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Characterization of Human Lysosomal Membrane Glycoproteins and Evidence for Their Differentiation-Related Expression in the Plasma Membrane of Myelomonocytic Leukemia Cells*

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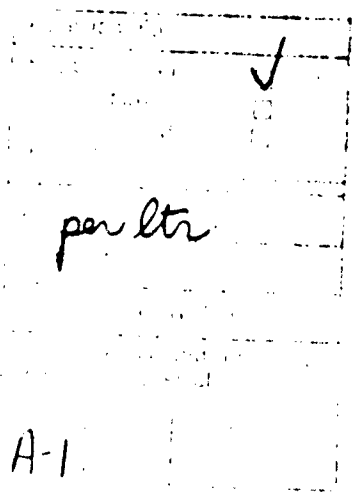

SUMMARY

Two human cell lysosomal membrane glycoproteins of ~120-kDa, hLAMP-1 and hLAMP-2, were identified by use of monoclonal antibodies prepared against U937 myelomonocytic leukemia cells or blood mononuclear cells. The two glycoproteins were purified by antibody affinity chromatography and each was found to be a major constituent of human spleen cells, representing ~0.05% of the total detergent-extractable protein. Both molecules were highly glycosylated, being synthesized as polypeptides of 40 to 45 kDa and cotranslationally modified by the addition of Asn-linked oligosaccharides. NH_2-terminal sequence analysis indicated that each was ~50% identical to the corresponding mLAMP-1 or mLAMP-2 of mouse cells. Electron microscopic studies of human blood monocytes, HL-60, and U937 cells demonstrated that the principal location of these glycoproteins was intracellular, in vacuoles and lysosomal structures but not in the peroxidase-positive granules of monocytes. Transport of the proteins between organelles was evidenced by their marked accumulation in the membranes of phagolysosomes. A fraction of each glycoprotein was also detected on the plasma membrane of U937 and HL-60 cells but not on a variety of other tissue culture cells. This cell-surface expression may be differentiation-related, since the proteins were not detected in the plasma membrane of normal blood monocytes and their expression on U937 and HL-60 cells was reduced when the cells were treated with differentiating agents. Cell-surface expression of both glycoproteins was markedly increased in blood monocytes but not in U937 cells after exposure to the lysosomotropic reagent methylamine HCL, indicating differences in LAMP-associated membrane flow in these cell types.

INTRODUCTION

Glycoproteins localized in lysosomal membranes represent a newly identified family of organelle-specific molecules whose study provides a means to approach some of the fundamental questions concerning the structure, function and biogenesis of lysosomes The number of proteins that act in this membrane is unknown; presumably they include the proton pump responsible for acidification of the organelle, as well as molecules involved in the structural, transport and receptor functions of the membrane. Studies of fractionated lysosomes suggest that there are as many as five principal molecules in the 60- to 110-kDa range (1,2), and recently several proteins of >100 kDa have been identified and characterized: mouse mLAMP-1 and mLAMP-2 (3-5), rat lpg 120 and lpg 100 (6), rat LIMP III (7), and chicken LEP100 (CV-24) (8,9). In addition, molecules of 70 to 80 kDa (lpg 80 and LIMP II) and 35 to 50 kDa (LIMP I) (6,7) and a molecule with properties related to an H^+,K^+-ATPase (10) have been reported.

A distinguishing feature of the >100-kDa molecules is their extensive glycosylation. mLAMP-1 has been shown to contain ~15% of the [^{3}H]glucosamine (11) and 6% of the [2-^{3}H]mannose[1] incorporated into an acid-insoluble fraction of NIH/3T3 and MDAY-D2 cells, respectively. Each of the >100-kDa glycoproteins is synthesized as a core polypeptide of about 40 kDa that is modified by the addition of up to 20 Asn-linked, complex-type and high mannose oligosaccharides (5-8). The modification of individual molecules is highly variable, resulting in a marked heterogeneity in apparent mass (100 to 150 kDa) of the mature glycoproteins. Kinetic analyses of the biosynthesis and movement of the newly synthesized glycoproteins (7,12) have shown that oligosaccharide processing occurs rapidly (30 to 60 min after synthesis) as the molecules pass through the endoplasmic reticulum and Golgi cisternae and that mature molecules are th-

targeted directly to lysosomes.

