteins of interest could be identified.

**Table 5.3 Total Amount of Plasma Proteins Eluted From LAMP-1 and Pre-Columns**

| Column | [a]Total Proteins 1 M NaCl (mg) | [a]Total Proteins pH 2.5 (mg) |
|---|---|---|
| CHO LAMP-1 (-Tail) Pre-Column[b] | 0.006 | 0.021 |
| CHO LAMP-1 (-Tail) | 1.098 | 0.02 |
| HT1080 LAMP-1 (-Tail) Pre-Column[b] | 0.061 | 0.011 |
| HT1080 LAMP-1 (-Tail) | 0.885 | 0.045 |
| Peptide Tail Pre-Column[b] | 0.012 | 0.009 |
| Peptide Tail | 0.511 | 0.154 |

[a] Total amount of plasma proteins eluted with 1 M NaCl/PBS (pH 7.4) and 0.1 M $H_3PO_4$/NaOH (pH 2.5) was calculated as an average of 4 runs where 10 mL of plasma loading was used. [b] Pre-column includes both Blank and BSA Affi-Gel 15 columns.

## 5.2 Identification of Plasma Proteins that Interact with LAMP-1 in Circulation

Plasma proteins eluted with 1 M NaCl/PBS and 0.1 M $H_3PO_4$/NaOH (pH 2.5) from the pre-column and LAMP-1 columns were run on SDS-PAGE and 2D gel electrophoresis (section 2.2.13). Plasma proteins of interest were isolated after comparing protein bands (on 1D gels) or spots (on 2D gels) from the pre-column and the LAMP-1 columns and identified by peptide mass fingerprinting.

### 5.2.1 SDS-PAGE of Eluted Plasma Proteins

From Figure 5.1B, two plasma proteins were of particular interest. These were eluted by the 0.1 M $H_3PO_4$/NaOH (pH 2.5) buffer, one from the CHO LAMP-1 (-Tail) column (32 kDa), the other from the LAMP-1 Peptide Tail column (14 kDa). The first of these proteins was suspected to be galectin-3 as galectin-3 (30 kDa) are known to interact with LAMP-1. To determine whether galectin-3 and E-selectin were amongst the plasma proteins that were purified using our affinity columns, western analysis were performed on the eluted plasma proteins (section 2.2.14). Membranes were probed with anti-galectin-3 and anti-E-selectin monoclonal antibodies. Neither galectin-3 nor E-selectin was detected (data not shown).

### 5.2.2 Two Dimensional Gel Electrophoresis of Eluted Plasma Proteins

To obtain better separation of plasma proteins 2D gel electrophoresis was performed (section 2.2.13.1). Figure 5.3 shows the total plasma proteins loaded onto the affinity columns. 2D gel electrophoresis of the flow through, plasma proteins that did not bind, showed no significant differences between the total plasma protein load and the flow through, as expected (data not shown). Figures 5.4 and 5.5 shows the plasma proteins eluted with 1 M NaCl and 0.1 M $H_3PO_4$/NaOH (pH 2.5) from the CHO LAMP-1 (-

Tail), HT1080 LAMP-1 (-Tail) and the LAMP-1 Peptide Tail columns, respectively. Although plasma were pre-cleared over the pre-column there was still a high percentage of non-specific plasma proteins bound to the LAMP-1 columns. As shown in Table 5.3, there was a low recovery of plasma proteins from the pre-column, indicating that non-specific proteins were not being pre-cleared sufficiently. However, by comparison of proteins found in all three LAMP-1 columns, plasma proteins that specifically interacted with the different forms of LAMP-1 were isolated. The CHO and HT1080 LAMP-1 (-Tail) columns do not have the same protein sequence as the LAMP-1 Peptide Tail column, thus they are mutually exclusive and proteins that bind to all three columns are likely to be non-specific. Plasma proteins that specifically interact with LAMP-1 will only bind to either one of the LAMP-1 (-Tail) columns or the LAMP-1 Peptide Tail column. These proteins are indicated by the arrows in Figures 5.4-5.6. Proteins of interest were excised from preparative 2D gels of the eluted plasma proteins (200 μg load) stained with Coomassie blue (section 2.2.13.3) and sent to APAF (North Ryde, Australia) for peptide mass fingerprinting.

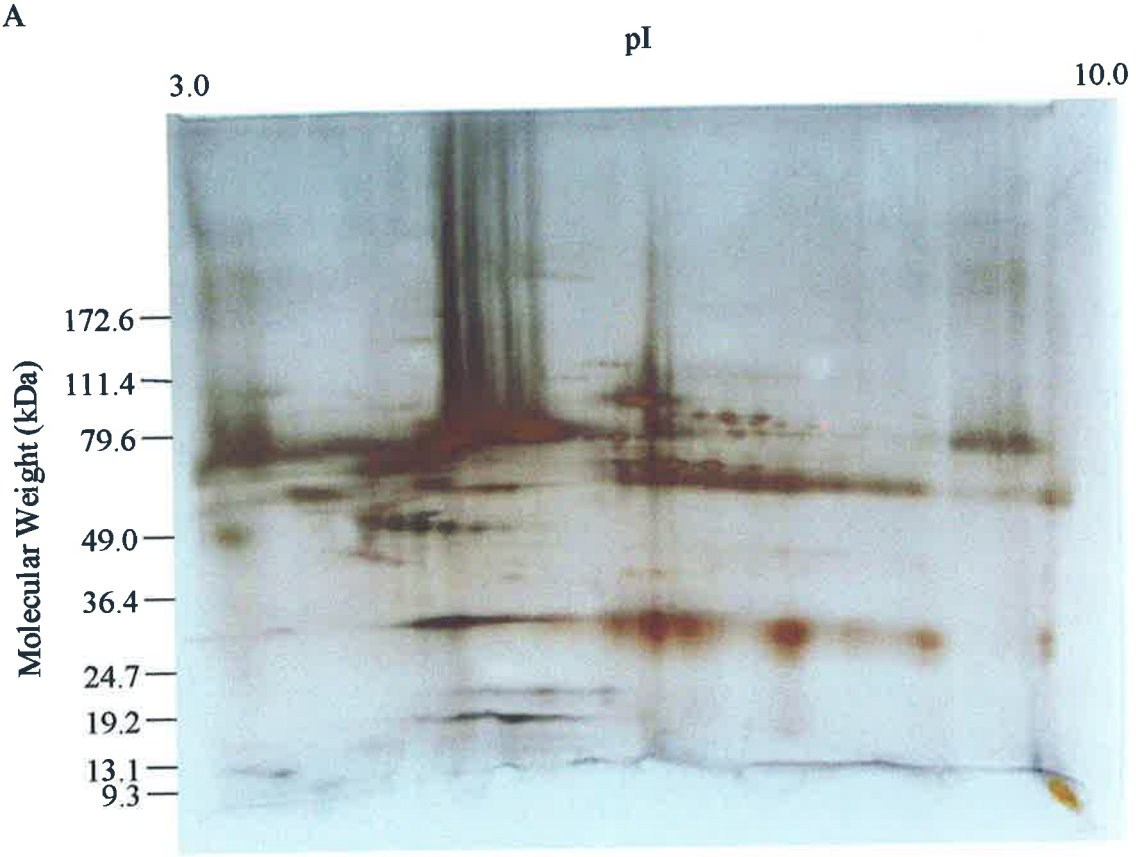
### 5.2.3 Peptide Mass Fingerprinting of Plasma Proteins

Samples sent to APAF were digested with trypsin (37°C, 16 hr) and resulting peptides were extracted from the gel with an aqueous solution of 50% (v/v) acetonitrile (AcN), 1% (v/v) TFA. An aliquot (1 μL) was spotted onto a sample plate with 1 μL of matrix (8 mg/mL a-cyano-4-hydorxycinnamnic acid in 50% (v/v) AcN, 1% (v/v) TFA) and air dried. Matrix assisted laser desorption ionisation (MALDI) was performed with a Micromass, MALDI-time of flight mass spectrometer. A 337 nm nitrogen laser was used to irradiate the sample and the spectra obtained in reflectron mode in the mass

***Figure 5.3 2D Gel Electrophoresis of the Total Plasma Proteins Applied onto LAMP-1 Affinity Columns***

Plasma (10 mL) from a healthy individual was applied to the BSA Affi-Gel 15 column connected to one of 3 LAMP-1 affinity columns at a flow rate of 0.5 mL/min then recirculated (5x). The columns were washed with PBS and the column flow through collected. The columns were separated and the plasma proteins were eluted from the columns successively with 1 M NaCl/PBS, 0.1 M $H_3PO_4$/NaOH (pH 2.5) and 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5). Plasma proteins were separated by 2D gel electrophoresis (section 2.2.13.1). Isoelectric focussing of plasma proteins, in the first dimension, was carried out on Immobiline$^{TM}$ DryStrips (pH 3-10, 7 cm). In the second dimension plasma proteins were run on 4-20% gradient polyacrylamide mini gels at a constant voltage (180 V) (section 2.2.13) then silver stained (section 2.2.13.2). Panel A, total plasma proteins applied onto the columns (50 μg load). Panel B, total plasma proteins applied onto the columns (5 μg load).

