The lysosomal membrane glycoproteins Lamp-1 and Lamp-2 are present in mobilizable organelles, but are absent from the azurophil granules of human neutrophils

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The subcellular localization of two members of a highly glycosylated protein group present in lysosomal membranes in most cells, the lysosome-associated membrane proteins 1 and 2 (Lamp-1 and Lamp-2), was examined in human neutrophil granulocytes. Antibodies that were raised against purified Lamp-1 and Lamp-2 gave a distinct granular staining of the cytoplasm upon immunostaining of neutrophils. Subcellular fractionation was used to separate the azurophil and specific granules from a light-membrane fraction containing plasma membranes and secretory vesicles, and Western blotting was used to determine

the presence of the Lamps in these fractions. The results show that Lamp-1 and Lamp-2 are present in the specific-granule-enriched fraction and in the light-membrane fraction, but not in the azurophil granules. Separation of secretory vesicles from plasma membranes disclosed that the light-membrane Lamps were present primarily in the secretory-vesicle-enriched fraction. During phagocytosis both Lamp-1 and Lamp-2 became markedly concentrated around the ingested particle and they both appear on the cell surface when the secretory organelles are mobilized.

INTRODUCTION

The neutrophil granulocyte is of great importance in eliminating invading micro-organisms [1]. Recognition of foreign material, as well as killing of microbes, is dependent on secretion of granule constituents and phagolysosome formation. The neutrophil contains classical lysosomes (termed primary or azurophil granules) as well as at least two other types of granule structures (secondary or specific granules and secretory vesicles) which store a variety of bactericidal products and mobilizable cell-surface receptors [2]. The antibacterial activity of neutrophils thus relies on different subcellular organelles to take part in the fusion processes in these cells. Such fusion processes are the formation of phagolysosomes by fusion of the phagocytic vesicles and the granules (azurophil and specific), and exocytosis occurring through fusion between the plasma membrane and intracellular vesicles. The sequential appearance of the different types of neutrophil granules is closely linked to different stages of myeloid differentiation [3,4]. The biosynthesis of azurophil granules is restricted to the promyelocytic stage, that of specific granules to the myelocytic stage, and the secretory vesicles appear first in band cells and segmented cells [5]. The azurophil granules are analogous to lysosomes [6,7] but in addition they contain constituents such as neutral proteases and myeloperoxidase (MPO) [8,9]. A class of highly glycosylated proteins has been identified as a major glycoprotein constituent in lysosomal membranes in many cells [10,11]. Within this class of lysosomal membrane glycoproteins, two members with similar structures, called lysosome-associated membrane protein-1 and -2 (Lamp-1 and Lamp-2) in human and mouse cells, have been characterized (for reviews see refs. [10] and [12]). The major portion of the Lamp molecules faces the luminal side of the organelle and carries several N-linked, as well as O-linked,

carbohydrate chains. In various cells, Lamp-1 and Lamp-2 are the major carriers of polylactosaminoglycans [11,13]. These unique high-molecular-mass carbohydrates can carry certain specific determinants, such as ABO and I/i blood groups [14]. In granulocytes and monocytes, the polylactosamines are enriched for Lewis^x and sialyl Lewis^x determinants [15], which serve as ligands for selectins (for a recent review see [16]). By immunocytochemistry, Lamp-1 and Lamp-2 (and their homologues in other species) have also been detected in small amounts in various vesicles in the endocytic pathway and at the cell surface. In fact, it seems that the distribution of Lamps may be cell specific [17–19]. The specific sorting events responsible for the movement of Lamps to their final destination also seems to differ between cells. In certain cells, the major transport pathway to the lysosome is intracellular, most likely mediated by a sorting step in the *trans*-Golgi network, whereas in other cells the transport seems to occur by transit through the cell surface [20–23]. In both cases the sorting is dependent on a tyrosine-containing sequence in the short cytoplasmic tails of Lamp-1 and Lamp-2 [21,23–25]. Since neutrophil granulocytes are highly specialized cells containing unique granules and vesicles, it is of interest to investigate the distribution of Lamps in these cells. We found that the Lamps are not primarily localized in the lysosomes of neutrophils, but are present in the secretory organelles of these cells. This finding may have implications for a role of Lamps during the inflammatory response.