Although these glycoproteins are localized primarily in the lysosomal membrane, a small fraction (~5%) of the molecules has been detected on the plasma membrane of certain cells, and a variety of data point to a potentially important role for lysosomal membrane proteins on the cell surface. LEP100 has been found on the surface of chick embryo fibroblasts, and studies of the movement of this molecule suggest a pathway of membrane flow from lysosomes through the plasma membrane and endosomes (8,9). mLAMP-1 and mLAMP-2 have been detected on the plasma membrane of the macrophage-like P388 cells but not on NIH 3T3 cells (4), and both mLAMP glycoproteins have been found to be closely similar, if not identical, to proteins described as cell-surface differentiation and oncogenesis antigens: The mouse gp130 (P2B) glycoprotein, a highly glycosylated molecule whose expression of specific carbohydrate epitopes at the cell surface is correlated with an increased metastatic potential of the tumor line MDAY-D2 (13), corresponds to mLAMP-1 in NH_2-terminal sequence (14);[1] and Mac-3, a cell-surface marker of mouse macrophages (15), corresponds in biochemical and antigenic properties to mLAMP-2 (3). Moreover, we have recently determined by cDNA cloning and sequencing that mLAMP-1 has high sequence similarity to a polylactosaminoglycan-containing glycoprotein of human leukemia cells that has been studied as an onco-differentiation antigen (16,17). These findings suggest a possible relationship between cell-surface expression of LAMP molecules, the state of differentiation of particular cell types, and the phenotypic properties of the cells.

In this report we describe the identification and characterization of two human cell lysosomal membrane glycoproteins, hLAMP-1 and hLAMP-2. The biochemical properties of these hLAMP molecules, including their NH_2-terminal amino acid sequences, are highly similar to the corresponding

murine mLAMP-1 and mLAMP-2 molecules (3,5). In addition, hLAMP-I has sequence identity to the human leukemia cell polylactosaminoglycan-containing glycoprotein (16), establishing this leukemia cell molecule as a lysosomal membrane component. We have also investigated the cell-surface expression of the hLAMP glycoproteins and find evidence for membrane flow from hLAMP-positive vesicles to the plasma membrane in U937 and HL-60 myelomonocytic leukemia cells that was not detected in a variety of other tissue culture cells or normal peripheral blood monocytes. The concentration of the glycoproteins on the plasma membrane of U937 cells was not affected by exposure to the lysosomotropic agent methylamine HCl but was reduced when the cells were converted to the Mac-1-positive, differentiated phenotype by treatment with phorbol myristate acetate (PMA).[2]

EXPERIMENTAL PROCEDURES[3]

RESULTS[3]

Cellular Localization of the Antigens - The localization of hLAMP-1 and hLAMP-2 in various human cells was analyzed by transmission electron microscopy and colloidal gold immunolabeling of ultrathin frozen sections. The hLAMP glycoproteins were predominantly localized in membranes of secondary lysosomes and in other smaller vacuoles and multivesicular bodies that may be precursors to the formation of secondary lysosomes. There was no significant difference observed between the localization of hLAMP-1 and hLAMP-2 in any of the cell types examined.

In monocytes the hLAMP antigens were present in moderately sized, clear vacuoles, some of which contained granular or membranous material (Fig. 5). The proteins were not detected in the plasma membrane, Golgi cisternae, or coated vesicles, nor were they found in the azurophil granules, which contain large amounts of lysosomal enzymes and correspond to primary lysosomes. The surprising absence of hLAMP antigens from the azurophil granules suggests that these structures are bounded by membranes characteristic of secretory granules or that they represent primary lysosomes with a biosynthetic pathway different from that of LAMP-positive vesicles.