A


pI
3.0
10.0
Molecular Weight (kDa)
172.6
111.4
79.6
49.0
36.4
24.7
19.2
13.1
9.3

B

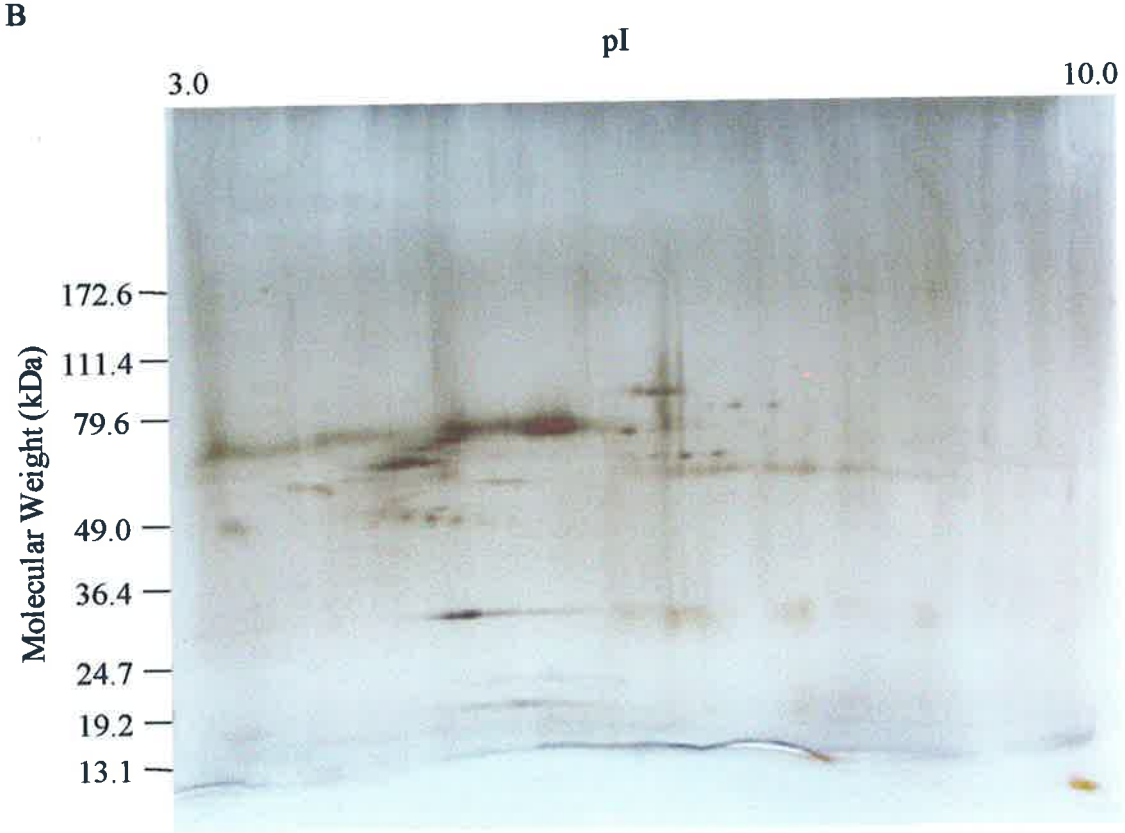
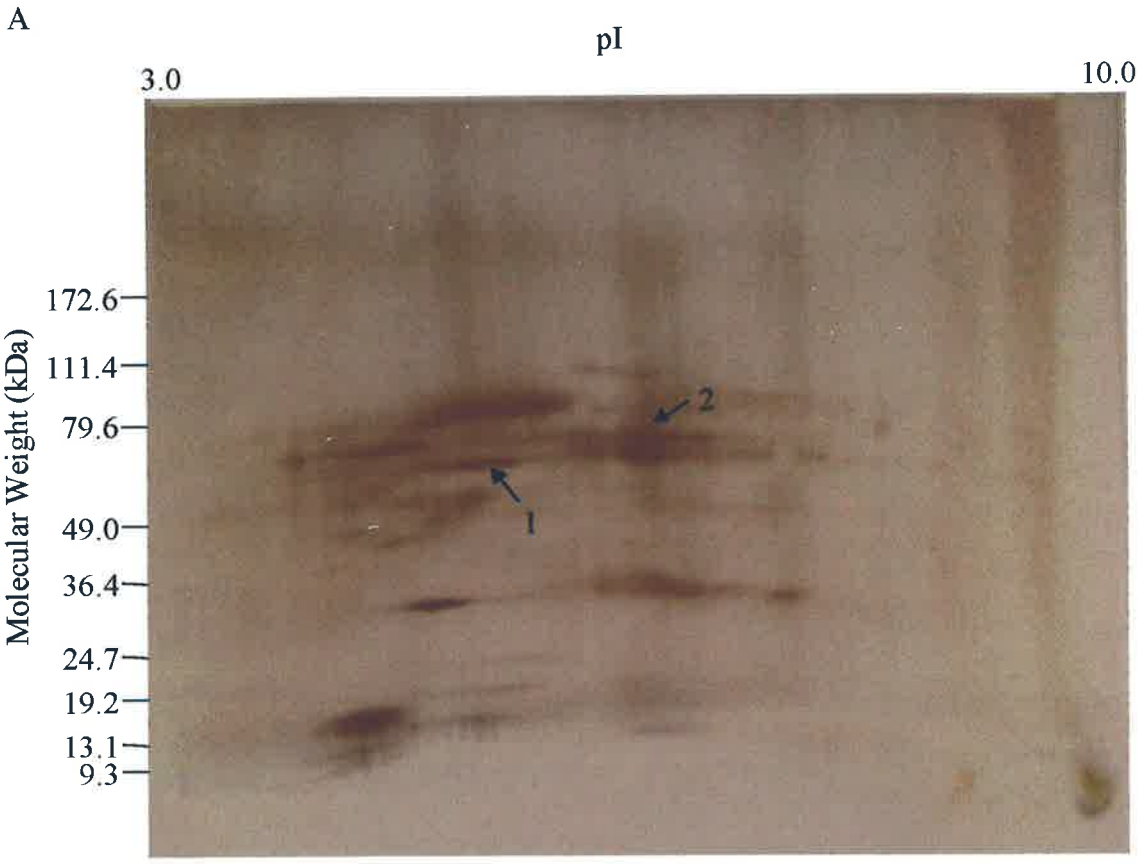

pI
3.0
10.0
Molecular Weight (kDa)
172.6
111.4
79.6
49.0
36.4
24.7
19.2
13.1

***Figure 5.4 2D Gel Electrophoresis of Plasma Proteins Eluted from the CHO LAMP-1 (-Tail) Column***

Plasma (10 mL) from a healthy individual was applied to the BSA Affi-Gel 15 column connected to the CHO LAMP-1 (-Tail) column at a flow rate of 0.5 mL/min then recirculated (5x). The columns were washed with PBS and the column flow through collected. The columns were separated and the plasma proteins were eluted from the columns successively with 1 M NaCl/PBS, 0.1 M $H_3PO_4$/NaOH (pH 2.5) and 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5). Plasma proteins were separated by 2D gel electrophoresis (section 2.2.13.1). Isoelectric focussing of plasma proteins, in the first dimension, was carried out on Immobiline$^{TM}$ DryStrips (pH 3-10, 7 cm). In the second dimension, plasma proteins were run on 4-20% gradient polyacrylamide mini gels at a constant voltage (180 V) (section 2.2.13) then silver stained (section 2.2.13.2). Panel A, plasma proteins eluted from the CHO LAMP-1 (-Tail) column with 1 M NaCl (10 μg load). Panel B, plasma proteins eluted from the CHO LAMP-1 (-Tail) column with 0.1 M $H_3PO_4$/NaOH (pH 2.5) (10 μg load); Arrows indicate plasma proteins of interest; From Table 5.4, 1 = CHO L1-TN1; 2 = CHO L1-TN2.


A
pI
3.0
10.0
Molecular Weight (kDa)
172.6
111.4
79.6
49.0
36.4
24.7
19.2
13.1
9.3
1
2

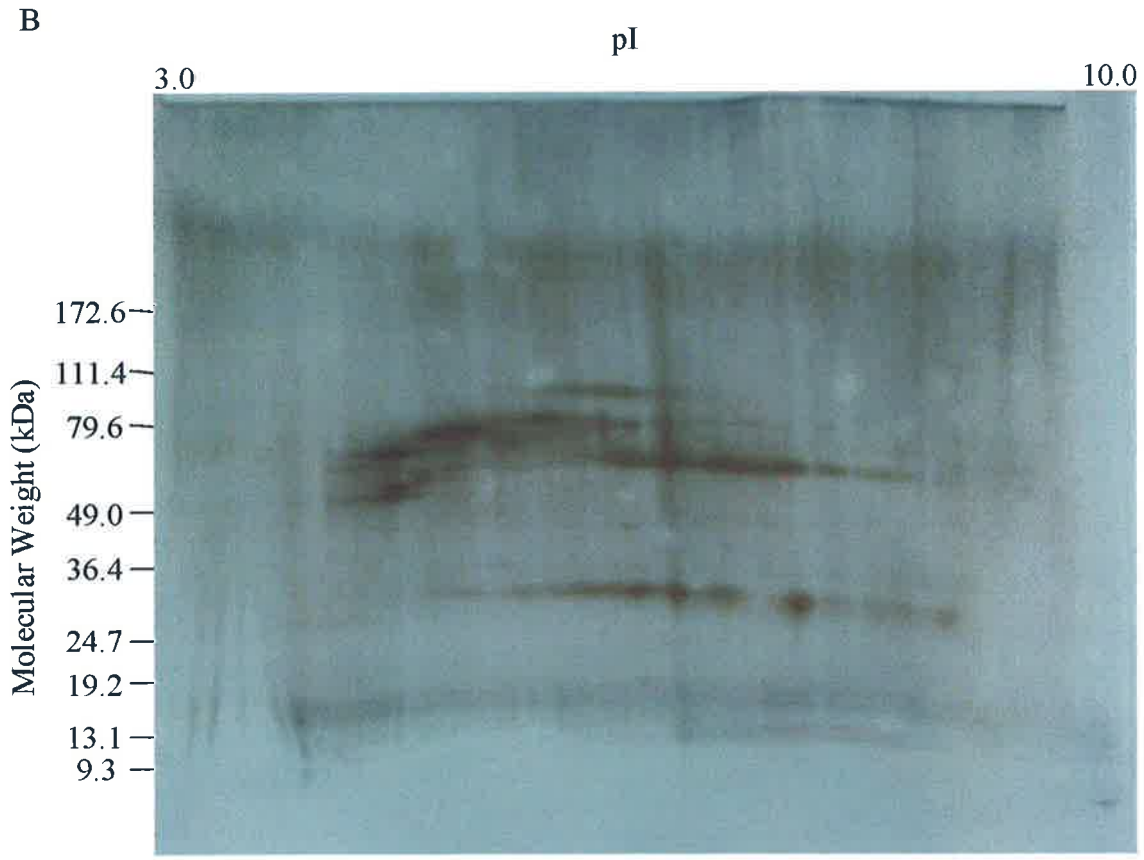
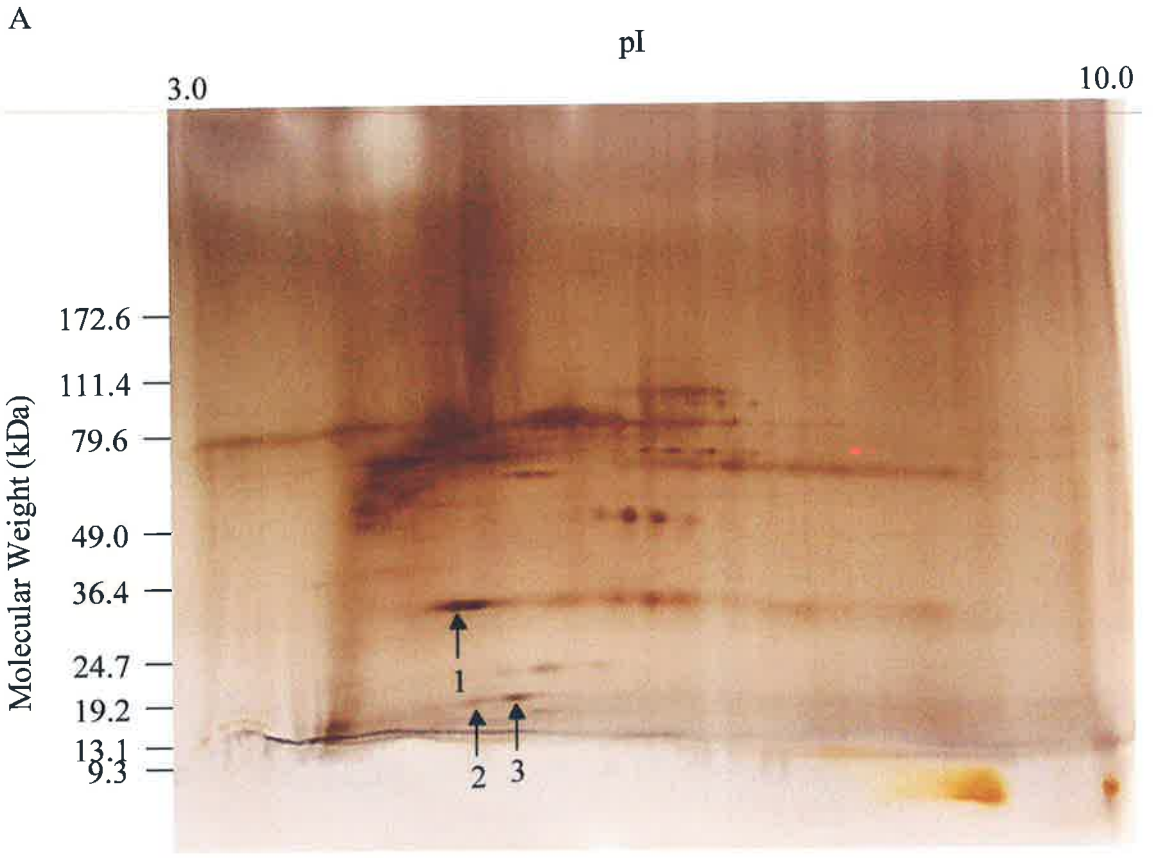

B
pI
3.0
10.0
Molecular Weight (kDa)
172.6
111.4
79.6
49.0
36.4
24.7
19.2
13.1
9.3

***Figure 5.5 2D Gel Electrophoresis of Plasma Proteins Eluted from the HT1080 LAMP-1 (-Tail) Column***

Plasma (10 mL) from a healthy individual was applied to the BSA Affi-Gel 15 column connected to the HT1080 LAMP-1 (-Tail) column at a flow rate of 0.5 mL/min then recirculated (5x). The columns were washed with PBS and the column flow through collected. The columns were separated and the plasma proteins were eluted from the columns successively with 1 M NaCl/PBS, 0.1 M $H_3PO_4$/NaOH (pH 2.5) and 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5). Plasma proteins were separated by 2D gel electrophoresis (section 2.2.13.1). Isoelectric focussing of plasma proteins, in the first dimension, was carried out on Immobiline™ DryStrips (pH 3-10, 7 cm). In the second dimension, plasma proteins were run on 4-20% gradient polyacrylamide mini gels at a constant voltage (180 V) (section 2.2.13) then silver stained (section 2.2.13.2). Panel A, plasma proteins eluted from the HT1080 LAMP-1 (-Tail) column with 1 M NaCl (10 μg load). Panel B, plasma proteins eluted from the HT1080 LAMP-1 (-Tail) column with 0.1 M $H_3PO_4$/NaOH (pH 2.5) (10 μg load); Arrows indicate plasma proteins of interest; From Table 5.4, 1 = HTL1-T28; 2 = HTL1-T16L; 3 = HTL1-T16R.