MATERIALS AND METHODS

Reagents

Percoll and Ficoll-Hypaque were from Pharmacia, Uppsala, Sweden. ATP, EGTA, Nitro-Blue Tetrazolium (NBT), 5-bromo-

Abbreviations used: BB6, monoclonal antibody directed against Lamp-1; BCIP, 5-bromo-4-chloro-3-indolylphosphate; CD3, monoclonal antibody directed against Lamp-2; CR3, complement receptor 3; FITC, fluorescein isothiocyanate; FMLP, formylmethionyl-leucyl-phenylalanine; HLA, histocompatibility locus antigen; KRG, Krebs–Ringer phosphate buffer; Lamp, lysosome-associated membrane protein; MPO, myeloperoxidase; NBT, Nitro-Blue Tetrazolium; PVDF, poly(vinylidene difluoride).

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Figure 1 Co-localization of Lamp-1 and Lamp-2 in intact neutrophils

Indirect immunofluorescence detection of Lamp-1 (polyclonal antibody) and Lamp-2 (monoclonal antibody) in human neutrophils using confocal laser scanning microscopy. Confocal horizontal optical sections ($0.5\ \mu\text{m}$) of the cells were analysed, and the fluorescence profile of one such section is shown for Lamp-1 (red fluorescence; upper left) and Lamp-2 (green fluorescence; upper right) respectively. An overlay technique was used to visualize areas of co-localization of Lamp-1 and Lamp-2. These areas are the yellow areas in the overlay picture.

4-chloro-3-indolylphosphate (BCIP) and *p*-nitrophenyl phosphate were products from Sigma, IL, U.S.A. The poly(vinylidene difluoride) (PVDF) membrane was from Millipore, Bedford, MA, U.S.A. Antibodies against lactoferrin, as well as pig anti-(rabbit or mouse Ig) were from Dakopatts, Copenhagen, Denmark and [⁵⁷Co]vitamin B₁₂ was from Amersham Laboratories, Amersham, Bucks., U.K.

Isolation of cells

Human polymorphonuclear leucocytes (neutrophil granulocytes) were isolated from buffy coats as described by Bøyum [26]. The purified neutrophils were washed twice in Krebs–Ringer medium (120 mM NaCl, 4.5 mM KCl, 1.2 mM MgSO₄, 1.0 mM CaCl₂, 1.7 mM KH₂PO₄, 8.3 mM Na₂HPO₄, 10 mM glucose, pH 7.3).

Antibodies

The monoclonal antibodies against Lamp-1 (BB6) and Lamp-2 (CD3) were the same as described previously [13,27]. In order to obtain antibodies with improved (different) characteristics with respect to, for example, immunostaining and immunoprecipitating properties, polyclonal rabbit antibodies were raised against human Lamp-1 and Lamp-2 purified from chronic myelogenous leukaemia (CML) cells. Detergent-solubilized cells were passed over a column of wheat germ-agglutinin–agarose, and sugar-eluted glycoproteins were applied to columns of immobilized monoclonal antibodies against Lamp-1 (BB6) and Lamp-2 (CD3), connected in series [28], and the Lamps were finally purified by preparative SDS/PAGE [13]. Special care was taken to avoid contamination from a cell-surface glycoprotein of 200 kDa, which was found to co-precipitate with the Lamps in small amounts, by use of previously prepared antisera [20]. The purified proteins were used for immunization of two rabbits, and the sera obtained are designated #931 (anti-Lamp-1) and #932 (anti-Lamp-2). Analysis by immunoprecipitation of [³⁵S]methionine/[³⁵S]cysteine-labelled HL-60 cells and Western blotting showed that the antisera were monospecific for Lamp-1 and Lamp-2 respectively. ELISA tests showed no cross-reactivity between Lamp-1 and Lamp-2. Peptide antibodies were raised against the large (gp91^{phox}; residues 546–558) and small (p22^{phox}; residues 162–174) subunits of the neutrophil cytochrome *b* as described by Quinn et al. [29] and Jesaitis et al. [30].

Fluorescence microscopy

Neutrophils were allowed to adhere (5 min at 37 °C) to microscope slides coated with human serum albumin. The cells were fixed in 4 % ice-cold paraformaldehyde, permeabilized with 0.4 % Triton X-100 in PBS and incubated with the antibodies as described [31]. The cells were examined in an ordinary fluorescence microscope (Axiovert, Zeiss) or in a confocal laser-scanning microscope (Zeiss).

