Transthyretin interacts with the lysosome-associated membrane protein (LAMP-1) in circulation

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LAMP-1 (lysosome-associated membrane protein), a major glycoprotein present in the lysosomal membrane, constitutes up to 50 % of total membrane proteins. LAMP-1, expressed at the plasma membrane, is reported to be the major molecule expressing the sialyl-Lewis X antigen. Two forms of LAMP-1 exist; the full-length LAMP-1 [LAMP-1 (+ Tail)] has a highly glycosylated luminal domain, a membrane-spanning domain and a short cytoplasmic tail, and the truncated LAMP-1 [LAMP-1 (– Tail)] contains only the luminal domain. Soluble LAMP-1 ($\pm$ Tail) has been reported in circulation. LAMP-1 at the cell surface has been shown to interact with E-selectin and galectin and is proposed to function in cell–cell interactions. However, the functional role(s) of soluble LAMP-1 in circulation is unclear. To investigate the functional role of soluble LAMP-1 in circulation, recombinant LAMP-1 (– Tail) and LAMP-1 (+ Tail) were produced in HT1080

cells. Two immune-quantification assays were developed to distinguish between the LAMP-1 forms. The interaction and aggregation properties of the different LAMP-1 forms were investigated using the immune-quantification assays. Only LAMP-1 (+ Tail) was found to aggregate and interact with plasma proteins. Plasma proteins that interact with LAMP-1 were isolated by affinity chromatography with either the recombinant LAMP-1 (– Tail) or a synthesized peptide consisting of the 14 amino acids of the LAMP-1 cytoplasmic tail. Transthyretin was found to interact with the cytoplasmic tail of LAMP-1. Transthyretin exists as a homotetramer in plasma, as such may play a role in the aggregation of LAMP-1 in circulation.

Key words: affinity chromatography, lysosome-associated membrane protein (LAMP-1), plasma protein, transthyretin.

INTRODUCTION

LAMP-1 (lysosome-associated membrane protein) is a major glycoprotein present in the lysosomal membrane, making up approx. 0.1–0.2 % of the total cell proteins. LAMP-1 has a polypeptide core of approx. 42 kDa and consists of 389 amino acids; the majority of these (350 residues) are found on the luminal side of lysosomal membrane [1]. This intraluminal domain is connected to a single transmembrane domain, which is extended to a short cytoplasmic tail. The transport of LAMP-1 from the *trans*-Golgi network to endosomes and lysosomes relies on the -G-Y-X-X-Ø motif at the C-terminus of the protein's cytoplasmic tail, where X is any amino acid and Ø is a hydrophobic amino acid. The luminal domain is extensively glycosylated with N- and O-linked carbohydrate chains; some of the N-linked oligosaccharides are terminated with poly-*N*-acetyl-lactosamine, which is composed of the repeat sequence (Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 3). The termini of poly-*N*-acetyl-lactosamine often contain unique structures, such as the sialyl-Lewis X antigen [NeuNAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4-(Fuc α 1 $\rightarrow$ 3)GlcNAc] [2,3]. The carbohydrate constitutes 55–65 % of the total mass in the LAMP-1 molecule, giving it a molecular mass of 100–120 kDa [4]. On the cell surface, LAMP-1 has been reported to interact with two adhesion molecules, galectin-3 and E-selectin [5,6]. Galectin-3, a mammalian lectin, is found in a wide range of tissues and cells, and its expression is developmentally regulated [7]. Galectin-3 has affinity for β -galactoside-containing glycoconjugates and preferentially binds to

poly-*N*-acetyl-lactosamines [8]. E-selectin is expressed on activated endothelial cells and recognizes the sialyl-Lewis X antigen, expressed on neutrophils, monocytes and certain T-lymphocytes [9,10]. Cell-surface LAMP-1 has therefore been proposed to assist in cell–cell interactions through association with these lectins.

LAMP-1 is also expressed as a soluble form. In a study of the trafficking and turnover of LAMP-1, we reported that approx. 25 % of LAMP-1 within the lysosome of skin fibroblasts from Pompe and control cells was in a soluble form [11]. The soluble LAMP-1 that was purified from within human skin fibroblasts contained little or no membrane domain and cytoplasmic tail. In contrast, soluble LAMP-1 that was secreted from the cells did contain the membrane domain and the cytoplasmic tail. The secreted LAMP-1 represented 5.6 % and 8.5 % of newly synthesized LAMP-1 in Pompe and control cells respectively. We have also reported on the elevated levels of soluble LAMP-1 in plasma from patients with a range of lysosomal storage disorders [12]. The role of soluble LAMP-1 in circulation is currently unclear. In addition, following reports on the aggregation properties of soluble membrane-associated proteins, particularly of LAMP-2, which is a close relative of LAMP-1 [13,14], LAMP-1 is postulated to form aggregates. However, it is unknown whether aggregation occurs in both LAMP-1 (– Tail) and LAMP-1 (+ Tail) or in only one form. In the present study, the aggregation properties and interactions of LAMP-1 in circulation were investigated by using immune assays, affinity chromatography and proteomic methods.

Abbreviations used: DEX, dexamethasone; DTT, 1,4-dithiothreitol; FCS, foetal calf serum; LAMP-1, lysosome-associated membrane protein; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight; MEM, modified Eagle's medium; SF, skin fibroblast; SV40, simian virus 40; 2D, two-dimensional.

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EXPERIMENTAL

Plasma samples

Control plasma samples of de-identified individuals were obtained from healthy volunteers within the laboratory (Women's and Children's Hospital, Adelaide).