A
pI
3.0
10.0
Molecular Weight (kDa)
172.6
111.4
79.6
49.0
36.4
24.7
19.2
13.1
9.3
1
2
3

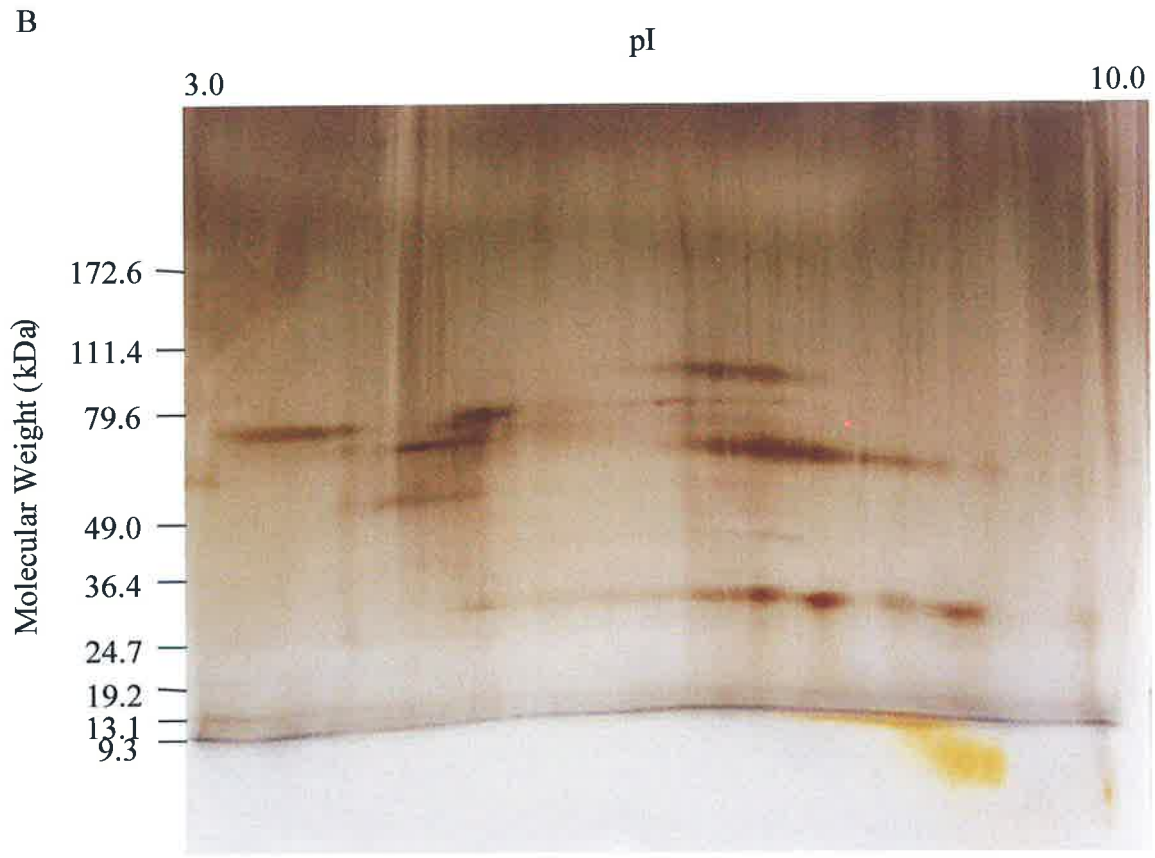
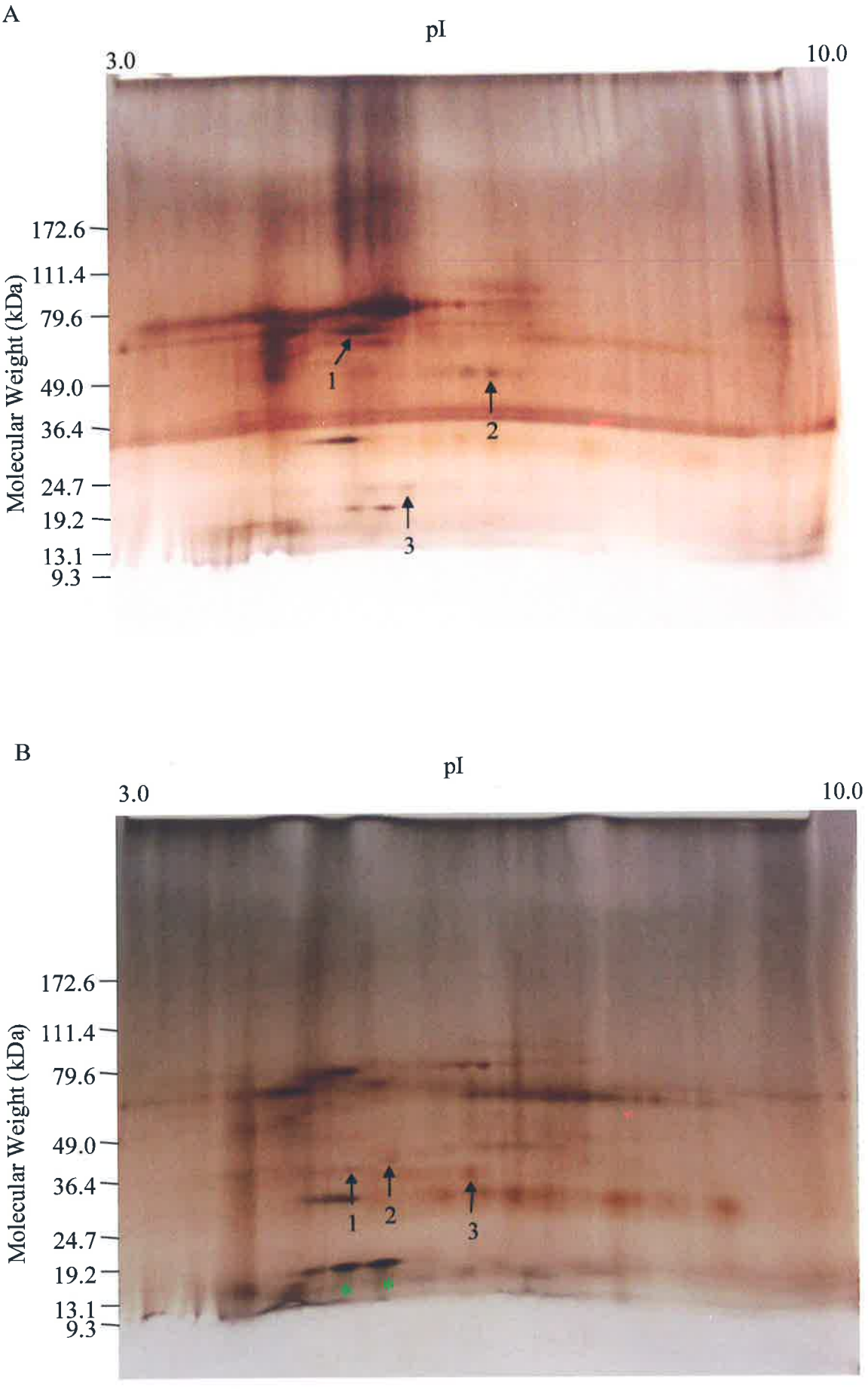

B
pI
3.0
10.0
Molecular Weight (kDa)
172.6
111.4
79.6
49.0
36.4
24.7
19.2
13.1
9.3

***Figure 5.6 2D Gel Electrophoresis of Plasma Proteins Eluted from the LAMP-1 Peptide Tail Column***

Plasma (10 mL) from a healthy individual was applied to the BSA Affi-Gel 15 column connected to the LAMP-1 Peptide Tail column at a flow rate of 0.5 mL/min then recirculated (5x). The columns were washed with PBS and the column flow through collected. The columns were separated and the plasma proteins were eluted from the columns successively with 1 M NaCl/PBS, 0.1 M $H_3PO_4$/NaOH (pH 2.5) and 3 M $MgCl_2$, 10 mM $NaH_2PO_4$/NaOH (pH 6.5). Plasma proteins were separated by 2D gel electrophoresis (section 2.2.13.1). Isoelectric focussing of plasma proteins, in the first dimension, was carried out on Immobiline™ DryStrips (pH 3-10, 7 cm). In the second dimension, plasma proteins were run on 4-20% gradient polyacrylamide mini gels at a constant voltage (180 V) (section 2.2.13) then silver stained (section 2.2.13.2). Panel A, plasma proteins eluted from the LAMP-1 Peptide Tail column with 1 M NaCl (10 μg load); Arrows indicate plasma proteins of interest; From Table 5.4, 1 = PepTN2; 2 = PepTN5; 3 = PepTN8. Panel B, plasma proteins eluted from the LAMP-1 Peptide Tail column with 0.1 M $H_3PO_4$/NaOH (pH 2.5) (10 μg load); Arrows indicate plasma proteins of interest; From Table 5.4, 1 = PepTp1; 2 = PepTp2; 3 = PepTp4. Asterisk (*) = transthyretin, refer to text (section 5.2.4.1).


A
pI
3.0
10.0
Molecular Weight (kDa)
172.6
111.4
79.6
49.0
36.4
24.7
19.2
13.1
9.3
1
2
3
B
pI
3.0
10.0
Molecular Weight (kDa)
172.6
111.4
79.6
49.0
36.4
24.7
19.2
13.1
9.3
1
2
3

range 800 to 3500 Da. Monoisotopic peak lists of the peptides from each sample and the spectra associated were generated.

### 5.2.4 Database Identification of Plasma Proteins Isolated from SDS Gels

The peptide masses of each sample obtained from APAF were searched against all human proteins in Swiss-Prot and trEMBL databases on the peptide identification program, PeptIdent (http://au.expasy.org/tools/peptident.html). A positive identification of eluted plasma proteins of interest was determined by taking into account how well peptide masses matched to known proteins in the database and by comparing the protein spot on the 2D gel to already identified proteins from human plasma in the Swiss-Prot database.

Table 5.4 shows the summary of the database search results of the plasma proteins of interest. The 32 kDa plasma protein excised from the 1D gel (Figure 5.1B) produced multiple peptide signal but no match to the database was obtained, presumably the protein band excised was not sufficiently pure. The peptide mass spectra of the 14 kDa plasma protein excised from the 1D gel (Figure 5.1B) is shown in Figure 5.7A as an example. This protein matched to transthyretin with an amino acid sequence coverage of 68.5% (Figure 5.7B). Figures 5.8-5.11 shows the amino acid sequence of the identified proteins.

Two plasma proteins eluted with 1 M NaCl from the CHO LAMP-1 (-Tail) column (Figure 5.4A), CHO L1-TN1 (63 kDa, pI 4.7-5.1) and CHO L1-TN2 (55 kDa, pI 5.7-6.2) were identified as fibrinogen γ-chain and fibrinogen β-chain, respectively (Figure

**Table 5.4 Results of Database Search of Plasma Proteins of Interests**

| Unknown Protein | Column Source | Elution Buffer | ~MW[a] (kDa) | ~pI[a] | No of Peptides[b] | No of Peptides Matched | Sequence Coverage (%) | Identified Protein | MW (kDa) | pI |
|---|---|---|---|---|---|---|---|---|---|---|
| CHOL1-TN1 | CHO L1-Tail | 1M NaCl | 63.3 | 4.7-5.1 | 16 | 13 | 43.6 | Fibrinogen γ-Chain | 48.5 | 5.2 |
| CHOL1-TN2 | CHO L1-Tail | 1M NaCl | 55.1 | 5.7-6.2 | 34 | 20 | 49 | Fibrinogen β-Chain | 50.8 | 8 |
| HTL1-T28 | HT1080 L1-Tail | 1M NaCl | 26.5 | 4.4-4.6 | 19 | 15 | 52.7 | Apolipoprotein | 28.1 | 5.3 |
| HTL1-T16L | HT1080 L1-Tail | 1M NaCl | 13.7 | 4.5 | 20 | 6 | 57.5 | Transthyretin | 13.8 | 5.4 |
| HTL1-T16R | HT1080 L1-Tail | 1M NaCl | 14.2 | 4.8 | 14 | 6 | 57.5 | Transthyretin | 13.8 | 5.4 |
| PepTN2 | Peptide Tail | 1M NaCl | 60.6 | 4.9 | 35 | 13 | 42.1 | α1-Antitrypsin | 44.3 | 5.4 |
| PepTN5 | Peptide Tail | 1M NaCl | 42.9 | 5.9 | 14 | 7 | 16.0 | Haptoglobin-2 | 45.2 | 6.3 |
| PepT16 | Peptide Tail | pH 2.5 | 14 | - | 10 | 5 | 68.5 | Transthyretin | 13.8 | 5.4 |
| PepTp1 | Peptide Tail | pH 2.5 | 31.4 | 4.8 | 13 | 5 | 56.7 | Transthyretin | 13.8 | 5.4 |
| PepTp2 | Peptide Tail | pH 2.5 | 34.7 | 5.1 | 14 | 6 | 56.7 | Transthyretin | 13.8 | 5.4 |
| PepTp4 | Peptide Tail | pH 2.5 | 30.3 | 5.7 | 13 | 4 | 15.2 | Poly(rC)- binding Protein 1 | 37.5 | 6.7 |

[a] Molecular weight and pI calculated from the 2D gel. [b] Number of peptide fragments used to search database.