Subcellular fractionation

Neutrophils (5 × 10⁸ cells) were resuspended in relaxation buffer (10 ml; 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 10 mM Pipes, 1 mM ATP(Na)₂, 0.5 mM PMSF, pH 7.4), disrupted in a nitrogen bomb [375 p.s.i. (2500 kPa); Parr Instruments, Moline, IL, U.S.A.] and the post-nuclear supernatants were layered on top of two-step (1.05 and 1.12 g/ml) Percoll density gradients and fractionated [32,33].

In order to separate secretory vesicles from plasma membranes, the postnuclear supernatants were mixed with an equal volume

of the heavy Percoll solution and this mixture (14 ml) was underlaid with 14 ml of a light (1.04 g/ml) solution followed by the heavy Percoll solution (5 ml) applied to the bottom of the centrifugation tubes, and relaxation buffer (5 ml) was applied on top. All gradients were centrifuged at 17 500 rev./min for 30 min (4 °C) using a fixed-angle Beckman JA-20 rotor. Gradient fractions (1.5 ml) were collected successively from the bottom of the gradients.

SDS/PAGE and Western blotting

SDS/PAGE was performed essentially according to Laemmli [34] using homogeneous gels of 10 % polyacrylamide. The gradient fractions were applied to the gels in volumes corresponding to the fractionated content of 2.5 × 10⁵ cells. The gels were electrophoretically blotted on to PVDF membranes [33]. The membranes were blocked and incubated with antibodies as described [33]. The blots were developed either with substrate buffer containing NBT (0.3 mg/ml) and BCIP (0.15 mg/ml) or in an enhanced chemiluminescence system (ECL-Western blotting, Amersham Laboratories). Densitometry was performed with an Ultrascan XL laser densitometer (Pharmacia-LKB, Sweden).

Quantification of granule markers

The Percoll fractions were assayed for MPO, vitamin B₁₂-binding protein and alkaline phosphatase as described [35]. The HLA class-I antigen was determined by a mixed ELISA assay [2] using a catching antibody directed against β₂-microglobulin and a detecting antibody that recognizes the heavy chain of HLA. The molecules analysed constitute markers for azurophil granules (MPO), specific granules (vitamin B₁₂-binding protein) and secretory vesicles/plasma membranes (alkaline phosphatase [2]) respectively. Alkaline phosphatase was assayed in the absence (non-latent pool) or presence (total pool) of Triton X-100 using *p*-nitrophenyl phosphate [36] as substrate.

Surface up-regulation of complement receptor 3 (CR3) and Lamps as determined by immunostaining and flow cytometry

Cells were either kept at 4 °C (resting cells) or stimulated for 10 min at 37 °C with formylmethionyl-leucyl-phenylalanine (FMLP; 10⁻⁷ M final concentration) in the absence or presence of cytochalasin B (5 μg/ml final concentration). The expression of Lamp-1 and Lamp-2 on the cell surface was analysed by adding 10 μl of mouse monoclonal antibodies (BB6 and CD3 for Lamp-1 and Lamp-2 respectively) to cell pellets (around 10⁶ cells). The cells were then incubated at 4 °C for 30 min, washed twice and incubated with a fluorescein isothiocyanate (FITC)-labelled secondary antibody (goat anti-mouse IgG, Dakopatts, Denmark), washed and examined in a flow cytometer (FACS Scan; Becton Dickinson, Mountain View, CA, U.S.A.). Phycoerythrin-conjugated monoclonal antibodies against CD11b (Becton-Dickinson) were used to determine mobilization of CR3 [37].

Phagocytosis

Yeast particles (*Saccharomyces cerevisiae*, bakers' yeast) were opsonized by mixing 1 × 10⁸ heat-killed yeast particles with normal human serum [25 % (v/v) in Krebs–Ringer phosphate buffer (KRG)]. The mixture was incubated for 30 min at 37 °C, and the particles were then washed twice and resuspended in

KRG to a concentration of 10^7 /ml. The yeast particles were added to microscope slides with surface adherent neutrophils and these cells were allowed to phagocytose the yeast particles (37°C for 15 min in a moist chamber). The cells were fixed, permeabilized, labelled with antibodies and mounted as described above (see the section Fluorescence microscopy). The primary antibodies were directed either against Lamps (rabbit antibodies) or lactoferrin (mouse antibodies) and the secondary antibodies were labelled with FITC [goat anti-(mouse IgG)] or tetramethylrhodamine-isothiocyanate [pig anti-(rabbit IgG)].