Construction of human LAMP-1 recombinant plasmid and transfection of human fibrosarcoma (HT1080) cells

The full-length human LAMP-1 [LAMP-1 (+ Tail)] cDNA was isolated as previously described [15]. The LAMP-1 sequence without the transmembrane coding and cytoplasmic tail sequence [LAMP-1 (– Tail)] (truncated at 1335 bp) was generated as described previously [11]. The LAMP-1 (+ Tail) and LAMP-1 (– Tail) cDNAs were cloned into the *EcoRI/NotI* site of the expression vector pGRE-neo.N-dhfr, which is a modification of the pCI-neo Mammalian Expression Vector (Promega, Anandale, NSW, Australia). Briefly, the human CMV (cytomegalovirus) promoter in pCI-neo was removed and replaced with a DEX (dexamethasone)-inducible SV40 (simian virus 40) promoter, new cloning sites were added, and also included was the amplification gene, DHFR (dihydrofolate reductase). The DEX-inducible SV40 promoter contained five copies of the rat tyrosine aminotransferase GRE (glucocorticoid-response element), the AdMLP (adenovirus 2 major late promoter) and the rabbit β -globin intron sequence, and was derived from the expression vector pGRE5-pGRE1 [16]. Two separate cell-expression systems were generated by transfecting the LAMP-1 (+ Tail) and LAMP-1 (– Tail) expression constructs in pGRE-neo.N-dhfr into HT1080 cells using a calcium phosphate procedure as described previously [17]. Stable clones were selected by resistance to G418 antibiotic. LAMP-1 (+ Tail) and LAMP-1 (– Tail) levels in the cell lysates and media of resultant clones were immune-quantified (see below) respectively. The clones that expressed the highest levels of LAMP-1 were selected for expansion.

Cell culture

HT1080 cells expressing LAMP-1 (+ Tail) were cultured in Iscove's medium containing 10 % (v/v) FCS (foetal calf serum), 570 mg/l G418 antibiotic and 4 μ M DEX, at 37 °C in a 5 % CO₂ humidified incubator. For large-scale production of LAMP-1 (+ Tail), cells were grown in 1700 cm² roller bottles (Corning Costar, Cambridge, MA, U.S.A.). HT1080 cells expressing LAMP-1 (– Tail) were cultured in MEM (modified Eagle's medium) containing 10 % (v/v) FCS, 4 μ M DEX, 570 mg/l G418, at 37 °C in a 5 % CO₂ humidified incubator. For large-scale production, cells were grown in two-tray 1264 cm² cell factories (Nalge Nunc International, Naperville, IL, U.S.A.). Once confluent, the cells were rinsed with PBS, and the medium was replaced with serum-free medium; this was alternated with serum-containing medium every 4–6 days.

FACS analysis

HT1080 cells expressing LAMP-1 (+ Tail) and LAMP-1 (– Tail), and wild-type HT1080 cells were harvested by methods described previously [18], and resuspended with 0.5 % (w/v) BSA/PBS at a final concentration of $(2.5\text{--}5) \times 10^6$ cells/ml. Non-specific binding of primary antibodies was blocked by incubating the cells with Intragam (CSL, Parkville, NSW, Australia) (20 μ l per tube) at 4 °C for 10 min. Cells were pelleted by centrifugation at 6000 g for 1 min at 4 °C, and resuspended in 0.5 % (w/v) BSA/PBS

(1 ml); this was repeated twice. Cells were incubated at 4 °C for 60 min with either FITC-conjugated anti-(sialyl Lewis X) CD15s monoclonal antibody (Pharmingen, San Diego, CA, U.S.A.) or unlabelled BB6 anti-LAMP-1 monoclonal primary antibody (100 μ g/ml). Cells were washed and blocked with Intragam as above. 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 at 4 °C for 30 min with 10 μ l (1:20 dilution) of rabbit anti-mouse phycoerythrin-conjugated antibody (Silenus Laboratories, Hawthorn, Vic, Australia). After a final wash with 0.5 % (w/v) BSA/PBS (2 $\times$ 1 ml), labelled cells were resuspended in 0.5 % (w/v) BSA/PBS (500 μ l) and analysed using Cellquest software (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

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

Immunofluorescence staining

HT1080 cells expressing LAMP-1 (+ Tail) and LAMP-1 (– Tail), and wild-type HT1080 cells (approx. 10⁵ cells) were grown in tissue culture chamber slides (Nalge Nunc International) in MEM, containing 10 % (v/v) FCS and 4 μ M DEX, at 37 °C in a 5 % CO₂ humidified incubator for 24 h. Cells on the slide were prepared, and immunofluorescence was carried out as described in [18]. The primary antibody used in this study was CD15s anti-sialyl-Lewis X monoclonal antibody.

Purification of LAMP-1 ($\pm$ Tail)

HT1080 cells expressing LAMP-1 (+ Tail) were grown in 6 $\times$ 1700 cm² roller bottles until confluent, harvested with 20 % (v/v) trypsin (in 20 ml) and washed twice with 40 ml of PBS. Total cellular membranes were prepared by resuspending cells in 20 ml of 1 M NaCl/PBS containing protease inhibitors (1 μ g/ml leupeptin, 1 μ M pepstatin and 200 μ M PMSF). Cells were placed in ice and lysed by forcing through a 26-gauge needle ten times. The suspension was clarified by centrifugation at 13 000 g for 10 min at 4 °C, and the resulting supernatant was retained. The cell pellet was resuspended in the same buffer (5 ml), and the cell rupture and centrifugation steps were repeated a total of six times. The membrane, containing LAMP-1 (+ Tail), in the pooled supernatant was pelleted by ultracentrifugation at 40 000 rev./min for 1 h at 4 °C in a Beckman L-90 ultracentrifuge in a Ti 70 rotor. LAMP-1 (+ Tail) was solubilized from the total cellular membrane by resuspending the membrane pellet in 1 % (w/v) CHAPS and 1 M NaCl in 20 ml of PBS containing protease inhibitors, Dounce-homogenized ten times, then freeze/thawed six times. The homogenate was clarified by ultracentrifugation at 40 000 rev./min for 1 h at 4 °C in a Beckman L-90 ultracentrifuge in a Ti 70 rotor. The supernatant containing LAMP-1 (+ Tail) was retained and dialysed overnight at 4 °C against PBS. LAMP-1 (+ Tail) in the dialysed supernatant was affinity-purified with an anti-LAMP-1 monoclonal antibody (BB6) [19] Affi-Gel 10 (Bio-Rad, Hercules, CA, U.S.A.) column (10 mg/ml). The solution was applied on to 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 H₃PO₄/NaOH, pH 2.5, at 0.25 ml/min. Eluted LAMP-1 proteins were dialysed overnight at 4 °C against PBS. The affinity-purified LAMP-1 (+ Tail) appeared as a homogeneous band on silver-stained SDS/PAGE with greater