5.8). Sequence coverage of 43.6% resulted for fibrinogen γ-chain and sequence coverage of 49% resulted for fibrinogen β-chain (Table 5.2).

Three proteins were eluted from the HT1080 LAMP-1 (-Tail) column with 1M NaCl (Figure 5.5A), HTL1-T28 (27 kDa, pI 4.4-4.6) was identified as apolipoprotein with sequence coverage of 52.7% (Table 5.2). The other two plasma proteins were HTL1-T16L (14 kDa, pI 4.5) and HTL1-T16R (14 kDa, pI 4.8), both were identified as transthyretin with sequence coverage of 57.5% (Figure 5.9).

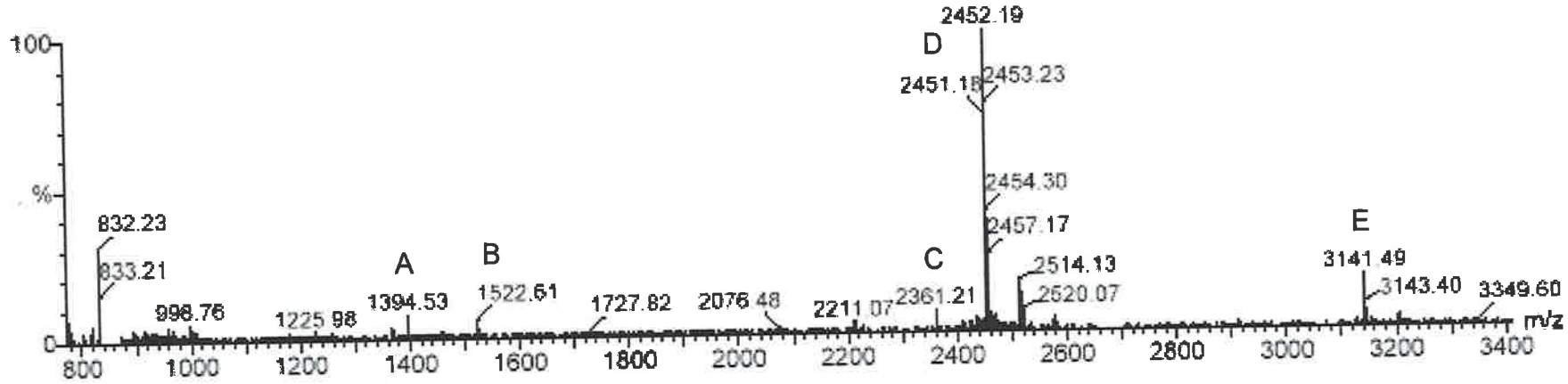
Three proteins were eluted with 1 M NaCl from the LAMP-1 Peptide Tail column (Figure 5.6A) PepTN2 (61 kDa, pI 4.9), PepTN5 (43 kDa, pI 5.9) and PepTN8 (16 kDa, pI 5.3). The first two were identified as α1-antitrypsin, with a sequence coverage of 42.1%, and haptoglobin-2, with a sequence coverage of 16%, respectively (Figure 5.10). The third did not result in a protein match. Three proteins were eluted with 0.1 M $H_3PO_4$,/NaOH pH 2.5 from the LAMP-1 Peptide Tail column (Figure 5.6B) PepTp1 (31 kDa, pI 4.8), PepTp2 (34 kDa, pI 5.1) and PepTp4 (30 kDa, pI 5.7). The first two were identified as transthyretin, both with a sequence coverage of 56.7%, and the third as poly(rC)-binding protein 1, with a sequence coverage of 15.2% (Figure 5.11). As PepTN5 resulted in a sequence coverage of 16% for haptoglobin-2 and PepTp2 resulted in a sequence coverage of 15.2% for poly(rc)-binding protein1, this gives only a low probability of a correct match.

Several molecular weights and pI values, calculated from the 2D gels, did not match with those of the identified proteins (Table 5.4). Mills *et al.* (Mills *et al.*, 2001) showed that isoforms of a plasma protein (α1-antitrypsin) with different molecular weights and

***Figure 5.7 APAF Results of Plasma Protein PepT16***

The plasma protein PepT16 was eluted from the LAMP-1 Peptide Tail column with 0.1 M $H_3PO_4$/NaOH (pH 2.5) and run on SDS-PAGE (section 2.2.13). The Coomassie blue stained (section 2.2.13.3) band was excised and sent to APAF for peptide mass fingerprinting. Panel A, mass spectra of the peptides resulting from in gel tryptic digest of the plasma protein of interest, PepT16. Panel B, amino acid sequence of transthyretin with the identified peptides shown in red. Corresponding peptides and mass spectra peaks are labelled A, B, C, D and E.

A


100
%
0
832.23
833.21
998.76
1225.98
1394.53
1522.61
1727.82
2076.48
2211.07
2361.21
2452.19
2451.18
2453.23
2454.30
2457.17
2514.13
2520.07
3141.49
3143.40
3349.60
A
B
C
D
E
m/z
800
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2000
2200
2400
2600
2800
3000
3200
3400

B

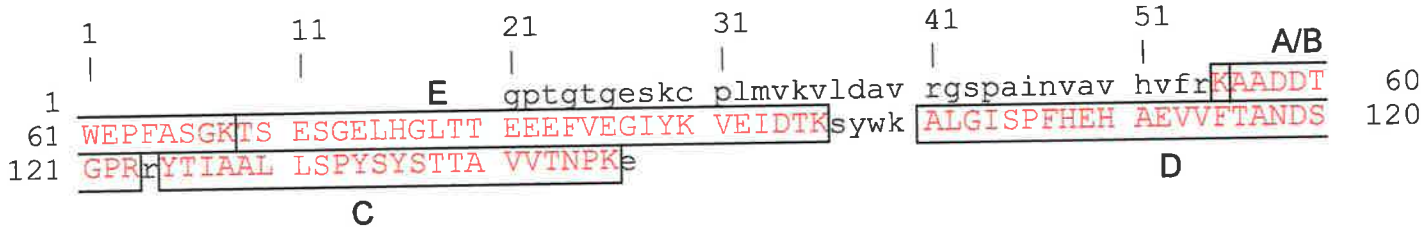
1
11
21
31
41
51
A/B
E
gptgtgeskc plmvkvldav rgspainvav hvfrKAADDT
WEPFASGKTS ESGELHGLTT EEEFVEGIYK VEIDTKsywk ALGISPFHEH AEVVFTANDS
GPRrYTIAAL LSPYSYSTTA VVTNPKe
C
D
1
61
121
60
120

***Figure 5.8 Amino Acid Sequence Matches of Plasma Proteins CHO L1-TN1 and CHO L1-TN2***

The plasma proteins CHO L1-TN1 and CHO L1-TN2 were eluted from the CHO LAMP-1 (-Tail) column with 1 M NaCl and run on 2D gel electrophoresis (section 2.2.13). The Coomassie blue stained (section 2.2.13.3) spots were excised and sent to APAF for peptide mass fingerprinting. Panel A, amino acid sequence of fibrinogen γ-chain, which matched to the peptide mass fingerprint of CHO L1-TN1. Panel B, amino acid sequence of fibrinogen β-chain, which matched to the peptide mass fingerprint of CHO L1-TN2. Matching peptides are shown in red.

A

```
    1          11         21         31         41         51
    |          |          |          |          |          |
  1                              yvat rdnccilder fgsycpttcg iadflstyqt    60
 61 kvdkdlqsle dilhqvenkt sevkqlikAI QLTYNPDESS KPNMIDAATL Ksrkmleeim   120
121 kYEASILTHD SSIRYLQEIY NSNNQKivnl kekVAQLEAQ CQEPCKDTVQ IHDITGKdcq   180
181 diankgakQS GLYFIKPLKa nqqflvycei dgsgngwtvf qkrldgsvdf kknwiqykEG   240
241 FGHLSPTGTT EFWLGNEKIH LISTQSAIPY ALRVELEDWN GRtstadyam fkVGPEADKY   300
301 Rltyayfagg dagdafdgfd fgddpsdkff tshngmqfst wdndndkfeg ncaeqdgsgw   360
361 wmnkCHAGHL NGVYYQGGTY SKASTPNGYD NGIIWATWKt rwysmkkttm kiipfnrLTI   420
421 GEGQQHHLGG AKqvrpehpa eteydslype ddl
```

B

```
    1          11         21         31         41         51
    |          |          |          |          |          |
  1                                          ghrpld kkrEEAPSLR    60
 61 PAPPPISGGG YRarpakaaa tqkkverkap daggclhadp dlgvlcptgc qlqeallqqe   120
121 rpirnsvdel nnnveavsqt ssssfqymyl lkdlwqkrqk qvkDNENVVN EYSSELEKHQ   180
181 LYIDETVNSN IPTNLRvlrs ilenlrskiq kLESDVSAQM EYCRtpctvs cnipvvsgke   240
241 ceeiirKGGE TSEMYLIQPD SSVKPYRVYC DMNTENGGWT VIQNRQDGSV DFGRkwdpyk   300
301 QGFGNVATNT DGKNYCGLPG EYWLGNDKis qltrmgptel liemedwkgd kvkAHYGGFT   360
361 VQNEANKyqi svnkyrgtag nalmdgasql mgenrTMTIH NGMFFSTYDR DNDGWLTSDP   420
421 RkqcskEDGG GWWYNRchaa npngrYYWGG QYTWDMAKhg tddgvvwmnw kGSWYSMRkm   480
481 smkIRPFFPQ Q
```

A

```
      1          11         21         31         41         51
      |          |          |          |          |          |
                             DEPPQS PWDRvkdlat vyvdvlkDSG RDYVSQFEGS     60
  1
 61 ALGKqlnlkL LDNWDSVTST FSKLREQLGP VTQEFWDNLE KETEGLRqem skdleevkak    120
121 VQPYLDDFQK KWQEEMELYR qkveplrael qegarqklhe lqeklsplge emrdrarahv    180
181 dalrTHLAPY SDELRqrlaa rlealkengg arlaeyhaka tehlstlsek AKPALEDLRQ    240
241 GLLPVLESFK VSFLSALEEY TKklntq
```

B

```
      1          11         21         31         41         51
      |          |          |          |          |          |
  1                        gptgtgeskc plmvkvldav rGSPAINVAV HVFRKAADDT     60
 61 WEPFASGKts esgelhgltt eeefvegiyk veidtksywk ALGISPFHEH AEVVFTANDS    120
121 GPRRYTIAAL LSPYSYSTTA VVTNPKe
```

***Figure 5.9 Amino Acid Sequence Matches of Plasma Proteins HTL1-T28, HTL1-T16L and HTL1-T16R***

The plasma proteins HTL1-T28, HTL1-T16L and HTL1-T16R were eluted from the HT1080 LAMP-1 (-Tail) column with 1 M NaCl and run on 2D gel electrophoresis (section 2.2.13). The Coomassie blue stained (section 2.2.13.3) spots were excised and sent to APAF for peptide mass fingerprinting. Panel A, amino acid sequence of apolipoprotein, which matched to the peptide mass fingerprint of HTL1-T28. Panel B, amino acid sequence of transthyretin, which matched to the peptide mass fingerprints of HTL1-T16L and HTL1-T16R. Matching peptides are shown in red.