RESULTS

Localization of Lamps in intact neutrophils

To localize Lamps morphologically in neutrophils, we used immunofluorescence microscopy on cells stained with rabbit anti-Lamp antibodies raised against purified Lamp-1 and Lamp-2. A distinct granular staining of the cytoplasm was observed when the localization of Lamp-1 and Lamp-2 in neutrophils was visualized under the fluorescence microscope (results not shown). By use of an overlay technique and confocal microscopy we found that the two Lamps are co-localized to a large extent on the same granule structures. Co-localization was determined using the polyclonal (rabbit) antibodies directed against Lamp-1 and the monoclonal (mouse) antibodies directed against Lamp-2 (Figure 1).

Localization of Lamps in neutrophil subcellular granules

By use of the rabbit antibodies raised against Lamp-1 and Lamp-2 we could detect a major 120 kDa band in Western blots of human neutrophils. To prove that a cell component is localized to a particular organelle, a biochemical approach has to be added to the morphological observation. In order to determine

Figure 2 Subcellular fractionation of neutrophils

A post-nuclear supernatant of disrupted neutrophils was fractionated on a discontinuous two-layer Percoll density gradient. Three bands were visible, which were denoted α , β and γ in order of decreasing density. Cytosol components were located in the upper portion of the gradient (S_2). Fractions were analysed for MPO (a marker for azurophil granules; $\square$), vitamin B_{12} -binding protein (marker for peroxidase-negative specific granules; $\blacklozenge$) and alkaline phosphatase (marker for plasma membrane/secretory vesicles; $\blacksquare$). Abscissa: fraction number; ordinate: amount of marker (arbitrary units).

Figure 3 Identification of Lamp-1 in subcellular fractions

A post-nuclear supernatant of disrupted neutrophils was fractionated on a discontinuous two-layer Percoll density gradient. The fractions (1.5 ml) were collected from the bottom of the tubes and assayed under non-reducing conditions in 10% (w/v) polyacrylamide gels which were immunoblotted using a polyclonal antiserum raised against Lamp-1 (a). The relative amount of Lamp-1 in the different fractions was determined in a laser scanning densitometer (b). Abscissa: fraction number (the fractions 1–4 and 18–22 were pooled in order to allow all samples to be analysed in the same gel/blotting membrane; no, or very low, binding was obtained to any of these fractions when they were investigated separately); ordinate: molecular mass (a) and absorbance (arbitrary units; b).

the subcellular locations of the Lamps, neutrophils were disintegrated and fractionated on discontinuous Percoll gradients. In an ordinary Percoll gradient four subcellular fractions were easily identified; the cytosol (S_2), plasma membrane/secretory vesicles (the γ -band), specific granules (the β -band) and azurophil granules (the α -band) (Figure 2). Neither of the Lamps were found in the upper part of the gradient (S_2), corresponding to the cytosol of the cells (Figures 3a and 4a). This was to be expected since the Lamps are membrane proteins. The Lamps were found both in the light-membrane fraction (the γ -band) and in the fraction enriched in specific granules (the β -band; Figures 3a and 4a), but very small amounts were detected in the azurophil granule fraction (the α -band; Figures 3a and 4a). The densitometry profiles (Figures 3b and 4b) were used to estimate roughly the relationship between the amount of Lamps in the specific-granule-enriched fraction (β -band) and the light-membrane (γ -band) fraction respectively. The β fraction (defined as fractions 7–10; Figure 2) contained around 40% of both Lamp-1 and Lamp-2 and roughly the same amounts of the Lamps were

Figure 4 Identification of Lamp-2 in subcellular fractions

A post-nuclear supernatant of disrupted neutrophils was fractionated on a discontinuous two-layer Percoll density gradient. The fractions (1.5 ml) were collected from the bottom of the tubes and assayed under non-reducing conditions in 10% (w/v) polyacrylamide gels which were immunoblotted using a polyclonal antiserum raised against Lamp-2 (a). The relative amount of Lamp-2 in the different fractions was determined in a laser scanning densitometer (b). Abscissa: fraction number (the fractions 1–4 and 18–22 were pooled in order to allow all samples to be analysed in the same gel/blotting membrane; no, or very low, binding was obtained to these fractions when they were investigated separately); ordinate: molecular mass (a) and absorbance (arbitrary units; b) respectively.

found in the γ fraction (defined as fractions 13–16; Figure 2). Even if the cellular content of Golgi, endoplasmic reticulum and mitochondria co-purifies with the γ -band in Percoll gradients [38] these organelles are scarce in the neutrophil [39], indicating that the major constituents of the γ -band are secretory vesicles and plasma membrane vesicles.