than 95 % purity, and was quantified by the bicinchoninic acid method with BSA as a calibrator [20].

The LAMP-1 (– Tail) was purified from 6–7-days-conditioned medium of HT1080 cells grown in serum-free medium. Conditioned medium was clarified by ultracentrifugation at 40 000 rev./min for 1 h at 4 °C in a Beckman L-90 ultracentrifuge in a Ti 70 rotor. Batches of conditioned medium were concentrated ($\times 10$) in a hollow-fibre concentrator (Amicon, Danvers, MA, U.S.A.). The concentrated medium was then subjected to octanoic acid precipitation as described in [21]. Octanoic-acid-precipitated medium was clarified by centrifugation at 6000 g for 15 min, and dialysed overnight at 4 °C against PBS. Dialysed medium was concentrated further ($\times 10$) using stirred-cell ultrafiltration units with YM10 membranes (Amicon). The LAMP-1 in the concentrated medium was affinity-purified on the BB6 anti-LAMP-1 monoclonal antibody Affi-Gel 10 column (as above). The affinity-purified LAMP-1 (– Tail) appeared as a homogeneous band on silver-stained SDS/PAGE with more than 98 % purity, and was quantified by the bicinchoninic acid method with BSA as a calibrator [20].

Production and purification of anti-LAMP-1 antibodies

The BB6 anti-LAMP-1 monoclonal antibody was produced as described previously [19]. The production of rabbit anti-LAMP-1 and rabbit anti-[LAMP-1 (Tail peptide)] polyclonal antibodies has been described previously [11,22]. Each antibody was purified on a 5 ml Hitrap™ Protein G column (Amersham Biosciences, Uppsala, Sweden). Rabbit anti-LAMP-1 polyclonal antibody was affinity-purified on an Affi-Gel 15 affinity column containing LAMP-1 (– Tail) (0.5 mg/ml gel) purified from the medium of a CHO (Chinese-hamster ovary) expression cell line, previously described by Isaac et al. [23], using the same loading and elution conditions as described for the purification of HT1080 LAMP-1 ($\pm$ Tail) proteins. Rabbit anti-[LAMP-1 (Tail peptide)] polyclonal antibody was affinity-purified on a matrix of synthetic LAMP-1 cytoplasmic tail peptide (CLVGRKRSHAGYQTI) (5 mg) coupled to agarose gel (5 ml) (Mimotopes, Melbourne, Vic, Australia), using the same conditions as for the rabbit anti-LAMP-1 polyclonal antibody. Anti-LAMP-1 monoclonal and polyclonal antibodies were quantified by measuring the absorbance at 280 nm (absorbance = 1.4 for 1.0 g/l antibody).

Europium labelling of rabbit anti-LAMP-1 polyclonal antibody

Purified rabbit anti-LAMP-1 polyclonal antibody was labelled with Eu^{3+} , using the DELFIA® Eu^{3+} -labelling kit, following the manufacturer's instructions (Wallac, North Ryde, NSW, Australia). Labelled antibodies were purified on a Superose 12 (1 cm $\times$ 30 cm) FPLC column (Amersham Biosciences) as previously described [12]. The amount of Eu^{3+} conjugated to each antibody molecule was determined by the fluorescence of a known antibody concentration compared with a 1 nM Eu^{3+} standard solution.

Immune-quantification of LAMP-1

Determination of LAMP-1 was performed using time-delayed fluorescence immune-quantification assays as described below. Before analysis, plasma samples were dialysed overnight at 4 °C against PBS, and cleared of cellular debris by ultracentrifugation at 86 000 rev./min for 15 min at 4 °C in a Beckman Coulter airfuge in an A-100/18 rotor.

LAMP-1 (– Tail) calibrator standard was obtained from affinity-purified conditioned medium of HT1080 cells expressing

LAMP-1 (– Tail) (see above). This was diluted in DELFIA® assay buffer to generate a calibration curve consisting of 0.039, 0.078, 0.156, 0.313, 0.625, 1.25 and 2.50 ng/well.

LAMP-1 (+ Tail) standard was obtained from human SFs (skin fibroblasts) by solubilization from a total cell membrane preparation as described for the purification of HT1080 LAMP-1 (+ Tail). The concentration of the SF LAMP-1 (+ Tail) standard was determined with the LAMP-1 (Total) assay (see below), using HT1080 LAMP-1 (– Tail) as a calibrator, and confirmed by Western blotting analysis of the SF LAMP-1 (+ Tail) standard compared with the HT1080 LAMP-1 (– Tail) standard. SF LAMP-1 (+ Tail) standard was diluted in DELFIA® assay buffer to generate a calibration curve consisting of 0.039, 0.078, 0.156, 0.313, 0.625, 1.25 and 2.50 ng/well.