A

```
      1           11          21          31          41          51
      |           |           |           |           |           |
   1                                edpqgd aaqkTDTSHH DQDHPTFNKi tpnlaefafs    60
  61 lyrqlahqsn stniffspvs iatafamlsl gtkadthdei leglnfnlte ipeaqihegf   120
 121 qellrTLNQP DSQLQLTTGN GLFLSEGLKl vdkfledvkK LYHSEAFTVN FGDTEEAKKq   180
 181 indyvekgtq gkivdlvkEL DRDTVFALVN YIFFKgkWER PFEVKDTEEE DFHVDQVTTV   240
 241 Kvpmmkrlgm fniqhckkls swvllmkylg nataifflpd egkLQHLENE LTHDIITKFL   300
 301 ENEDRRsasl hlpklsitgt ydlkSVLGQL GITKVFSNGA DLSGVTEEAP LKlskavhka   360
 361 vltidekgte aagamfleai pmsippevkF NKPFVFLMIE QNTKsplfmg kvvnptqk
```

B

```
      121         131         141         151         161         171
      |           |           |           |           |           |
                                                     ilgghldak GSFPWQAKmv   180
 121
 181 shhnlttgat lineqwlltt aknlflnhse natakDIAPT LTLYVGKkql veiekvvlhp   240
 241 nysqvdigli klkqkvsvne rvmpiclpsk dyaevgrVGY VSGWGRnanf kftdhlkYVM   300
 301 LPVADQDQCI Rhyegstvpe kktpkspvgv qpilnehtfc agmskyqedt cygdagsafa   360
 361 vhdleedtwy atgilsfdkS CAVAEYGVYV KVTSIQDWVQ Ktiaen
```

***Figure 5.10 Amino Acid Sequence Matches of Plasma Proteins PepTN2 and PepTN5***

The plasma proteins PepTN2 and PepTN5 were eluted from the LAMP Peptide Tail column with 1 M NaCl and run on 2D gel electrophoresis (section 2.2.13). The Coomassie blue stained (section 2.2.13.3) spots were excised and sent to APAF for peptide mass fingerprinting. Panel A, amino acid sequence of α1-antitrypsin, which matched to the peptide mass fingerprint of PepTN2. Panel B, amino acid sequence of haptoglobin-2 β-chain, which matched to the peptide mass fingerprints of PepTN5. Matching peptides are shown in red.

A

```
      1          11         21         31         41         51
      |          |          |          |          |          |
                            gptgtgeskc plmvkvldav rGSPAINVAV HVFRKAADDT    60
  61 WEPFASGKts esgelhgltt eeefvegiyk veidtksywk ALGISPFHEH AEVVFTANDS   120
 121 GPRrYTIAAL LSPYSYSTTA VVTNPKe
```

B

```
      1          11         21         31         41         51
      |          |          |          |          |          |
   1 MDAGVTESGL NVTLTIRllm hgkevgsiig kkgesvkrir eesgarINIS EGNCPERiit    60
  61 ltgptnaifk afamiidkle edinssmtns taasrppvtl rlvvpatqcg sligkGGCKI   120
 121 KEIRestgaq vqvagdmlpn steraitiag vpqsvtecvk qiclvmletl sqspqgrvmt   180
 181 ipyqpmpass pvicaggqdr csdavgypha thdlegppld aysiqgqhti spldlaklnq   240
 241 varqqshfam mhggtgfagi dssspevkgy wasldastqt theltipnnl igciigrQGA   300
 301 NINEIRQMSG AQIKianqve gssgrqvtit gsaasislaq ylinarlsse kgmgcs       360
```

***Figure 5.11 Amino Acid Sequence Matches of Plasma Proteins PepTp1, PepTp2 and PepTp4***

The plasma proteins PepTp1, PepTp2 and PepTp4 were eluted from the LAMP Peptide Tail column with 0.1 M $H_3PO_4$/NaOH (pH 2.5) and run on 2D gel electrophoresis (section 2.2.13). The Coomassie blue stained (section 2.2.13.3) spots were excised and sent to APAF for peptide mass fingerprinting. Panel A, amino acid sequence of transthyretin, which matched to the peptide mass fingerprint of PepTp1 and PepTp2. Panel B, amino acid sequence of poly(rC)-binding protein, which matched to the peptide mass fingerprints of PepTp4. Matching peptides are shown in red.

pI value were the result of glycan heterogeneity. This may also explain the differences observed in some of the proteins in this study. There was an obvious discrepancy in the molecular weights for proteins PepTp1 and PepTp2 (Table 5.4). PepTp1 and PepTp2 had calculated molecular weights of 31.4 and 34.7 kDa, respectively, but both were matched to transthyretin, which has a molecular weight of 13.8 kDa. However, transthyretin with a molecular weight of 34.3 kDa in cerebrospinal fluid (Christmanson *et al.*, 1991) and 35.5 kDa in human plasma (Mita *et al.*, 1984) have been reported. These are presumably dimers of the transthyretin molecule with glycan heterogeneity.

#### *5.2.4.1 Determination of Plasma Proteins that Specifically Interact with LAMP-1*

Plasma proteins eluted from the CHO and/or HT1080 LAMP-1 (-Tail) columns as well as the LAMP-1 Peptide Tail column indicated that these proteins do not specifically interact with LAMP-1. These proteins include fibrinogen and apolipoprotein, which were eluted from all three LAMP-1 columns. Also, α1-antitrypsin and haptoglobin-2 were eluted from the HT1080 LAMP-1 (-Tail) and LAMP-1 Peptide Tail columns at equal amounts, suggesting that they do not specifically bind to LAMP-1. However, transthyretin was enriched significantly over the LAMP-1 Peptide Tail column (Figure 5.6B, indicated by *) as compared to the HT1080 LAMP-1 (-Tail) column (Figure 5.5A, indicated by 2 and 3), indicating that transthyretin has a higher specificity for the cytoplasmic tail of LAMP-1.

## 5.3 Discussion

To date, studies of LAMP-1 interactions with extracellular proteins have been confined to LAMP-1 expressed on the cell surface. Sawada *et al.* (Sawada *et al.*, 1993) found that in cells genetically manipulated to increase the level of surface LAMP-1, there was an

enhanced adherence to E-selectin expressing cells. Sarafian *et al.* (Sarafian *et al.*, 1998) observed accumulation of LAMP-1 at the edges and extensions of A2058 human metastasizing melanoma cells, suggesting that these glycoproteins could participate in cell adhesion. Enhanced adherence of LAMP-1 to galectin-3 was shown when cells were treated with butyrate to increase the cell surface expression of LAMP-1 (Sarafian *et al.*, 1998). LAMP-1 expressed on the cell surface has been proposed to assist in cell-cell interactions and specific localisation of LAMP-1 could facilitate cancer-cell spread and locomotion during tumour progression. Very little is known of LAMP-1 in circulation. In order to examine possible functions of LAMP-1, we have identified several plasma proteins that interact with LAMP-1.

Three affinity LAMP-1 columns, CHO and HT1080 LAMP-1 (-Tail) and LAMP-1 Peptide Tail, were made to isolate plasma proteins that could be interacting with LAMP-1 in circulation. Galectin-3 and E-selectin were not amongst the proteins that were identified to interact with LAMP-1 in this study. It is possible that galectin-3 may have bound to the LAMP-1 columns but at levels that were too small to detect. Galectin-3 is found expressed in the cytosol of various cell types including epithelial cells, activated macrophages, and some sensory neurons and is secreted into the intercellular space at tissue wound sites (Inohara *et al.*, 1998; Gonen *et al.*, 2000). Therefore, it is likely that galectin-3 was not identified in this study because it is not expressed in circulation. E-selectin was not identified due to the fact that the recombinant LAMP-1 (-Tail) proteins we used did not contain the sialyl-Lewis X antigen (Chapter 3), the ligand for E-selectin.

The plasma proteins that bound to the LAMP-1 columns were identified as transthyretin, haptoglobin-2, α1-antitrypsin, poly(rc)-binding protein 1, fibrinogen γ and β-chains, and

apolipoprotein (Table 5.2). The majority of these proteins were eluted with 1 M NaCl, indicating that the interaction is not of high affinity. Proteins that bound equally to both the LAMP-1 (-Tail) column and the LAMP-1 Peptide Tail column were considered to be the result of non-specific binding, as the columns did not contain any similar peptide sequence. Poly(rc)-binding protein 1 and transthyretin were identified as interacting with LAMP-1. The functions of these proteins were also examined to determine the probability that they interact specifically with LAMP-1.

Poly(rc)-binding protein 1 was eluted with 0.1 M $H_3PO_4$/NaOH (pH 2.5) only from the LAMP-1 Peptide Tail column. This protein is expressed abundantly in skeletal muscle, thymus and peripheral blood leucocytes and is a single-stranded nucleic acid binding protein that binds preferentially to oligo DC (Swiss Prot). Given the identified functions of this protein, it is not apparent what role, if any, it would have in binding to LAMP-1. Although transthyretin was eluted from both HT1080 LAMP-1 (-Tail) and LAMP-1 Peptide Tail columns (Figures 5.5A and 5.6), it was significantly more enriched in the pH 2.5 eluate from the LAMP-1 Peptide Tail column. Moreover, transthyretin from the HT1080 LAMP-1 (-Tail) column was eluted totally with 1 M NaCl (Figure 5.5A) whereas it required the pH 2.5 buffer to elute from the LAMP-1 Peptide Tail column (Figure 5.6B), indicating that it had greater affinity for the cytoplasmic tail of LAMP-1.

Transthyretin, a plasma protein that exists as a homotetramer of 54 kDa, functions to transport the hormone thyroxine from the blood stream to the brain across the blood brain barrier of the choroid plexus blood. It also transports vitamin A indirectly by forming a complex with the retinol-binding protein. Given that transthyretin interacts with the cytoplasmic tail peptide of LAMP-1, it is possible that in plasma LAMP-1

aggregation may be formed through its association with the homotetramer of transthyretin.

From this study no plasma proteins were identified that interact specifically with the soluble LAMP-1 (-Tail). However, the recombinant LAMP-1 (-Tail) produced for this study was not identical to endogenous LAMP-1 (-Tail) found in circulation, in terms of the carbohydrate side chains. The carbohydrate side chains of LAMP-1 constitutes 55-65% of the total mass of the protein (Fukuda, 1991), therefore making the carbohydrate content of LAMP-1 the most likely variable between endogenous and recombinant LAMP-1 (-Tail). Indeed, the recombinant LAMP-1 (-Tail) does not have the sialyl-Lewis X antigen found on endogenous LAMP-1 (-Tail) (Chapter 3). Thus it is likely that other differences would exist. Therefore, plasma proteins that would normally recognise certain forms of carbohydrate side chains on LAMP-1 (-Tail) *in vivo* may not have recognised the recombinant form.

# CHAPTER SIX:

# Concluding Discussion and Future Work

## 6.1 Concluding Discussion

Lysosomal storage disorders (LSD) represents a group of more than 45 distinct genetic diseases with a collective incidence of approximately 1:5,000 births. LSD have a progressive degenerating nature with symptoms that include central nervous system dysfunction, skeletal abnormalities, coarse facial features, organomegaly, and corneal clouding (Neufeld and Meunzer, 1995). If current and proposed therapies are to achieve maximum efficacy then it will be imperative that the disorders are detected early, before the onset of irreversible pathology, particularly if there is central nervous system and/or bone pathology involvement. Except in cases where a family history is available, pre-symptomatic diagnosis of LSD can only be achieved by newborn screening.

A common feature of all LSD is the accumulation and storage of lysosomal substrates within the lysosome. This results in an increase in the number and size of lysosomes within the cell from approximately 1% to as much as 50%. Meikle *et al.* (Meikle *et al.*, 1997) hypothesised that the levels of certain lysosomal proteins would be increased as a result of storage and these proteins were potential screening markers for LSD. The lysosome-associated membrane protein, LAMP-1, was reported to be elevated up to 7 fold in some LSD affected cell lines (Karageorgos *et al.*, 1997), which led to its evaluation as a screening marker for LSD (Meikle *et al.*, 1997). The concentration of soluble LAMP-1 was increased in the plasma in approximately 72% of LSD patients,

suggesting that it was a useful marker for LSD. If optimal use of LAMP-1 as a screening marker for LSD is to be achieved then a better and clearer understanding of LAMP-1 will be required, in particular the biology and functions of soluble LAMP-1 in circulation.