HL-60 cells (Fig. 6) contained small amounts of the antigens in tubular and vesicular structures and higher amounts in large vacuoles. Some of these vacuoles resembled multivesicular bodies and others contained numerous membrane whorls; the latter have been shown to be autophagic vacuoles because they contain endogenously synthesized peroxidase (45).

The hLAMP proteins in U937 cells were predominantly associated with cytoplasmic vacuoles that were often grouped in clusters and contained electron-dense material. The diameter of these vacuoles ranged from 0.1 to

0.4 μm. By criteria of size, shape, and lumenal content these vacuoles resembled typical lysosomal dense bodies and a heterogeneous population of vacuoles that were best visualized in glutaraldehyde-fixed conventional ultrathin plastic sections.

The lysosomal localization of the glycoproteins was also indicated by the typically perinuclear staining pattern observed by immunofluorescence microscopy, the selective labeling of phase-dense granules of cells from patients with mucolipidosis II (I-cell disease), and by the colocalization of the antigens to sites of acridine orange uptake, confirming their presence in acidic vacuoles (data not shown). This lysosomal specificity has made it possible to use the anti-hLAMP monoclonal antibodies to distinguish lysosomal from peroxisomal membranes (46).

Membrane Flow of hLAMP Glycoproteins During Phagocytosis - Evidence for vertical flow of hLAMP-positive vesicles to phagosomes was obtained from studies of the phagocytosis of opsonized sheep erythrocytes by U937 cells (Fig. 7). A marked accumulation of the anti-hLAMP antibodies was observed at the membrane surrounding phagocytosed erythrocytes. Many of the phagolysosomes contained quantities of antigen far in excess of the apparent number of antigenic sites present in individual LAMP-positive vacuoles, suggesting the incorporation of multiple hLAMP-positive membranes into the phagosomes. Some vacuoles containing sheep erythrocytes were unlabeled despite the presence of contiguous labeled lysosomes; these unlabeled vacuoles may represent the early prelysosomal stage of phagocytic uptake. Antibody did not bind to extracellular sheep erythrocytes, and there was no significant labeling of other cellular structures, including the plasma membrane. No labeling was observed when control serum or buffer replaced the specific antibody.

hLAMP Expression in the Plasma Membrane of Myelomonocytic Leukemia Cells - Although neither of the hLAMP antigens was found on the plasma membrane by electron microscopy, both molecules were readily detected on the surface of intact U937 cells by flow immunocytofluorimetry (see below), by indirect immunofluorescence microscopy, and by vectorial labeling with ^{125}I followed by immunoprecipitation (data not shown). The fraction of the total hLAMP-1 or hLAMP-2 antigen present on the surface of U937 cells was small; quantitative antibody binding studies showed that the amount of antigen detected on intact cells was ~5 % of that present in detergent extracts of cells or in cells treated with saponin to permit entry of antibody. Failure to detect the antigens on the plasma membrane by electron microscopy can be attributed to their relatively low concentration at this site, combined with the low efficiency (~15%) of immunogold labeling of the plasma membrane when frozen sections are used (47,48).

The concentration of hLAMP molecules present in the plasma membrane of U937 cells was markedly reduced in cells treated with the phorbol ester PMA, which induces differentiation of these myelomonocytic leukemia cells to macrophage-like cells (49,50) (Fig. 8). In the absence of PMA, U937 cells analyzed by flow cytofluorimetry showed a well-defined shift in fluorescence intensity when incubated with anti-hLAMP-1 or anti-hLAMP-2 monoclonal antibodies and fluoresceinated rabbit anti-mouse IgG, with ~50% of the cells moving out of the negative gate. The low intensity staining of these cells was similar to that seen after incubation with antibodies recognizing the myeloid differentiation antigen CD11b (Mac-1 α/CR3) (51) or the lymphocyte antigen CD18 (LFA-1 β) (Fig. 8). In U937 cells treated with PMA, the plasma membrane expression of hLAMP-1 and hLAMP-2 was reduced to background level. This decreased expression of the hLAMP antigens contrasted sharply with the differentiation-related increase in cell-surface CD11b (Mac-1 α) and CD18

(LFA-1 β) produced by PMA treatment (as previously described [50]) and with the reported increase in plasma membrane expression of a variety of cell receptors resulting from increased fusion of intracellular membrane pools with the cell surface (52).