Localization of Lamps in neutrophil secretory vesicles

In order to clarify further the identity of the Lamp-containing membranes in the γ -band, the post-nuclear material was fractionated on a modified Percoll gradient in which the light membranes were separated by floatation. The gradient disclosed two light-membrane bands, and based on the distribution profiles for the latent alkaline phosphatase activity (the secretory vesicle marker) and the HLA class-I antigen (the plasma membrane marker), we conclude that the modified gradient permits a clear separation of secretory vesicles (γ 2) from the plasma membranes (γ 1; Figure 5). The Lamps were found primarily in the fraction enriched for secretory vesicles (Figure 5).

Figure 5 Identification of Lamp-1 and Lamp-2 in secretory vesicles and plasma membranes

A post-nuclear supernatant of disrupted neutrophils was fractionated on a modified Percoll density gradient. Fractions were analysed for latent alkaline phosphatase (ALP) (marker for secretory vesicles; ■) calculated by subtraction of the ALP activity determined without any detergent from the total activity as determined in the presence of Triton X-100. The HLA class-1 antigen determined by a mixed ELISA (MELISA; [2]) technique was used as a marker for plasma membranes (◆). The positions in the gradient of the azurophil granules and the specific granules are indicated by one or two arrows respectively. The plasma membrane and secretory vesicle fractions were assayed under non-reducing conditions in 10% (w/v) polyacrylamide gels which were immunoblotted using polyclonal antisera raised against Lamp-1 (A) and Lamp-2 (B) respectively. Abscissa: fraction number; ordinate: amount of marker (arbitrary units).

Protease resistance of Lamps

We had anticipated that the Lamps would be found in the azurophilic granules, the granules that resemble lysosomes. Azurophil granules contain a wide range of hydrolytic enzymes (involved in microbicidal activity and degradation of foreign substances). It could therefore be argued, that the reason why no Lamps are detected in the azurophil fraction is that the proteolytic enzymes present in this granule fraction attack and degrade Lamps during the preparation. In order to investigate the sensitivity of the Lamps to such degradation, we separated the different membrane fractions of neutrophils, added a freeze-thawed preparation of azurophil granules to the Lamp-containing fractions and incubated these mixtures for 24 h. The membrane components (p22^{phox} and gp91^{phox}) of the neutrophil NADPH-oxidase present in the specific granules and in the plasma membrane/secretory vesicle fractions were degraded by this procedure (Figure 6). In contrast both Lamp-1 and Lamp-2 were found to be totally resistant to protease degradation (Figure 6), which strongly supports the conclusion that the membranes of the azurophil granules lack the Lamps *in situ*.

Mobilization of Lamps to the cell surface

Mobilization of neutrophil secretory vesicles can be determined indirectly by the exposure of membrane receptors stored in the secretory vesicles. In neutrophils the adhesion protein CR3 (Mac1) has been shown to be localized to a very low degree in the plasma membrane, the major fraction of these molecules is found in the secretory vesicles and the specific granules [40,41]. As

Figure 6 Protease resistance of Lamps

A post-nuclear supernatant of disrupted neutrophils was fractionated on a discontinuous two-layer Percoll density gradient. The α -band (azurophil granules) was freeze-thawed (four times) and added to the γ -band (plasma membranes/secretory vesicles). SDS/PAGE samples were prepared directly after mixing (lanes A) of the fractions and after a 24 h incubation period at 4 °C (lanes B). The samples were assayed under non-reducing conditions in 10% (w/v) polyacrylamide gels which were immunoblotted using polyclonal antisera raised against Lamp-1, Lamp-2, p22^{phox} (the small subunit of cytochrome *b*) and gp91^{phox} (the large subunit of cytochrome *b*).