Although LAMP-1 and its structural relative, LAMP-2, are the best characterised lysosomal membrane proteins and are the most abundant lysosomal membrane glycoproteins, making up approximately 0.1-0.2% of the total cell proteins (Carlsson and Fukuda, 1989), their exact functions are unclear. There have been many studies on LAMP-1 and LAMP-2 expressed on the cell surface, which gave rise to several propositions as to the function of these proteins. The most favoured of these propositions is that LAMP-1 and LAMP-2 provide ligands to facilitate cell-cell interactions through their associations with galectin-3 and E-selectin. Moreover, cancer researches have shown that LAMP-1 and LAMP-2 are involved in metastasis of cancer cells. While there have been many studies directed towards understanding the function of membrane bound LAMPs there are relatively few studies of the soluble forms of LAMPs. This thesis, therefore, focuses on the study of soluble LAMP-1 with the aim to characterise and investigate its possible function(s) in circulation.

Two forms of recombinant human LAMP-1, LAMP-1 (-Tail) and LAMP-1 (+Tail), were expressed in CHO-K1 and HT1080 cells (section 3.1). Two immunoassays were developed and optimised to detect these forms of LAMP-1 (section 4.3). The LAMP-1 (Tail) 1-step assay only detected LAMP-1 (+Tail) whereas the BB6 2-step assay detected both forms. The challenges of purifying LAMP-1 (+Tail) from HT1080 cell membranes limited the investigations into LAMP-1 (+Tail) (section 3.2.1). Pure forms

of LAMP-1 (+Tail) and LAMP-1 (-Tail) were to be used as calibration standards in the LAMP-1 immunoassays (section 4.3). However, as we were only able to affinity purify very little LAMP-1 (+Tail) protein from HT1080 cells, membrane preparations of SF LAMP-1 (+Tail) was used instead (section 4.1.5) as a calibration standard. In contrast, a sufficient amount of LAMP-1 (-Tail) protein was purified from the media of HT1080 cells (section 3.2.2) and was thus used as a calibration standard.

Using the BB6 and LAMP-1 (Tail) assays, the LAMP-1 form that predominated in the plasma from 12 unaffected and 48 LSD affected individuals representing 8 LSD was determined (section 4.4.1). Approximately 67% of all the plasma samples assayed contained mainly LAMP-1 (+Tail). The control plasma samples investigated in this study showed a wide variation in the total amount of LAMP-1 (8-50 ng/mL) and percentage of LAMP-1 (+Tail) form (8-100%). Similarly the plasma samples from LSD patients showed a wide, albeit higher, range in the total amount of LAMP-1 (14-323 ng/mL) and percentage of LAMP-1 (+Tail) form (6-100%). Of note are two LSD groups, Gaucher and Niemann-Pick A/B that had predominantly LAMP-1 (+Tail) in their plasma (Table 4.2). Both disorders are sphingolipidosis (Table 1.1), lipid storage disorders, and have been reported to have elevated levels of lipid in plasma (Strasberg *et al.*, 1983; Dawson and Oh, 1997; Whitfield *et al.*, 2001). It is not known whether the secretion of lipids into circulation is the result of their accumulation within lysosomes or whether lipids are secreted in a controlled manner. Likewise the mechanism(s) by which LAMP-1 is secreted are unclear. Soluble LAMP-2 has been reported to form aggregates with lipids in acidic pH, conditions similar to that in the lysosome (Jadot *et al.*, 1997). It is therefore possible that LAMP-1 may also form similar aggregates with lipid rafts within the lysosome. In the process of exocytosis, lysosome derived vesicles fuse with

the plasma membrane, thus both LAMP-1 (+Tail) and lipids could be released from lipid rafts and dissociate in the higher pH of the plasma membrane. The mechanism by which LAMP-1 (-Tail) may be secreted from the cell has previously been speculated to be the result of proteolytic processing of the membrane-bound form. Whereas secretion of LAMP-1 (+Tail) has been speculated to be the result of alternatively spliced forms with different transmembrane domains (Meikle *et al.*, 1999b), such as for LAMP-2. Three alternatively spliced variants of LAMP-2 have been reported with one targeting to the lysosome and the other two to the plasma membrane (Konecki *et al.*, 1995; Gough and Fambrough, 1997). The observed LAMP-1 (+Tail) levels in Gaucher and Niemann-Pick A/B may be a contributing factor to the pathology of these disorders, however it is more likely that LAMP-1 (+Tail) in the plasma of these disorders is the result of their accumulation within the lysosome. In this case LAMP-1 (+Tail) may potentially be an important marker for diagnosis and monitoring of therapy in Gaucher and Niemann-Pick A/B disorders. Whitfield *et al.* (Whitfield *et al.*, 2002) found that Gaucher patients displaying the highest 16:0-glucosylceramide/16:0-lactosylceramide ratios and saposin C and LAMP-1 levels were among the most severely affected in the study group. This indicates that LAMP-1 levels in plasma may be able to predict disease severity and monitor patients undergoing therapy. With the observations in this study, LAMP-1 (+Tail) levels, rather than total LAMP-1, may provide a better marker for disease severity and therapy efficacy. The proposed strategy for the newborn screening of LSD (section 1.2.1) involves a primary screening assay to detect elevations in total LAMP-1 {LAMP-1 (±Tail)} and saposin C in blood spots from Guthrie cards (Meikle *et al.*, 1999a). Those identified to be in the high risk population will then undergo a second tier screen. In this case, LAMP-1 (+Tail) may provide a better and more specific marker for the first tier screen than total LAMP-1 and may give more of an advantage for the

targeting of specific LSD, in particular Gaucher and Niemann-Pick A/B disorders. Further investigation into the level of LAMP-1 (+Tail) will be required in a cohort of plasma from a greater range of LSD-affected individuals to confirm this.

During the affinity purification process of LAMP-1 (+Tail) approximately 30% was lost after CHAPS detergent was dialysed out of the LAMP-1 (+Tail) supernatant (Table 3.1). It was thought that the elimination of CHAPS caused LAMP-1 (+Tail) to aggregate and become insoluble leading to the protein precipitating out of solution. This was confirmed when the aggregation properties of LAMP-1 were investigated (section 4.5). The addition of CHAPS in the LAMP-1 immunoassays (section 4.5.1) enhanced the detection of the SF LAMP-1 (+Tail) calibration standard but not the HT1080 LAMP-1 (-Tail) calibration standards, indicating that only the LAMP-1 (+Tail) was affected by self aggregation or interaction with other proteins. Moreover, whilst the addition of a small volume of plasma caused a great reduction in the fluorescent signal obtained by the SF LAMP-1 (+Tail) calibration curve, the addition of a relatively large volume of plasma only slightly inhibited the HT1080 LAMP-1 (-Tail) calibration (section 4.3.2). The inhibition observed indicates that LAMP-1 (+Tail) interacts with specific plasma proteins while LAMP-1 (-Tail) inhibition is likely to be caused by non-specific inhibition of the immunoassay by high concentrations of plasma proteins, rather than aggregation or interaction of LAMP-1 (-Tail). Through size exclusion chromatography experiments (section 4.5.2) of affinity purified HT1080 LAMP-1 (±Tail), it was concluded that self aggregation of LAMP-1 only occurred in LAMP-1 (+Tail) (Figure 4.15). These results indicated that self aggregation does occur in addition to interaction with other plasma proteins.

In Chapter 5, plasma proteins that interact with LAMP-1 (±Tail) were identified to investigate possible functions of soluble LAMP-1 in circulation. Plasma proteins were isolated over three LAMP-1 affinity columns CHO and HT1080 LAMP-1 (-Tail) and LAMP-1 Peptide Tail. Immunofluorescence staining and flow cytometry characterisations of the LAMP-1 expression cell lines (section 3.3.3 and 3.3.4) showed that sialyl-Lewis X was not being expressed on the recombinant LAMP-1 proteins. Thus E-selectin was not amongst the plasma proteins that were isolated and identified to interact with LAMP-1 in this study. Plasma proteins that were identified to interact with LAMP-1 were transthyretin, haptoglobin-2, α1-antitrypsin, poly(rc)-binding protein 1, fibrinogen gamma and beta chains, and apolipoprotein (Table 5.2). Of these only transthyretin was found to interact specifically with LAMP-1, in particular the cytoplasmic tail of LAMP-1.

Transthyretin, also known as prealbumin, circulates in the serum of all mammals (Schreiber *et al.*, 1995). It transports 15-20% of serum thyroxine and complexes with retinol-binding protein to transport vitamin A. In normal human serum transthyretin exists as a 54 kDa tetramer of four identical subunits arranged to form a cylindrical channel (Blake *et al.*, 1974). Thyroxine binds to an internal channel of the transthyretin tetramer and retinol-binding protein to the exterior (van Jaarsveld *et al.*, 1973; Blake *et al.*, 1978). The channel can theoretically accommodate two molecules of thyroxine, but most studies have found that either one molecule is bound or two with different affinities (Curtis *et al.*, 1994). It is as yet unknown where the binding site of LAMP-1 (+Tail) is on transthyretin.

The identification of transthyretin as interacting specifically with the cytoplasmic tail of LAMP-1 supports the results of the LAMP-1 immunoassays (section 4.3.2) that showed LAMP-1 (+Tail) interacts more specifically and with greater affinity to plasma proteins than LAMP-1 (-Tail). Furthermore, the aggregation observed only in LAMP-1 (+Tail) may be formed through the binding of LAMP-1 (+Tail) to the tetrameric form of transthyretin. It is also possible that this is the cause of the inhibition observed in the LAMP-1 immunoassays when plasma was added to the SF LAMP-1 (+Tail) calibration curve. As LAMP-1 (+Tail) interacts specifically with a plasma protein and has the ability to form aggregates, which are not seen for LAMP-1 (-Tail), LAMP-1 (+Tail) is suspected to have a function in circulation. It may be that LAMP-1 (+Tail) is released from cells into circulation in a selective manner to serve a specific function, whilst the release of LAMP-1 (-Tail) is a random event.

Sawada *et al.* (Sawada *et al.*, 1993) demonstrated that soluble LAMP-1 generated from CHO cells was able to inhibit E-selectin mediated adhesion of cell surface LAMP-1, but only when the soluble LAMP-1 was prepared from cells expressing the sialyl-Lewis X antigen. Thus, by considering all the results obtained during this study, soluble LAMP-1 in circulation may function to competitively inhibit the cell-cell interactions facilitated by cell-surface LAMP-1, presumably to maintain cell binding and releasing events. Furthermore, self-aggregation of LAMP-1 in circulation and its interaction with tetrameric transthyretin may result in greater avidity to cell surfaces through cell adhesion molecules thereby increasing the inhibition of cell surface LAMP-1 interactions. As LAMP-2 is similar to LAMP-1, LAMP-2 in circulation may have a similar function.

LAMP-1 has been found in greater levels on highly metastatic colonic carcinoma tumour and melanoma cells than poorly metastatic cells (Saitoh *et al.*, 1992; Ohyama *et al.*, 1999; Chakraborty *et al.*, 2001). Moreover, high metastatic cells were found to bind activated human endothelial cells expressing E-selectin more efficiently than low metastatic cells (Sawada *et al.*, 1994). As such LAMP-1 expressed on the cell surface has been suggested to facilitate tumour metastasis. However, pancreatic carcinoma patients were found to live longer when their tumours exhibit higher LAMP-1 levels than patients whose tumours exhibited low LAMP-1 levels (Kunzli *et al.*, 2002). It is possible that the over expression of LAMP-1 on tumour cells will lead to increased secretion of LAMP-1 into circulation. This increased level of LAMP-1 in the circulation of patients with tumours exhibiting higher LAMP-1 levels will therefore result in the inhibition of tumour metastasis. The level of LAMP-1 in the plasma of the pancreatic carcinoma patients was not reported. Soluble LAMP-1 may therefore prove to be useful as a therapeutic agent for the inhibition of E-selectin-mediated binding to tumour cells. Aggregated recombinant LAMP-1 (+Tail) displaying the sialyl-Lewis X antigen could be produced as a therapeutic agent to increase this inhibition.