The extent of hLAMP expression on the surface of U937 cells was not affected by the lysosomotropic agent methylamine HCl, which accumulates in lysosomes and other acidic intracellular compartments, producing an increase in intralysosomal pH and inhibition of vacuolar function (53). In repeated experiments, a range of concentrations of the drug did not appreciably alter the fluorescence intensity observed for the hLAMP antigens, in the presence or absence of PMA (Table IV). In contrast, approximately 90% of peripheral blood monocytes, which showed only background levels of plasma membrane hLAMP-1 and hLAMP-2 in the untreated state, became strongly positive at the cell surface for both antigens after treatment with low concentrations of methylamine HCl. This response of monocytes to methylamine was similar to the increase in cell-surface expression of LEP100 produced by treatment of chick embryo fibroblasts with chloroquine, another lysosomotropic agent (9). Based on these studies of LEP100, it has been suggested that a fraction of the lysosomal membrane molecules flows through the plasma membrane and that lysosomotropic agents, by perturbing the endocytotic mechanism, cause an accumulation of the lysosomal membrane glycoproteins on the cell surface. Our results provide evidence that the localization and movement of LAMP molecules differ from one cell type to another and that, at least in U937 cells, the equilibrium of membrane exchange between LAMP-positive vesicles and the plasma membrane is not detectably altered by lysosomotropic agents.

A variety of cells, including cultured fibroblasts, peripheral blood cells (T cells, B cells, and monocytes), and other hematopoietic cell lines showed little or no cell-surface antigen when analyzed by flow

immunocytofluorimetry (Table V). Only the myelomonocytic leukemia cell line HL-60 showed cell-surface antibody binding comparable to that of U937 cells. Antigen expression at the plasma membrane was reduced to background levels (data not shown) when the HL-60 cells were treated with 1.25% dimethyl sulfoxide, which induces differentiation of these cells to granulocytic precursors (54).

DISCUSSION

Characterization of two glycoproteins of human cells that are localized primarily in the lysosomal membrane, hLAMP-1 and hLAMP-2, has shown these molecules to be highly similar in biochemical properties to the previously described mLAMP-1 and mLAMP-2 of mouse cells (3,5). This similarity was confirmed by amino acid sequence analysis, which showed approximately 50% sequence identity between the NH_2-terminal residues of the corresponding molecules. The human and mouse proteins therefore appear to be homologous molecules of different species or closely related proteins that have arisen by gene duplication. Sequence data and antigenic cross-reactivity indicate that the >100-kDa lysosomal membrane glycoproteins of rat cells (7) are also similar to LAMP-1 or LAMP-2 (unpublished data). In addition, the protein sequence deduced from a cDNA clone of chicken LEP100 (55) has ~40% identity with those of the mouse and human LAMP-1 molecules (14). The LAMP-1 and LAMP-2 classes of molecules thus appear to be immunodominant antigens among the lysosomal membrane proteins (due to their high concentrations or antigenicity), or they may be more readily identified than other lysosomal membrane components by the immunochemical procedures used to select the monoclonal antibodies.

A possible role of LAMP antigens on the plasma membrane has been suggested by the observation that three cell-surface markers of differentiation or oncogenesis are highly similar or identical to the LAMP molecules. We have shown that Mac-3, originally described as a cell-surface differentiation antigen of macrophages (15), corresponds in antigenic and biochemical properties to mLAMP-2 (3). We have also observed that mLAMP-1 is very similar in amino acid sequence and other biochemical properties to gp130 (P2B), a 130-kDa glycoprotein of mouse tumor cells (14).[1] P2B is a major lectin-binding protein (leucoagglutinin) of the MDAY-2 lymphoid tumor