For the quantification and detection of both LAMP-1 (+ Tail) and LAMP-1 (– Tail) the LAMP-1 (Total) assay was used. Microtitre plates (Immulon 4; Dynatech Laboratories, Chantilly, VA, U.S.A.) were coated overnight at 4 °C with BB6 monoclonal antibody (5 $\mu\text{g/ml}$) diluted in 0.1 M NaHCO_3 (100 $\mu\text{l/well}$) and then washed once with DELFIA® wash buffer. Samples and standards were diluted in DELFIA® assay buffer and added to the wells (100 $\mu\text{l/well}$), shaken for 10 min at 20 °C, and incubated overnight at 4 °C. Plates were then washed six times with DELFIA® wash buffer. Eu^{3+} -labelled rabbit anti-LAMP-1 polyclonal antibody (0.2 mg/l) was added to the wells (100 $\mu\text{l/well}$) and incubated at 37 °C for 4 h. Plates were washed six times with DELFIA® wash buffer, and DELFIA® enhancement solution (200 μl) was added to each well. Plates were shaken for 10 min at 20 °C, and the fluorescence was read on a Wallac 1234 DELFIA® Research Fluorometer.

To specifically detect LAMP-1 (+ Tail), the LAMP-1 (Tail) assay was used. Microtitre plates were coated overnight at 4 °C with anti-[LAMP-1 (Tail peptide)] polyclonal antibody (5 $\mu\text{g/ml}$) diluted in 0.1 M NaHCO_3 (100 $\mu\text{l/well}$) and then washed once with DELFIA® wash buffer. Samples and standards were diluted in DELFIA® assay buffer and added to the wells (50 $\mu\text{l/well}$) together with Eu^{3+} -labelled rabbit anti-LAMP-1 polyclonal antibody (0.4 mg/l; 50 $\mu\text{l/well}$), shaken for 10 min at 20 °C, and incubated overnight at 4 °C. Plates were washed six times with DELFIA® wash buffer, DELFIA® enhancement solution (200 μl) was added to each well, shaken for 10 min at 20 °C, and the fluorescence was read on a Wallac 1234 DELFIA® Research Fluorometer.

LAMP-1 concentrations in the samples were calculated using a spline fit of the LAMP-1 calibration curve that covered the range 40–2500 pg/well. All points on the LAMP-1 calibration curve, and samples, were assayed in duplicate.

Preparation of affinity columns

For the preparation of affinity columns, HT1080 LAMP-1 (– Tail) protein or BSA was coupled to Affi-Gel 15 (Bio-Rad) at 1 mg/ml of gel, according to the manufacturer's instructions. The BB6 anti-LAMP-1 monoclonal antibody was coupled to Affi-Gel 10 at 10 mg/ml of gel, according to the manufacturer's instructions. Columns were stored in 0.02 % sodium azide/PBS at 4 °C, when not in use.

For the LAMP-1 (Tail peptide) column, synthetic LAMP-1 cytoplasmic tail peptide (H-CLUGRKRSHAGYQTI-OH) was bound to Sepharose CL-6B at 0.5 mg/ml (Mimotopes).

Affinity chromatography of plasma proteins

A pre-column consisting of BSA coupled to Affi-Gel 15 (2 ml) was connected directly to each HT1080 LAMP-1 (– Tail) and

LAMP-1 (Tail peptide) columns (2 ml). Plasma (10 ml), collected from healthy individuals, was applied on to 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 on to the BSA and LAMP-1 columns five times. After 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. The columns were separated and plasma proteins were eluted from each column 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 regenerate the LAMP-1 columns and ensure complete removal of plasma proteins, columns were washed with pre-elution buffer (10 mM NaH_2PO_4 /NaOH, pH 6.5), followed by 3 M MgCl_2 (pH 6.5). Eluted plasma proteins were analysed by SDS/PAGE and two-dimensional (2D) gel electrophoresis.

SDS/PAGE

Purified LAMP-1 (– Tail) were analysed by SDS/PAGE (12.5 % gel) according to Laemmli [24]. Proteins were visualized using periodate treatment followed by silver staining [25].

2D gel electrophoresis

Plasma proteins eluted from the LAMP-1 columns (10 μg) were solubilized in 50 μl of 25 mM Tris/HCl, pH 8.8, 1 % (w/v) SDS and 20 mM DTT (1,4-dithiothreitol) at 37 °C for 30 min. The sample was then centrifuged at 13 000 *g* for 15 min, and made up to a final volume of 200 μl with 5 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 0.4 % (v/v) Triton X-100, 1.7 % Pharmolyte 3-10 (Bio-Rad), 10 mM DTT and 0.01 % (w/v) Bromophenol Blue.

These samples were applied on to Immobiline™ DryStrips (pH 3–10, 7 cm) (Amersham Biosciences) in the rehydration apparatus (Bio Technology Services, Adelaide, SA, Australia), incubating on a rocking platform overnight at 20 °C. Isoelectric focusing of the sample was carried out on a flat-bed Metaphor II apparatus (Amersham Biosciences) using the following program: step 1, 0–300 V gradient for 1 min; step 2, constant 300 V for 3 h; step 3, 300–3500 V gradient for 5 h; step 4, constant 3500 V for 13 h; step 5, 3500–200 V gradient for 30 min; and step 6, constant 200 V for 4 h.

Following isoelectric focusing, Immobiline strips were incubated for 15 min at 20 °C in 5 ml of buffer containing 50 mM Tris/HCl, pH 6.8, 30 % (v/v) glycerol, 2 % (w/v) SDS and 0.25 % (w/v) DTT. The strips were then transferred into 5 ml of the same solution that had been pre-heated to 95 °C, and incubated for 1 min on a rocking platform. Strips were then incubated for 15 min at 20 °C in 5 ml of buffer containing 50 mM Tris/HCl, pH 8.8, 30 % (v/v) glycerol, 2 % (w/v) SDS, 5 M urea, 2 M thiourea and 1 grain of Bromophenol Blue. The strips were then fitted on to 4–20 % mini 2D gradient gels (Bio-Rad), and the samples were electrophoresed at 180 V for 60 min in a Bio-Rad MiniProtean II electrophoresis unit.