In summary, a wide range of the amount of total LAMP-1 and percentage of LAMP-1 (+Tail) was shown in the plasma from control and LSD-affected individuals. LAMP-1 (+Tail) but not LAMP-1 (-Tail) was shown to form self aggregations and to interact specifically with a plasma protein, transthyretin.

## 6.2 Future Work

A more extensive investigation into the forms of LAMP-1 in circulation of control and LSD-affected individuals will be essential to gain an understanding of the mechanisms

involved in the secretion of LAMP-1 and the potential use of LAMP-1 (+Tail) as an LSD marker. The use of pure HT1080 LAMP-1 (+Tail) as a calibration standard and the addition of CHAPS to the calibration standards and samples to prevent aggregation and alleviate inhibition will be employed.

A better purification procedure for LAMP-1 (+Tail) would be beneficial. This can possibly be achieved by incorporating size exclusion chromatography to isolate the solubilised membrane protein rather than dialysis which leads to the protein precipitating out of solution. Development of a better anti-LAMP-1 (+Tail) monoclonal antibody with greater avidity to the cytoplasmic tail of LAMP-1 for use in the affinity purification of LAMP-1 (+Tail) is also a possibility.

Future work may involve production of recombinant LAMP-1 (±Tail) expressing the sialyl-Lewis X antigen. It will be interesting to see whether the presence of sialyl-Lewis X will alter any of the findings in this research, such as the interactions in the immunoassays and whether new plasma proteins that interact with LAMP-1 will be isolated, particularly with LAMP-1 (-Tail).

Further characterisation of the recombinant LAMP-1 produced in this research will lead to a better understanding of the interactions of LAMP-1 with other proteins, as well as LAMP-1 with itself. This may involve deglycosylation of the LAMP-1 proteins to determine the oligosaccharides carried by the two forms of the glycoproteins. Also characterisation of the interaction between LAMP-1 (+Tail) and transthyretin will be required to find the binding site on tranthyretin and whether LAMP-1 (+Tail) binds to transthyretin at the same site as the thyroxine or retinol-binding protein.

In depth investigations into the role of LAMP-1 in cancer progression will also be beneficial. A first step will be to determine whether cancer patients have an elevated LAMP-1 level in their plasma. In addition to this, the inhibition theory given in this study of soluble LAMP-1, in the form of non-aggregated LAMP-1 (-Tail) and aggregated LAMP-1 (+Tail), inhibiting cell-cell interactions, should be investigated to determine whether soluble LAMP-1 (+Tail) is a better therapeutic agent than LAMP-1 (-Tail).

Work still to be done outlined above would be necessary to confirm and expand the findings of this thesis as to the function of LAMP-1 in circulation. This, in turn, will be critical for a complete understanding of the relevance of elevated LAMP-1 levels in LSD, their use as biochemical markers and possible contribution to the pathology of this group of disorders. In addition, this study will help to further elucidate the role of LAMP-1 in cancer and possibly help in developing a novel therapy for cancer.

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# APPENDICES

## APPENDIX 1: COMPONENTS OF MARKERS

BenchMark™ Protein Ladder — Invitrogen™ life technologies. San Diego, CA, USA

The components are:

| Band Number | Approximate Molecular Weight (kDa) |
|---|---|
| 1 | 220 |
| 2 | 160 |
| 3 | 120 |
| 4 | 100 |
| 5 | 90 |
| 6 | 80 |
| 7 | 70 |
| 8 | 60 |
| 9 | 50 |
| 10 | 40 |
| 11 | 30 |
| 12 | 25 |
| 13 | 20 |
| 14 | 15 |
| 15 | 10 |

BenchMark™ Prestained Protein Ladder GibcoBRL® life technologies. San Diego, CA, USA

The components are:

| Band Number | Approximate Molecular Weight (kDa) |
|---|---|
| 1 | 172.6 – 184.2 |
| 2 | 111.4 – 115.5 |
| 3 | 79.6 – 83.6 |
| 4 | 61.3 – 61.5 |
| 5 | 49.0 – 50.8 |
| 6 | 36.4 – 37.6 |
| 7 | 24.7 – 25.4 |
| 8 | 19.2 – 20.0 |
| 9 | 13.1 – 14.0 |
| 10 | 9.3 |

2-D SDS-PAGE Standards Bio-Rad Laboratories. Richmond, CA, USA

The components are:

| Protein | Approximate Molecular Weight (kDa) | pI (pH) |
|---|---|---|
| Hen egg white conalbumin type 1 | 76.0 | 6.0, 6.3, 6.6 |
| Bovine serum albumin | 66.2 | 5.4, 5.6 |
| Bovine muscle actin | 43.0 | 5.0, 5.1 |
| Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase | 36.0 | 8.3, 8.5 |
| Bovine carbonic anhydrase | 31.0 | 5.9, 6.0 |
| Soybean trypsin inhibitor | 21.5 | 4.5 |
| Equine myoglobin | 17.5 | 7.0 |

Spp1 (*EcoR I*) size marker Bresatec. Adelaide, SA, Australia

The fragment sizes are:

| Fragment Number | Size (kb) | Concentration (ng/µl) |
|---|---|---|
| 1 | 8.51 | 98.0 |
| 2 | 7.35 | 84.0 |
| 3 | 6.11 | 70.0 |
| 4 | 4.84 | 55.5 |
| 5 | 3.59 | 41.0 |
| 6 | 2.81 | 32.0 |
| 7 | 1.95 | 22.0 |
| 8 | 1.86 | 21.0 |
| 9 | 1.51 | 17.0 |
| 10 | 1.39 | 16.0 |
| 11 | 1.16 | 13.0 |
| 12 | 0.98 | 11.0 |
| 13 | 0.72 | 8.0 |
| 14 | 0.48 | 5.5 |
| 15 | 0.36 | 4.0 |

# APPENDIX 2: MATERIALS AND THEIR SOURCES

## APPENDIX 2A: CHEMICALS AND COMPOUNDS

(All reagents are analytical grade unless otherwise specified)

| | |
|---|---|
| Acetic acid | Ajax. Auburn, NSW, Australia |
| Ammonium persulphate | BIO-RAD Labarotories. Hercules, CA, USA |
| Ampicillin | Boehringer Mannheim. Mannheim, Germany |
| Bacto agar | Difco Laboratories. Detroit, MI, USA |
| Bacto tryptone | Difco Laboratories. Detroit, MI, USA |
| Bacto yeast extract | Difco Laboratories. Detroit, MI, USA |
| Boric acid | BDH Chemicals Pty. Ltd. Kilsyth, Vic., Australia |
| CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) | Sigma Chemical Co. St. Louis, MO, USA |
| Calcium choloride tissue culture grade | Sigma Chemical Co. St. Louis, MO, USA |
| CHAPS (3-{(3-cholamidopropyl)-dimethylammonio}-1-propanesulfonate) | Sigma Chemical Co. St. Louis, MO, USA |
| di-Sodium hydrogen orthophosphate anhydrous | BDH Chemicals Pty. Ltd. Kilsyth, Vic., Australia |
| DMSO (dimethyl sulphoxide) | Sigma Chemical Co. St. Louis, MO, USA |
| DNA grade agarose | Progen Industries Ltd. Dara, Qld., Australia |
| DTPA (diethylenetriamine-pentaacetic acid) | Sigma Chemical Co. St. Louis, MO, USA |
| DTT | Sigma Chemical Co. St. Louis, MO, USA |
| EDTA (Ethylenediamine-tetraacetic acid) | Ajax. Auburn, NSW, Australia |
| Ethanol | BDH Chemicals Pty. Ltd. Kilsyth, Vic., Australia |
| Ethidium bromide | Boehringer Mannheim. Mannheim, Germany |
| Formaldehyde | Sigma Chemical Co. St. Louis, MO, USA |

| | |
|---|---|
| Formamide | Ajax. Auburn, NSW, Australia |
| Glutaraldehyde | Sigma Chemical Co. St. Louis, MO, USA |
| Glycine | BDH Chemicals Pty. Ltd. Kilsyth, Vic., Australia |
| Glycerol | Biolab Scientific. Clayton, Vic. Australia |
| HEPES (1-[2-hydroxyethyl]-1-piperazinethansulfonsäure | Boehringer Mannheim. Mannheim, Germany |
| Hydrochloric acid | BDH Chemicals Pty. Ltd. Kilsyth, Vic., Australia |
| Magnesium Chloride | BDH Chemicals Pty. Ltd. Kilsyth, Vic., Australia |
| Methanol | BDH Chemicals Pty. Ltd. Kilsyth, Vic., Australia |
| Nonidet P-40 | Sigma Chemical Co. St. Louis, MO, USA |
| Orthophosphoric acid | M & B Australia. West Footscray, Vic., Australia |
| Phenol | Sigma Chemical Co. St. Louis, MO, USA |
| PIPES (piperazine-N, N1-bis[2-hydoxy-propanesulfonic acid]) | Boehringer Mannheim. Mannheim, Germany |
| Potassium hydrogen phthalate | Sigma Chemical Co. St. Louis, MO, USA |
| Isopropanol | Ajax. Auburn, NSW, Australia |
| Sodium acetate | BDH Chemicals Pty. Ltd. Kilsyth, Vic., Australia |
| Sodium azide | Sigma Chemical Co. St. Louis, MO, USA |
| Sodium chloride | Ajax. Auburn, NSW, Australia |
| Sodium carbonate anhydrous | Ajax. Auburn, NSW, Australia |
| Sodium di-hydrogen orthophosphate monohydrate | BDH Chemicals Pty. Ltd. Kilsyth, Vic., Australia |
| SDS (sodium dodecyl sulphate) | Sigma Chemical Co. St. Louis, MO, USA |
| Sodium hydrogen carbonate | Ajax. Auburn, NSW, Australia |
| Sodium hydroxide | Ajax. Auburn, NSW, Australia |

| | |
|---|---|
| Silver nitrate | Calbiochem-Novabiochem Corp. La Jolla, CA, USA |
| Sodium thiosulphate | Ajax. Auburn, NSW, Australia |
| Tris (Tris [hydroxymethyl aminomethane]) | Boehringer Mannheim. Mannheim, Germany |
| Tween 20 | BDH Chemicals Pty. Ltd. Kilsyth, Vic., Australia |
| TEMED (N, N, $N^1$,$N^1$-Tetramethylethylenediamine) | Bio-Rad Laboratories. Richmond, VA, USA |
| Thiourea | Sigma Chemical Co. St. Louis, MO, USA |
| Urea | Sigma Chemical Co. St. Louis, MO, USA |

## APPENDIX 2B: ENZYMES AND ENZYME BUFFERS

| | |
|---|---|
| Alkaline phophatase | Boehringer Mannheim. Mannheim, Germany |
| AP Buffer | Boehringer Mannheim. Mannheim, Germany |
| dNTP (deoxynucleotide triphosphates) | New England BioLabs Inc. Beverly, MA, USA |
| *EcoR I* | Boehringer Mannheim. Mannheim, Germany |
| *Eco RV* | Boehringer Mannheim. Mannheim, Germany |
| Ligation buffer | Bresatec. Adelaide, SA, Australia |
| *Not I* | Boehringer Mannheim. Mannheim, Germany |
| *Sal I* | Boehringer Mannheim. Mannheim, Germany |
| *Sau 3AI* | Boehringer Mannheim. Mannheim, Germany |
| T4-DNA ligase | Bresatec. Adelaide, SA, Australia |
| T4-DNA polymerase | New England BioLabs Inc. Beverly, MA, USA |
| Buffer H | Boehringer Mannheim. Mannheim, Germany |