cell line and contains a large fraction (>50%) of the complex-type Asn-linked oligosaccharides of this cell.[1] An increase in ß1-6-linked branching of the Asn-linked oligosaccharides of P2B has been correlated with increased metastatic potential in certain tumor cell lines (13). Furthermore, our antisera against hLAMP-1 and hLAMP-2 have been used to precipitate purified polylactosaminoglycan-containing glycoproteins of human chronic myelogenous leukemia cells that are under investigation as onco-differentiation antigens, and the deduced protein sequence of the leukemia cell glycoprotein reactive with anti-hLAMP-1 is identical to hLAMP-1 in 23 amino acids near the NH_2-termini of the molecules (16,17). As expected, this human leukemia cell glycoprotein is also very similar to mLAMP-1: the total sequences of both proteins, as deduced from cDNA clones, are 66% identical (252 of 382 residues), with almost complete identity of the 35 amino acid residues of the putative membrane-spanning and cytoplasmic domains in the carboxyl-termini of the proteins (14).

The correlations between LAMP molecules and cell-surface onco-differentiation markers suggest that both the antigenic determinants defining these markers and the oligosaccharide moieties that may affect the phenotypic properties of certain tumor cells are borne by glycoproteins whose predominant site of expression is the lysosomal membrane. The structures of mLAMP-1 and hLAMP-1 (as deduced from sequencing of cDNA clones) (14,16) indicate that 90% of each molecule, beginning at the NH_2-terminus and including all 18 to 20 glycosylation sites, resides in the lumen of the lysosome (56). The intralysosomal domain is followed in both proteins by a hydrophobic membrane-spanning region of 24 amino acids and a short carboxyl-terminal cytoplasmic domain. Fusion of the lysosomal membrane with the plasma membrane would place the highly glycosylated NH_2-terminal domain in the extracellular compartment. The cell type-specific

expression of the LAMP antigens in the plasma membrane that we have observed could therefore result from a selective movement of a fraction of the molecules to the cell surface or from alterations in the oligosaccharide composition of the molecules, or both. These possibilities are consistent with the differences in the amount of hLAMP molecules present in the plasma membrane of various human blood cells and with the reduction in cell-surface expression induced by treatment with differentiating agents. They are also consistent with the marked heterogeneity in oligosaccharide composition of the mature LAMP glycoproteins seen in different cell types (4) and the occurrence of β1-6 linkages and polylactosaminoglycan structures (16)[1] that appear to function as differentiation-specific antigenic epitopes in some cells (13,17).

The mechanisms influencing the changes in cell-surface concentration and oligosaccharide composition of the LAMP molecules may be relevant to a variety of cell processes. Protein glycosylation is altered in many differentiating and oncogenically transformed cells (57-60), and changes in NH_2-linked oligosaccharide composition are reported to affect cell adhesion, metastasis, and immune recognition (13,17,61-63). Although the specific relationships between the surface expression of lysosomal membrane glycoproteins and these processes are not defined at present, the identification of the LAMP glycoproteins as molecules corresponding to onco-differentiation antigens of both mouse and human cells suggests the need for a more extensive analysis of the presence and function of these glycoproteins on the surface of human cells.

TABLE IV

Flow cytofluorimetric analysis of hLAMP antigen expression on U937 cells and peripheral blood monocytes and the effects of PMA and methylamine HCl

The experiment was performed as described in Fig. 8, using the H4A3 (anti-hLAMP-1) and H4B4 (anti-hLAMP-2) monoclonal antibodies. Cell isolation and culture conditions are given in Experimental Procedures.

TABLE IV, con't.

Cells	Treatment[a]	Antigen Expression	
		hLAMP-1	hLAMP-2
		%[b]	
U937	None	56 ± 18[c]	25 ± 8[c]
U937	PMA (1.6×10^{-9} M)	10 ± 1[c]	8 ± 1[c]
U937	Methylamine HCl:		
	10 mM	35	26
	15 mM	40	19
	20 mM	53	39
	25 mM	62	43
U937	PMA (1.6×10^{-9} M) and Methylamine HCl:		
	10 mM	15	14
	15 mM	19	13
	20 mM	19	15
	25 mM	18	14
Monocytes	None	7 ± 3[d]	4 ± 3[d]
Monocytes	Methylamine HCl:		
	10 mM	91	85
	15 mM	94	91