Peptide mass fingerprinting

Proteins of interest were excised from preparative gels of the eluted plasma proteins stained with Brilliant Blue G-Colloidal stain (Sigma Chemical Co., St. Louis, MO, U.S.A.). Proteins were digested with trypsin, and peptide mass fingerprints were generated by the Australian Proteome Analysis Facility (APAF, North Ryde, NSW, Australia) using MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS, as described in

Figure 1 Flow cytometry analysis of cell-surface LAMP-1 on HT1080 cells expressing LAMP-1 (– Tail)

HT1080 cells expressing LAMP-1 ($\pm$ Tail) were grown to confluence in 75 cm^2 flasks, harvested and labelled with BB6 anti-LAMP-1 monoclonal antibody, and detected with a phycoerythrin-conjugated rabbit anti-mouse IgG antibody. Wild-type (a), LAMP-1 (– Tail) (b) and LAMP-1 (+ Tail) (c) HT1080 cells were stained with BB6 anti-LAMP-1 monoclonal antibody. The shift in each histogram (solid lines on the right) correlates with cell-surface expression of LAMP-1 above background fluorescence (dotted line on the left).

[26]. The peptide masses were then used to search Swiss-Prot and TrEMBL databases (<http://au.expasy.org/tools/peptident.html>).

RESULTS

Characterization of the HT1080 expression cell lines and recombinant LAMP-1

The levels of LAMP-1 expressed by HT1080 cells transfected with pGRE-neo.N-dhfr-LAMP-1 (+ Tail) and pGRE-neo.N-dhfr-LAMP-1 (– Tail) were analysed with the LAMP-1 (Total) assay. This assay showed that 95 % of the expressed LAMP-1 (+ Tail) was retained in the cell membrane, and approx. 95 % of the expressed LAMP-1 (– Tail) was secreted into the culture medium. The maximum expression of LAMP-1 (+ Tail) from transfected cells was 20 $\mu\text{g}/\text{mg}$ of total cell protein, compared with 1.9 $\mu\text{g}/\text{mg}$ of total cell protein in wild-type cells. LAMP-1 (– Tail) secreted into the medium from transfected cells was up to 4 mg/l, compared with 0.1 mg/l in wild-type cells. This showed that overexpression of recombinant LAMP-1 ($\pm$ Tail) was achieved in transfected HT1080 cells.

Labelling of cells with CD15s to characterize the expression of the sialyl-Lewis X antigen showed no difference between HT1080 cells expressing LAMP-1 (+ Tail) or LAMP-1 (– Tail) and HT1080 wild-type cells (results not shown).

FACSscan cell staining with BB6 and CD15s of HT1080 wild-type and the expression cell line were carried out to specifically examine cell-surface expressions of LAMP-1 ($\pm$ Tail) and sialyl-Lewis X antigen. HT1080 wild-type cells labelled with BB6 resulted in little surface staining, whereas HT1080 LAMP-1 ($\pm$ Tail) showed a significant increase in surface staining for LAMP-1 (Figure 1). Staining with CD15s gave negative results.

Tables 1 and 2 outline the average HT1080 LAMP-1 (+ Tail) and LAMP-1 (– Tail) protein recoveries for each of the purification steps respectively. SDS/PAGE with silver staining performed on 50 ng of affinity-purified HT1080 LAMP-1 (+ Tail) showed that it was approx. 95 % pure, with an average molecular mass of 120 kDa, and HT1080 LAMP-1 (– Tail) was approx.

Table 1 Recovery of LAMP-1 (+ Tail) from HT1080 expression cell membrane during purification

Purification step	Total HT1080 LAMP-1 (+ Tail) (μ g)	Recovery in each step (%)	Recovery of total LAMP-1 (%)
Lysed cells	86	—	100
Frozen-thawed membranes	54	63	63
Dialysis	41	76	48
Affinity chromatography	9.5	23	11

Table 2 Recovery of LAMP-1 (– Tail) from HT1080 expression cell media during purification

Purification step	Total HT1080 LAMP-1 (– Tail) (μ g)	Recovery in each step (%)	Recovery of total LAMP-1 (%)
6-Day conditioned medium	362	—	100
Concentration	362	100	100
Octanoic acid precipitation	261	72	72
Dialysis	261	100	72
Affinity chromatography	150	57	41

98 % pure, with an average molecular mass of 100 kDa (results not shown).

Aggregation properties of LAMP-1: effects of CHAPS and plasma on the LAMP-1 immune-quantification assays

In order to quantify LAMP-1 and to distinguish between LAMP-1 (+ Tail) and LAMP-1 (– Tail), two LAMP-1 immune-quantification assays were developed: the LAMP-1 (Total) assay and the LAMP-1 (Tail) assay.

The final parameters of the immune-quantification assays were optimized by generating a series of calibration curves under different test conditions. The assays were optimized for concentrations of the coating and Eu^{3+} -labelled antibodies, and the incubation times of the samples and secondary antibodies. Figure 2 shows the final calibration curves of the two LAMP-1 immune-quantification assays generated using optimized parameters. Due to the difficulty in purifying sufficient amounts of the membrane LAMP-1 (+ Tail) protein, a crude preparation of LAMP-1 (+ Tail) from cultured skin fibroblasts was used as a calibration standard. Both the LAMP-1 (Total) and the LAMP-1 (Tail) assays gave near linear responses up to 2.5 ng/well LAMP-1; all assays were performed within this range. All standards and samples were assayed in duplicate with a coefficient of variance always less than 10 %. In all Figures, the averages of the duplicate assays are shown.