illustrated by the surface exposure of CR3 (Figure 7), the secretory vesicles are mobilized to the plasma membrane following neutrophil interaction with the chemotactic peptide FMLP. Mobilization of the secretory vesicles was accompanied by an increased exposure of Lamp-2 (Table 1). Less than 5% of the specific-granule marker vitamin B₁₂-binding protein is released in response to FMLP, but if neutrophil actin assembly is inhibited by cytochalasin B the chemoattractant mobilizes almost completely the specific granules also (results not shown). This mobilization is associated with exposure of more CR3 molecules (Figure 7; Table 1) and, in agreement with the localization of the Lamps to the specific granules, a further increased exposure of both Lamp-1 and Lamp-2 was obtained on the surface of FMLP/cytochalasin B-treated cells (Table 1). These experiments

Figure 7 Mobilization of CR3

Surface up-regulation of CR3 on human neutrophils, expressed as mean fluorescence intensity of cells labelled with monoclonal antibodies against CD11b. Cells were either kept at 4 °C (resting cells) or stimulated at 37 °C with FMLP (10⁻⁷ M final concentration) in the absence or presence of cytochalasin B (5 µg/ml final concentration). Abscissa: fluorescence intensity (arbitrary units); ordinate: number of neutrophils.

Table 1 Surface up-regulation of CR3 (CD11b/CD18), Lamp-1 and Lamp-2 on human neutrophils, expressed as mean fluorescence intensity of cells labelled with monoclonal antibodies against the three antigens

Cells were either kept at 4 °C (resting cells) or stimulated at 37 °C with FMLP (10⁻⁷ M final concentration) in the absence or presence of cytochalasin B (5 µg/ml final concentration). Results are mean ± S.E.M. of data from 15 experiments.

	Surface expression		
	CR3	Lamp-1	Lamp-2
Resting cells	315 ± 45	42 ± 6	106 ± 21
FMLP-activated cells (no cytochalasin B present)	1086 ± 120**	56 ± 7*	154 ± 29*
FMLP-activated cells (activated in the presence of cytochalasin B)	1703 ± 184**	117 ± 20**	231 ± 37**

* $P < 0.05$; ** $P < 0.01$; t -test for paired samples.

were performed using earlier described monoclonal antibodies directed towards Lamp-1 (BB6) and Lamp-2 (CD3) respectively. We interpret the lack of specific cell-surface binding with our rabbit antisera (#931 and #932) as being due to the fact that epitopes for these antibodies are hidden on Lamps exposed on the cell surface.

Distribution of Lamps in phagocytosing neutrophils

After ingestion of a yeast particle, the ring-like enrichment of the specific-granule-marker lactoferrin (Figure 8) associated with the prey marks the formation of a phagolysosome. Similar to the pattern observed with lactoferrin, the fluorescence pattern of Lamps changed from an even granular cytoplasmic staining to a ring-like enrichment around the phagosome after ingestion of yeast particles (results shown for Lamp-2 in Figure 8).

DISCUSSION

Human neutrophils contain numerous intracellular granules [2,3,5] and it is customary to classify the two major granule types as peroxidase-positive (azurophil or primary granules) and peroxidase-negative (specific or secondary granules) granules. The azurophil granules serve primarily an intracellular role, featuring the characteristics of classical lysosomes. Neither the specific granules nor the newly described secretory vesicles are classical lysosomes; they resemble storage granules present in secretory organs (for reviews see [2] and [5]). The Lamps belong to a class of proteins present in lysosomal membranes of many cell types. We expected to find the Lamp glycoproteins in the membrane of the azurophil granules. To our surprise we did not find the Lamps in these granules, but found that Lamp-1 as well as Lamp-2 were present in the specific-granule fraction and in the light-membrane fraction enriched for secretory vesicles. The sorting determinant of the Lamps resides in a short C-terminal tail (10–11 amino acids) which is exposed on the cytoplasmic side of the membrane. A tyrosine-containing four-amino-acid motif is located at the very end of the peptide chain [12]. It has been suggested that the lysosomal sorting specificity of the tyrosine motif is due to an adjacent glycine molecule and/or the proximity of the motif to the membrane [21,25]. This suggestion is based partly on experimental evidence and partly on the fact that other lysosomal membrane proteins, e.g. CD63,