[a] Cells were treated with PMA as described in Fig. 8. For methylamine HCl treatment, U937 cells or normal monocytes were washed with C-RPMI and then suspended at 10^6 cells/ml in C-RPMI. Ten ml of cell suspensions containing varying concentrations of methylamine HCl were incubated overnight in 5% CO_2 at 37°C. The cells were then washed three times with

C-RPMI, and dead cells and debris were removed by Ficoll-Hypaque density gradient centrifugation. Viable cells were suspended at 2 X 10^7 cells/ml in C-RPMI and then analyzed as described in Fig. 8.

[b] Antigen expression is given as the % of total cells that move above a negative gate established at the approximate upper limit of fluorescence in the absence of primary antibody, minus the background reactivity observed with a non-relevant control IgG of the same isotype. Percentages were corrected to the nearest whole number.

[c] Mean and standard deviation of four experiments.

[d] Mean and standard deviation of two experiments.

TABLE V

Flow cytofluorimetric analysis of hLAMP antigen expression on various cell types

Cells were reacted with H4A3 (anti-hLAMP-1) or H4B4 (anti-hLAMP-2) as described in Table IV. Cell isolation and culture conditions are given in Experimental Procedures.

Cell Type	Antigen Expression	
	hLAMP-1	hLAMP-2
	(%)	
PBMC	4	2
Monocytes	4	1
T cells	1	1
B cells and monocytes	6	3
U937 (myelomonocytic leukemia cells)	76	27
HL-60 (myelomonocytic leukemia cells)	44	11
HSB-2 (T cell lymphoma)	16	2
Molt-4 (T cell lymphoma)	3	5
K562 (erythroleukemia cells)	4	3
JR (human EBV transformed B cells)	6	4

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FOOTNOTES

1. Laferte, S. and Dennis, J.W., 1987. Purification of two glycoproteins expressing β1-6 branched Asn-linked oligosaccharides commonly associated with the malignant phenotype (manuscript submitted).

2. The abbreviations used are: PMA, 12-O-tetradecanoylphorbol-13-acetate; FCS, fetal calf serum; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; NHS, normal human serum; C-RPMI, complete RPMI 1640 medium; PMSF, phenylmethylsulfonyl fluoride.

3. Portions of this paper (including "Experimental Procedures," part of "Results," Tables I-III, and Figs. 1-4) are presented in the miniprint at the end of the paper.

4. J.W. Chen and J.T. August, unpublished data.

FIGURE LEGENDS

Figure 5. Transmission electron microscopy of frozen thin sections of human blood monocytes, illustrating the immunogold localization of H4B4. Heparinized normal human leukocytes were sedimented in dextran and washed in Hank's balanced salt solution (Gibco). The cells were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C or in 4% paraformaldehyde in the same buffer for 4 to 6 h at 4°C. They were then washed in the same buffer containing 5% (w/v) sucrose and stained for peroxidase activity (40) to identify peroxidase-positive granules (41). The cells were then pelleted, embedded in 2.1 M sucrose, frozen, and stored in liquid N_2. Frozen thin sections were cut on a Reichert Ultracut E microtome (42). The sections were treated with purified H4B4 IgG primary antibody followed by goat anti-mouse Ig-gold (GAM-5), 1:50 dilution (Janssen Pharmaceutica, Beerse, Belgium); nonimmune purified mouse IgG was used as a control. Grids were stained with uranyl acetate and embedded in methylcellulose as described by Tokuyasu (43) and modified by Griffiths et al. (44). Note the presence of gold label in 3 clear vacuoles of various sizes (V1, V2, V3). V1 is closely associated with one of the stacks of Golgi cisternae that do not show detectable levels of label. Several of the peroxidase-positive storage granules (g) are evident. The inset is a higher magnification of the area containing V2 and a peroxidase-positive granule. N, nucleus; m, mitochondria; cv, coated vesicles; Gc, Golgi complex. Magnification: X 46,000; inset, X 85,000.