The effect of detergent on these assays was evaluated to investigate how LAMP-1 may interact with other proteins. Figure 3(A) shows that while 0.1 % CHAPS has little effect, 1–2 % CHAPS suppresses the signal from LAMP-1 (+ Tail) in the LAMP-1 (Total) assay, presumably by inhibition of the LAMP-1 protein/anti-LAMP-1 antibody interaction. Figure 3(B) shows that 0.1 % CHAPS results in a marked signal increase from the LAMP-1 (+ Tail) in the LAMP-1 (Tail) assay. This is thought to be the result of exposure of the LAMP-1 (+ Tail) by disassociation from other proteins, or possibly itself, by the CHAPS. Higher concentrations of CHAPS (1–2 %) resulted in signal suppression, presumably also as a result of inhibition of the LAMP-1 protein–anti-LAMP-1 antibody interaction. Thus the LAMP-1 (Tail) assay provides us with a sensitive method to detect the exposure of the LAMP-1 cytoplasmic tail peptide in solution.

Figure 2 Calibration curves for LAMP-1 immune-quantification assays

Microtitre plates were coated with either BB6 anti-LAMP-1 monoclonal antibody (A) or anti-LAMP-1 (Tail peptide)] polyclonal antibody (B) (5 μ g/ml) overnight at 4 °C. In the LAMP-1 (Total) assay (A), standards were incubated overnight at 4 °C, and detected with Eu^{3+} -labelled rabbit anti-LAMP-1 polyclonal antibody (200 ng/ml) at 37 °C for 4 h. In the LAMP-1 (Tail) assay (B), standards were incubated overnight at 4 °C with Eu^{3+} -labelled rabbit anti-LAMP-1 polyclonal antibody (200 ng/ml). The standards used were HT1080 LAMP-1 (– Tail) (○) and SF LAMP-1 (+ Tail) (Δ).

When the effect of plasma on the LAMP-1 assays was investigated, we observed no effect on the LAMP-1 (Total) assay (results not shown). However, the addition of plasma inhibited the LAMP-1 (Tail) assay (Figure 4), indicating that plasma proteins were masking the LAMP-1 tail peptide. The addition of CHAPS to this assay system reversed the effect of the plasma, although 1 % CHAPS was required to give maximal signal, compared with 0.1 % when plasma was not present (Figure 4, compared with Figure 3B).

Identification of plasma proteins that interact with LAMP-1 in circulation

In order to identify proteins that specifically interact with either the luminal or cytoplasmic domains of LAMP-1 in circulation, affinity chromatography was performed with plasma samples (10 ml) on columns containing either HT1080 LAMP-1 (– Tail) or the peptide sequence of the cytoplasmic tail of LAMP-1 respectively. Plasma proteins, eluted with 1 M NaCl/PBS and 0.1 M $\text{H}_3\text{PO}_4/\text{NaOH}$ (pH 2.5) from the BSA pre-column and LAMP-1 columns, were run on SDS/PAGE and 2D gel electrophoresis. Plasma proteins of interest were isolated after comparing protein bands (on one-dimensional gels) or spots (on 2D gels) from the BSA pre-column and the LAMP-1 columns. The specificity of a

Figure 3 Effects of CHAPS on the LAMP-1 (Total) and LAMP-1 (Tail) assays

Microtitre plates were coated with either BB6 monoclonal antibody (5 μ g/ml) for LAMP-1 (Total) assays (A) or anti-[LAMP-1 (Tail peptide)] polyclonal antibody (5 μ g/ml) for LAMP-1 (Tail) assays (B) overnight at 4 °C. SF LAMP-1 (+ Tail) standards without (○) and with 0.1% (×), 1% (Δ) and 2% (+) CHAPS were detected with Eu³⁺-labelled rabbit anti-LAMP-1 polyclonal antibody (200 ng/ml) overnight at 37 °C.

Figure 4 Effects of plasma on the LAMP-1 (Tail) assay

Microtitre plates were coated with anti-[LAMP-1 (Tail peptide)] polyclonal antibody (5 μ g/ml) overnight at 4 °C. SF LAMP-1 (+ Tail) standard without (○) or with 2 μ l of control plasma (□) or plasma plus 0.1% (×), 1% (Δ) and 2% (+) CHAPS were detected with Eu³⁺-labelled rabbit anti-LAMP-1 polyclonal antibody (200 ng/ml) at 37 °C for 4 h.

plasma protein towards LAMP-1 (– Tail) or the cytoplasmic tail of LAMP-1 was determined as those not found eluting from the BSA pre-column, but solely from their respective columns.

Figure 5 SDS/PAGE of plasma proteins eluted from LAMP-1-affinity columns

Plasma (10 ml) was applied on to the BSA Affi-Gel 15 pre-column and LAMP-1 (Tail peptide) column at a flow rate of 0.5 ml/min. The columns were washed with PBS, separated, and plasma proteins were eluted successively with 1 M NaCl/PBS and 0.1 M H₃PO₄/NaOH (pH 2.5). Eluted plasma proteins (250 ng) were run on 12.5% polyacrylamide gels at a constant voltage (180 V), then silver stained. Lanes 1–3 show proteins eluted from the pre-column; lanes 4–5 show proteins eluted from the LAMP-1 (Tail peptide) column. Lane 1, plasma load; lanes 2 and 4, plasma proteins eluted with 1 M NaCl; lanes 3 and 5, plasma proteins eluted with 0.1 M H₃PO₄/NaOH, pH 2.5. The arrow indicates plasma protein of interest. Molecular mass is indicated (in kDa) to the left of the gel.