Figure 8 Phagosomal enrichment of lactoferrin and Lamp-2

Phase-contrast micrograph of a phagocytosing neutrophil (a), and distribution of lactoferrin (b) and Lamp-2 (c). The yeast particle that was phagocytosed is indicated by an arrow, and double immunostaining was performed to visualize the distribution of the two molecules. Lamp-1 and lactoferrin is enriched in the periphery of the phagosome, but some out-of-focus fluorescence is also seen in the rest of the phagosome. The fluorescence micrographs are magnified 1.25 times compared with the phase-contrast micrograph.

have exactly these features in the cytoplasmic tail [42]. CD63 and the Lamps are therefore considered to follow the same intracellular pathway and to have the same subcellular distribution. Hence it is of interest that CD63 has been shown to be a constituent of the azurophil-granule membrane in neutrophils [43]. The molecular background to this difference in subcellular distribution between CD63 and the Lamps is either that some additional signalling part of the molecules, in addition to the cytoplasmic tail, is of importance for the targeting process, or that CD63 and the Lamps are produced during different stages of cell differentiation. Further studies on neutrophil precursor cells are necessary to determine the state of maturation at which CD63 and the Lamps appear. Our finding that the Lamps are absent in the azurophil granules is in agreement with results recently presented by Cieutat et al. [44] using an immunoelectron microscopy technique. They show that the Lamps are absent from all granule structures (including the azurophil granules) but present in non-identified vesicular membrane

structures. We show that Lamp-1 as well as Lamp-2 is present in the membranes of an organelle found in the β -band of the Percoll gradient (enriched for specific granules) and the organelle resembles the specific granules in that it is redistributed to the cell surface during secretion and to the phagosomal area during phagocytosis (Figure 8). There are at least three different subpopulations of the peroxidase-negative specific granules [45], and the fact that the Lamps are peaking one fraction higher in the β -band than the vitamin B₁₂-binding protein, indicates that the Lamps are present mainly in one of the subpopulations of the specific granules. The marker for the gelatinase-containing granule has been shown to have a similar distribution [45], making this granule subset a good candidate. It could, however, not be excluded that the Lamps are stored in a not yet identified granule subpopulation that resembles the other granule subsets with respect to mobilization. The Lamps are also found in the light-membrane (γ) fraction containing secretory vesicles. In order to separate the secretory vesicles from the plasma membranes and examine this purified organelle for the presence of the Lamps, we developed a new, modified floatation gradient. Using this gradient technique we obtained a clear separation of the HLA class-I antigen (a first-class plasma membrane marker; [2]) and the latent alkaline phosphatase activity (a secretory vesicle marker; [5]). FMLP stimulation results in a nearly selective and complete exocytosis of secretory vesicles [46,47]. Since FMLP stimulation of granulocytes is associated with a mobilization of Lamps to the cell surface, our suggestion that the Lamps are present also in the membranes of the secretory vesicles gains further support.

Taken together, the data suggest that Lamp molecules are present both in a peroxidase-negative specific granule and the secretory vesicle of human neutrophils. Despite the fact that both types of Lamp-containing organelles behave like regulated storage granules in being actively secreted upon stimulation, there are no similarities between the two vesicles with respect to the route of formation. Specific granules are formed during the myelocyte stage by the pathway defined for other secretory organelles (rough endoplasmic reticulum $\rightarrow$ Golgi complex $\rightarrow$ granules; [4]), whereas the secretory vesicles are formed at a later stage by an endocytic process [5]. This suggests that the Lamps are transported to the storage granules by two alternative pathways, which finds support in experiments performed with HL-60 cells in which the majority of Lamp-1 is directly transported to storage organelles; a minor part of Lamp-1 is transported to the cell surface where it is internalized through an endocytic process [20]. The HL-60 cells share many properties with normal neutrophils, but it should be pointed out that the HL-60 cells are granule-deficient (lacking the specific granules [48,49]; and probably also the secretory vesicles (C. Dahlgren, unpublished work)), and therefore supportive results obtained with HL-60 cells should be interpreted cautiously. The fact that the Lamps are localized partly in secretory organelles in neutrophils, suggests that these glycoproteins may have potentially important roles on the surface of activated/extravasating phagocytes. In this context, it is intriguing that the Lamps carry polylactosaminoglycans, structures which are known to contain selectin ligands in granulocytes. Furthermore, since recognition of micro-organisms most often involves microbial lectins that bind to neutrophil glycoconjugate receptors [50], the Lamps could be part of a mobilizable system designed to recognize and eliminate microbial intruders.

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