Figure 6. Transmission electron microscopy of a frozen thin section of HL-60 cells labeled with H4B4. Cells were prepared as described in Fig. 5, except that they were fixed in 4% paraformaldehyde and processed without the peroxidase procedure. The plasma membrane (pm) has no label, whereas

abundant label is found in adjacent vacuoles (v), which contain some membranes. A low level of gold labeling is seen in tubular structures (upper right hand corner). Inset: Depiction of another organelle which is frequently labeled in HL-60 cells. These vacuoles contain whorls of membranes (arrows) and are believed to be autophagic vacuoles. N, nucleus, Magnification: X 70,000; inset, X 73,000.

Figure 7. Immunogold labeling of hLAMP-1 and hLAMP-2 in ultrathin frozen sections of U937 cells incubated with sheep erythrocytes. U937 cells were incubated for 90 min at 37°C with sheep erythrocytes coated with rabbit anti-sheep red blood cell antibody. The cells were then washed twice with Hank's solution, fixed in 2% paraformaldehyde for 48 h at 4°C, and embedded in 10% gelatin. Specimen blocks (1 x 3 mm) were either immediately processed or stored for 2 weeks at 4°C. Specimen blocks were infiltrated with 2.3 M sucrose for 3 h and frozen in liquid propane cooled by a liquid N_2 bath. Sections of approximately 100 nm were cut with a DuPont-Sorvall ultramicrotome at -110°C and transferred with 2 M sucrose to formvar-coated nickel grids. The grids were incubated in the following reagents (all diluted in PBS), with two to three PBS washes between incubations: 5% NHS with 5% normal goat serum, 30 min; monoclonal antibody H4A3 (A) or H4B4 (B) (10 ug/ml), 1 h; and goat anti-mouse Ig coupled to gold particles of 40nm (A) or 15nm (B) average diameter (Janssen Pharmaceutica, Beerse, Belgium), 90 min. The grids were immersed in 3% glutaraldehyde in 0.1 M cacodylate buffer for 30 min, rinsed and incubated in 1% osmium tetroxide in 0.1 M cacodylate buffer for 15 min at 4°C. The sections were then embedded in ice-cold 0.5% methylcellulose containing 0.3% uranyl acetate. The dried sections were examined with a JEOL CX-1 transmission electron microscope at an accelerating voltage of 60 kV. Both H4A3 (hLAMP-1) and H4B4 (hLAMP-2)

antigens were exclusively localized in vacuoles (arrowheads, inset) that resembled the typical lysosomal dense bodies seen in the glutaraldehyde-fixed conventional ultrathin plastic sections and in secondary lysosomes (arrows) marked by the presence of phagocytosed sheep red blood cells. Gold particles were not detected on other cellular structures, such as the plasma membrane (pm), nucleus (Nu), endoplasmic reticulum, Golgi complex, or mitochondria. Some phagosomes (plate A, double arrow) were devoid of gold particles and may represent early prelysosomal phagosomes. Magnification: A, X 13,250 (inset, X 25,000); B, X 25,250 (inset, X 26,500).

Figure 8. Flow cytofluorimetric analysis of the expression of hLAMP and other cell-surface proteins on the plasma membrane of U937 cells and the effect of PMA. Flow cytofluorimetry was carried out as described under Experimental Procedures. U937 cells were induced to differentiate into a monocyte pathway by incubation for 24 h at 37°C in C-RPMI with 10% FCS and 1.6×10^{-9} M PMA. U937 cells (1×10^6 in 50 µl of FACS medium) were incubated with 50 µl of a 1/50 dilution of either H5G11 (anti-hLAMP-1), H4B4 (anti-hLAMP-2), H4C2 (anti-Mac-1 α), or H52 (anti-LFA-1 β) ascites fluid and then incubated with 2.5 µg of fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody in 50 µl FACS medium. The fluorescence signal without primary antibody (AUTO) was identical to that obtained with non-relevant control antibody. The arrowhead indicates the position of the negative fluorescence gate, and the % values represent the fraction of cells with a fluorescence intensity greater than the value of the negative gate.

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