Figure 5 shows SDS/PAGE of plasma proteins eluted from the pre-column (lanes 1–3) and the LAMP-1 (Tail peptide) column (lanes 4 and 5). A protein of 14 kDa eluted by the 0.1 M H₃PO₄/NaOH (pH 2.5) buffer from the LAMP-1 (Tail peptide) column was identified for further characterization. To determine whether galectin-3 and E-selectin were amongst the plasma proteins that were purified using our affinity columns, Western blot analysis was performed on the eluted plasma proteins. Membranes were probed with anti-galectin-3 and anti-E-selectin monoclonal antibodies. Neither galectin-3 nor E-selectin was detected (results not shown).

To obtain better separation of plasma proteins, 2D gel electrophoresis was performed. Protein spots generated from plasma proteins, eluted from the LAMP-1 (– Tail) column (Figure 6A) with 0.1 M H₃PO₄/NaOH (pH 2.5), were the same as those eluted from the BSA pre-column (results not shown); thus there were no proteins of interest isolated from the LAMP-1 (– Tail) column. Comparison of plasma proteins eluted from the BSA pre- and LAMP-1 (– Tail) columns with proteins eluted from the Tail peptide column, with 0.1 M H₃PO₄/NaOH (pH 2.5), identified two proteins that were specific for the LAMP-1 peptide tail (indicated by the arrows, Figure 6B).

The peptide fingerprints of each protein were searched against all human proteins in Swiss-Prot and TrEMBL databases with the online peptide-identification software, PeptIdent (<http://au.expasy.org/tools/peptident.html>). The peptide mass spectra of the 14 kDa plasma protein excised from the one-dimensional gel (Figure 5, lane 5) matched to transthyretin with an amino acid sequence coverage of 68.5%. Both proteins isolated from the 2D gel in Figure 6(B) were identified as transthyretin, with sequence coverage of 56.7% each.

There were no plasma proteins identified that bound specifically to the HT1080 LAMP-1 (– Tail).

DISCUSSION

To date, studies of LAMP-1 interactions with extracellular proteins have been confined to LAMP-1 expressed on the cell surface.

Figure 6 2D gel electrophoresis of plasma proteins eluted from the LAMP-1-affinity columns

Plasma samples (10 ml) from a healthy individual were applied to the BSA pre-column connected to the LAMP-1 columns at a flow rate of 0.5 ml/min, then recirculated five times. The columns were washed with PBS, separated, and the plasma proteins were eluted from the columns successively with 1 M NaCl/PBS, then 0.1 M $\text{H}_3\text{PO}_4/\text{NaOH}$ (pH 2.5). Plasma proteins were separated by 2D gel electrophoresis, then silver stained. (A) Plasma proteins eluted from the HT1080 LAMP-1 (– Tail) column with 0.1 M $\text{H}_3\text{PO}_4/\text{NaOH}$ (pH 2.5) (10 μg load). (B) Plasma proteins eluted from the LAMP-1 (Tail peptide) column with 0.1 M $\text{H}_3\text{PO}_4/\text{NaOH}$ (pH 2.5) (10 μg load); arrows indicate plasma proteins of interest.

Sawada et al. [27] 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. [5] 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 [5]. LAMP-1 expressed on the cell surface has been proposed to assist in cell–cell interactions, and may be involved in metastasis of cancer cells.

Although LAMP-1 and its structural analogue, LAMP-2, are the most abundant lysosomal membrane proteins, making up approx. 0.1–0.2 % of the total cell proteins [19], the soluble forms of these proteins have not been well characterized. We, and others, have reported previously the occurrence of a truncated soluble form of LAMP-1 that does not contain the membrane span or cytoplasmic domain [LAMP-1 (– Tail)] [11,28]. 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 either at the cell surface or within the lysosome, followed by exocytosis from the cell [11]. We have also reported on a soluble form of LAMP-1 with the membrane span and cytoplasmic tail intact. Through pulse–chase experiments, we found that this soluble LAMP-1 was released directly from the

cells without trafficking to the lysosome and was not re-interalized by the cell [11].

In order to examine possible interactions of these soluble forms of LAMP-1 in circulation, we have investigated the aggregation properties of soluble LAMP-1 and have identified a plasma protein that interacts with the full-length form of LAMP-1.

Characterization of recombinant LAMP-1 ($\pm$ Tail)

The recombinant LAMP-1 (+ Tail) and LAMP-1 (– Tail) proteins produced from HT1080 cells were 120 and 100 kDa in size respectively. FACSscan analysis and immunoassay of expression in the HT1080 LAMP-1 expression cell lines showed that LAMP-1 ($\pm$ Tail) was overexpressed on the cell surface and that the sialyl-Lewis X antigen was not being expressed. There was also a marked elevation in the level of LAMP-1 (– Tail) secreted from the cells into the culture medium. As the cytoplasmic tail is missing in the LAMP-1 (– Tail) form of the protein, the signalling motif that traffics this protein to the lysosome is also missing. Therefore we observed an increase in LAMP-1 secreted from the cells, but no increase in lysosomal LAMP-1 (– Tail). The elevated level 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 also been removed, thus providing no anchor to the plasma membrane. It is thought that this may be the result of the interaction of this LAMP-1 with other surface proteins on the HT1080 cells. Despite the increased expressions of LAMP-1 ($\pm$ Tail), there was no increase in the level of the sialyl-Lewis X antigen. This may have been the result of the over expression of LAMP-1 ($\pm$ Tail) and/or the absence of the membrane anchor in the LAMP-1 (– Tail) protein to facilitate the action of the glycosyltransferases required for the biosynthesis of the sialyl-Lewis X antigen.