## APPENDIX 2C: ANTIBODIES

| | |
|---|---|
| 1B1 anti-LAMP-1 monoclonal antibody | Elaine Ravenscroft, Department of Chemical Pathology, Women's and Children's |

| | |
|---|---|
| | Hospital, SA, Australia |
| 4F5 anti-LAMP-1 monoclonal antibody | Miao Yan, Department of Chemical Pathology, Women's and Children's Hospital, SA, Australia |
| Hybridoma cell line expressing BB6 anti-LAMP-1monoclonal antibody | Sven Carlsson, University of Umea, Umea, Sweden |
| Mouse anti-CD15s FITC conjugated monoclonal antibody | Pharmingen. San Diego, CA, USA |
| Sheep anti-mouse IgG FITC conjugated antibody | Silenus Laboratories. Hawthorn, Australia |
| Rabbit anti-LAMP-1 polyclonal antibody | Institute of Medical and Veterinary Science (IMVS), Vet Services Division, SA, Australia |
| Rabbit anti-LAMP (Tail) polyclonal Antibody | IMVS, Vet Services Division, SA, Australia |
| Rabbit anti-mouse IgG PE conjugated antibody | Silenus Laboratories. Hawthorn, Australia |
| Sheep anti-rabbit IgG HRP conjugated antibody | Silenus Laboratories. Hawthorn, Australia |

## APPENDIX 2D: CELLS AND VECTORS

| | |
|---|---|
| CHO-K1 | CSL Limited. Melbourne, Vic., Australia |
| HT1080 fibrosarcoma | CSL Limited. Melbourne, Vic., Australia |

| | |
|---|---|
| PANC-1 adenocarcinoma | American Type Culture Collection (ATCC), CRL 1469. Rockville, MD, USA |
| Control skin fibroblasts: SF5075, SF5241, SF5263 SF5264, SF5294 | Chemical Pathology, Women's and Children's Hospital, SA, Australia |
| E. coli MC1061 F-ara D139 ρ (ara-leu) 7697 gal E15 gal K16 ρ (lac) X74 tsp1 ($Str^R$) Hsd R2 ($RkM_k$) mcrA MC1B1 | New England Biolabs Inc. Beverley, MA, USA |
| pBluescript transfer vector containing LAMP-1 cDNA | Minoru Fukuda, La Jolla Cancer Research Foundation, CA, USA. |
| pFRNEE.2 expression vector containing LAMP-1 cDNA | E. Melville, Department of Chemical Pathology, Women's and Children's Hospital, SA, Australia |
| pCI-neo.N-dhfr expression vector | Tom Litjens, Department of Chemical Pathology, Women's and Children's Hospital, SA, Australia |

## APPENDIX 2E: TISSUE CULTURE REAGENTS

| | |
|---|---|
| Ampicillin | Boehringer Mannheim. Mannheim, Germany |
| Dexamethosone | Sigma Chemical Co. St Louis, MO, USA |
| Dulbecco's Modified Eagle Medium | Gibco BRL, Life Technology Inc. Grand Island, NY, USA |
| Foetal calf serum | CSL Ltd. Melbourne, Vic., Australia or Gibco BRL, Life Technology Inc. Grand Island, NY, USA |
| G418 | Boehringer Mannheim. Mannheim, Germany |

| | |
|---|---|
| HT (hypoxanthine, thymidine) | Gibco BRL, Life Technology Inc. Grand Island, NY, USA |
| Iscoves Medium | Gibco BRL, Life Technology Inc. Grand Island, NY, USA |
| Methotrexate | Sigma Chemical Co. St Louis, MO, USA |
| Minimum Essential Medium, alpha medium minus nucleosides | Gibco BRL, Life Technology Inc. Grand Island, NY, USA |
| Penicillin/Streptomycin sulphate | CSL Ltd. Melbourne, Vic., Australia |
| Phosphate buffered saline (PBS) | CSL Ltd. Melbourne, Vic., Australia |
| Trypsin-versene | CSL Ltd. Melbourne, Vic., Australia |

## APPENDIX 2F: COMMERCIAL KITS

| | |
|---|---|
| BRESApure™ Plasmid Midi Kit (50) | Bresatec. Thebarton, SA, Australia |
| DELFIA® Assay buffer, DELFIA® Wash buffer, DELFIA® Enhancement Solution | Wallac. North Ryde, NSW, Australia |
| DELFIA® Eu-Labelling Kit 1244-302 | Wallac. North Ryde, NSW, Australia |
| Renaissance® Western Blot Chemiluminescence Reagent | NEN™ Life Science Products. Boston, MA, USA |
| The GENECLEAN II® Kit | BIO 101 Inc. La Jolla, CA, USA |

## APPENDIX 2G: EQUIPMENTS

| | |
|---|---|
| Beckman Airfuge™ | Beckmann Instruments Inc. Palo Alto, CA, USA |
| Beckmann JA-20 centrifuge | Beckmann Instruments Inc. Palo Alto, CA, USA |
| Benchtop Megafuge 1.0 R | Heraeus Sepatech Instruments, Radiometer Pacific. Adelaide, SA, Australia |
| Biofuge 13 | Heraeus Sepatech Instruments, Radiometer Pacific. Adelaide, SA, Australia |

| | |
|---|---|
| BioMax QS 710 agarose electrophoresis tanks | Eastman Kodak Company. New Haven, CT, USA |
| Bio-Rad Miniprotean II electrophoresis unit | Bio-Rad Laboratories. Hercules, CA, USA |
| Ceres 900 HDI plate reader | Bio Teck Instruments Inc. Highland Park, Vinooski, VT, USA |
| DELFIA 1234 research fluorometer | Wallac. North Ryde, NSW, Australia |
| Econo system (low pressure liquid chromatography system) | Bio-Rad Laboratories. Richmond, VA, USA |
| Electronic UV transilluminator Model 44-16 | Polaroid Ltd. Cambridge, MA, USA |
| Electrophoresis power supply EPS 600 | Pharmacia Biotech. Uppsala, Sweden |
| Hoefer Mighty Small II electrophoresis unit | Hoefer Scientific Instruments. SF, CA, USA |
| HPLC system | Pharmacia Biotech. Uppsala, Sweden |
| TE Series Transphor electrophoresis unit | Hoefer Scientific Instruments. SF, CA, USA |
| UV 1201 Spectrophotometer | Shimadzu Corporation. Japan |

## APPENDIX 2H: MISCELLANEOUS

| | |
|---|---|
| 4-20% mini 2D gradient gels | Bio-Rad Laboratories. Hercules, CA, USA |
| 4-20% mini gradient gels | Gradipore Ltd. Pyrmont, NSW, Australia |
| 40% Acrylamide/Bis Solution (37.5:1) | Bio-Rad Laboratories. Hercules, CA, USA |
| Bio-Rad Affi-Gel 10 and Affi-Gel 15 | Bio-Rad Laboratories. Hercules, CA, USA |
| Brilliant Blue G-Colloidal stain | Sigma Chemical Co., MO, USA |
| Corning® 25 $cm^2$, 75 $cm^2$, 175 $cm^2$ tissue culture flasks | Corning Costar corporation. Cambridge, MA, USA |
| Corning® 1700 $cm^2$ roller bottles | Corning Costar corporation. Cambridge, MA, USA |

| | |
|---|---|
| Corning® 96 and 24 well tissue culture plates | Corning Costar corporation. Cambridge, MA, USA |
| Corning® 100mm tissue culture dishes | Corning Costar corporation. Cambridge, MA, USA |
| Filter Paper Model 583 Gel Dryer (34 x 45cm) | Bio-Rad Laboratories. Richmond, VA, USA |
| Hitrap™ Protein G column (5 ml) | Pharmacia Biotech. Uppsala, Sweden |
| Hollow Fibre Concentrator | Amicon., MA, USA |
| Immobiline™ DryStrips (pH 3-10, 7 cm) | Amersham Pharmacia Biotech. NJ, USA |
| Immunlon® 4HBX Removawell® Strips | Dynatech Laboratories. Chantilly, VA, USA |
| Kodak Diagnostic Film (18 x 24 cm) | Eatman Kodak Co. Rochester, NY, USA |
| LAMP-1 biotinylated peptides | Mimotopes Pty Ltd. Clayton, Vic., Australia |
| LAMP-1 peptide Tail column (1 x 5 cm) | Mimotopes Pty Ltd. Clayton, Vic. Australia |
| Ministart® single use syringe filter (0.2µm) | Sartorius. Goettingen, Germany |
| Nunc 1264 $cm^2$ 2-tray cell factory | Nalge Nunc International. IL, USA |
| Nunc chamber slides | Nalge Nunc International. IL, USA |
| PolyScreen® PVDF Transfer Membrane | NEN™ Life Science Products. Boston, MA, USA |
| Positive land film, type 667 | Polaroid Ltd. Hertfordshire, England |
| Removawell® Strip Holder | Dynatech Laboratories. Chantilly, VA, USA |
| Superose 12 FPLC column (1.5 x 30cm) | Pharmacia Biotech. Uppsala, Sweden |
| YM10 ultrafilter membranes | Amicon®. Beverly, MA, USA |

## APPENDIX 3: BUFFERS, SOLUTIONS AND MEDIA

{All buffers, solutions and media were prepared in millipore distilled water and sterilised by autoclaving at 125°C, 120 kPa, 5 mins or filtration (0.2 μm)}

### APPENDIX 3A: BUFFERS AND SOLUTIONS

| | |
|---|---|
| 1% Agarose gel | 1 x TBE buffer, 1% (w/v) agarose |
| 2 x HEPES buffered saline | 0.28M NaCl, 0.05M HEPES, 1.5mM $Na_2HPO_4$/NaOH, pH 7.05 |
| 10 x HT | 10mM sodium hypoxanthine, 1.6mM thymidine |
| 2 x loading buffer (SDS-PAGE) | 125 mM Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol |
| 10 x loading buffer (agarose gel electrophoreses) | 92.5% (v/v) formamide, 20 mM EDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue |
| Resolving gel buffer | 3M Tris/HCl, pH 8.8 |
| 12.5% SDS polyacrylamide gel | 12.5% (v/v) Resolving gel buffer, 0.1% (w/v) SDS, 0.075% (w/v) Ammonium persulphate, 0.75% (v/v) TEMED, 31.25% (v/v) Acrylamide/bisacrylamide (37.5:1) |
| Stacking gel buffer | 0.5M Tris/HCl, pH 6.8 |
| 4% Stacking gel | 25% (v/v) Stacking gel buffer, 0.1% (w/v) SDS, 0.075% (w/v) Ammonium persulphate, |

| | |
|---|---|
| | 0.75% (v/v) TEMED, 0.1% (v/v) Acrylamide/bisacrylamide (37.5:1) |
| 10 x TBE buffer | 90mM Tris/HCl, pH 8.74, 90mM, 2mM EDTA |
| TE buffer | 10mM Tris/HCl, pH 8.0, 1mM EDTA |
| Phosphate Buffered Saline (PBS) | 10mM $H_3PO_4$/NaOH, pH 7.4, 0.15M NaCl |
| 2D SDS PAGE: | |
| Equilibrium solution 1 | 50 mM Tris/HCL, pH 6.8, 30% (v/v) glycerol, 2% (w/v) SDS, 0.25% (w/v) DTT |
| Equilibrium solution 2 | 50 mM Tris/HCL, pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS, 5 M urea, 2 M thiourea, 1 grain of bromophenol blue |
| Lysis buffer 1 | 25 mM Tris/HCL, pH 8.8, 1% (w/v) SDS, 20 mM DTT |
| Lysis buffer 2 | 13% (w/v) CHAPS, 0.65% (v/v) Triton X-100, 43 mM DTT |
| Rehydration solution | 5 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.4% (v/v) Triton X-100, 1.7% pharmolytes 3-10, 10 mM DTT, 1 grain of bromophenol blue |

## APPENDIX 3B: CELL CULTURE MEDIA

| | |
|---|---|
| CHO-K1 Medium (for wild type) | α-MEM minus nucleosides, 10% FCS, 2xHT |
| CHO-K1 Selection Medium | α-MEM minus nucleosides, 10% dialysed |

| | |
|---|---|
| (for transformed clones) | FCS, 550 mg/L G418, 25 μM MTX |
| HT1080 Medium (for wild type) | Iscoves, 10% FCS |
| HT1080 Selection Medium | Iscoves, 10% FCS, 570 mg/L G418 |
| (for transformed clones) | (4 μM DEX when inducing expression) |
| LB Broth | 1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 1% (w/v) NaCl, NaOH, pH 7.5 |
| LB agar | LB broth, 1.5% (w/v) Bacto agar |
| LB-Amp | LB agar, 100 mg/L ampicillin |
| PANC Medium (for wild type) | DMEM, 10% FCS |
| PANC Selection Medium | DMEM, 10% FCS, 570 mg/L G418 |
| (for transformed clones) | (4 μM DEX when inducing expression) |
| Skin Fibroblast Medium | DMEM, 10% FCS |