Aggregation properties of LAMP-1 ($\pm$ Tail)

Two immune-quantification assays were developed and optimized to detect the two forms of LAMP-1. The LAMP-1 (Total) assay detected both forms, whereas the LAMP-1 (Tail) assay only detected LAMP-1 (+ Tail) by using an antibody specific for the tail peptide of LAMP-1. The interactions of LAMP-1 were investigated through these LAMP-1 immune-quantification assays. The detection of SF LAMP-1 (+ Tail) was inhibited with the addition of 1 and 2 % CHAPS in the LAMP-1 (Total) assay (Figure 3A), suggesting that CHAPS may inhibit the LAMP-1 (Total) assay by disrupting the binding of LAMP-1 to the immobilized antibody on the plate.

The effects of CHAPS in the LAMP-1 (Tail) assay (Figures 3B and 4) suggested that SF LAMP-1 (+ Tail) is able to form aggregates, through interaction with either itself or other proteins. When CHAPS was included in the assay at 0.1 %, disruption of SF LAMP-1 (+ Tail) aggregation was seen to enhance the assay; this enhancement was offset, however, when the 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 3B). This observation would indicate that SF LAMP-1 (+ Tail) is able to aggregate, and thus the enhancement in its detection when 0.1 % CHAPS was added. However, as the SF LAMP-1 (+ Tail) calibration standard was not an affinity-pure protein, but a membrane protein preparation, it is possible that the aggregation observed may not be self-aggregation, but interaction with other membrane proteins. Moreover, the addition of plasma caused substantial inhibition of the SF LAMP-1 (+ Tail) determination in the LAMP-1 (Tail) assay (Figure 4), suggesting that LAMP-1 (+ Tail) interacts

specifically with plasma proteins. This interaction could also be reduced by the addition of 1% CHAPS, although the higher concentration required indicates a higher affinity interaction than that observed in Figure 3.

Plasma protein that interacts with LAMP-1 (± Tail)

Two affinity-chromatography columns, HT1080 LAMP-1 (– Tail) and LAMP-1 (Tail peptide), were used to isolate plasma proteins that could be interacting with LAMP-1 (± Tail) in circulation. Galectin-3 and E-selectin were not among the proteins that were identified to interact with LAMP-1 in the present study. 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 [29,30]. Therefore it is likely that galectin-3 was not identified in the present study because it is not expressed in circulation at levels high enough to detect. E-selectin was not identified, as the recombinant LAMP-1 (– Tail) protein used in the present study did not contain the sialyl-Lewis X antigen, the ligand for E-selectin.

A plasma protein (transthyretin) was identified to interact specifically with LAMP-1 through the cytoplasmic tail, supporting our results from the LAMP-1 immune-quantification assays. The high affinity of transthyretin to the cytoplasmic tail of LAMP-1 is indicated by its elution with 0.1 M $\text{H}_3\text{PO}_4/\text{NaOH}$ (pH 2.5), but not 1 M NaCl. In normal human serum, transthyretin, also known as prealbumin, exists as a 54 kDa tetramer of four identical subunits arranged to form a cylindrical channel [31]. Each subunit of the transthyretin homotetramer has a cysteine residue at position 10. Several transthyretin isoforms have been identified in human plasma and cerebrospinal fluid by MALDI-TOF MS, most of which are caused by disulphide linkage with Cys¹⁰ [32,33]. These isoforms would explain the result shown in Figure 6(B), in which both plasma proteins of interest were identified as transthyretin, although they differed in pI values.

Transthyretin is found in the serum of all mammals, and functions to transport the hormone thyroxine from the blood stream to the brain across the blood–brain barrier of the choroid plexus. It also transports vitamin A indirectly by forming a complex with the retinol-binding protein. Thyroxine binds to an internal channel of the transthyretin tetramer and retinol-binding protein to the exterior [34,35]. 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 [36].

The dissociation and misfolding of transthyretin resulting in insoluble amyloid fibrils has been implicated with familial amyloid polyneuropathy and senile systemic amyloidosis. It was proposed that transthyretin could self-assemble into amyloid-like fibrils in acidic environments, such as the lysosome [37]. Moreover, it was shown that stabilization of the tetrameric form of transthyretin by the binding of thyroxine prevented the formation of fibril formation *in vitro* [37]. Given that transthyretin interacts with the cytoplasmic tail peptide of LAMP-1, it is possible that LAMP-1 may bind to transthyretin within the lysosome, preventing fibril formation, and this formation is secreted into circulation. Furthermore, LAMP-1 aggregation may also form through its association with the homotetramer of transthyretin.

From the present study, no plasma proteins were identified that interact specifically with soluble LAMP-1 (– Tail). However, the recombinant LAMP-1 (– Tail) produced for the present study was not identical with endogenous LAMP-1 (– Tail) found in circulation, in terms of the carbohydrate side chains. The carbohydrate side chains of LAMP-1 constitute 55–65% of the total mass of the protein [4], therefore making the carbohydrate content

of LAMP-1 the most likely variable between endogenous and recombinant LAMP-1 (– Tail). Therefore plasma proteins that would normally recognize certain forms of carbohydrate side chains on LAMP-1 (– Tail) *in vivo* may not have recognized the recombinant form.

LAMP-1 has been found in greater levels on highly metastatic colonic carcinoma tumour and melanoma cells than on poorly metastatic cells [2,38]. Moreover, high-metastatic cells were found to bind activated human endothelial cells expressing E-selectin more efficiently than low-metastatic cells [39]. 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 [40]. It is possible that the overexpression 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 would therefore result in the inhibition of tumour metastasis and the observed prolonged life. The level of LAMP-1 in the plasma of the pancreatic carcinoma patients was not reported. Soluble LAMP-1 therefore may prove to be a useful therapeutic agent for the inhibition of E-selectin-mediated binding to tumour cells. Moreover, this effect may be modulated by the interaction of LAMP-1 (+ Tail) with other plasma proteins.

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