

LAMP-1 and LAMP-2, but not LAMP-3, Are Reliable Markers for Activation-Induced Secretion of Human Mast Cells

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Background: Mast cells are resident tissue cells that induce anaphylactic reactions by rapidly releasing mediators after antigen-mediated cross-linking of immunoglobulin E receptors. In the similarly active peripheral blood basophilic leukocyte, lysosome-associated membrane protein 3 (LAMP-3; CD63) has been described as an activation marker, but LAMPs have not been investigated in normal tissue mast cells.

Methods: Intra- and extracellular expressions of LAMP-1 (CD107a), LAMP-2 (CD107b), and LAMP-3 (CD63) were analysed by flow cytometry, immunocytochemistry, and functional assays in unstimulated and stimulated leukemic human mast cell line 1 (HMC-1) and skin mast cells.

Results: On flow cytometry, all mast cells expressed LAMP-3 at their cell membranes, whereas LAMP-1 and LAMP-2 were barely detectable (HMC-1 cells) or ex-

pressed at low levels (<10% of skin mast cells). After fixation and permeabilisation, high intracellular levels of all three LAMPs were noted in both cell types. After stimulation, a rapid translocation of intracellular LAMPs to the cell membrane, with an associated release of histamine, leukotriene C₄ and prostaglandin D₂, was ascertained in skin mast cells only.

Conclusion: These results show that LAMP-1 and LAMP-2 are activation markers for normal mast cells. The lack of LAMP translocation after activation of leukemic mast cells may be related to maturation or malignancy-associated defects of these cells. © 2004 Wiley-Liss, Inc.

Key terms: lysosome-associated membrane proteins; CD63; CD107; mast cells; mast cell activation; human mast cell line 1; lysosomes; histamine release

The lysosome-associated membrane proteins (LAMPs) LAMP-1 (CD107a), LAMP-2 (CD107b), and LAMP-3 (CD63) are transmembrane lysosomal glycoproteins that share the common Gly-Tyr motif as a lysosomal targeting signal (1). Structurally, the molecules differ because LAMP-1 and LAMP-2 contain a type 1 transmembrane and LAMP-3 contains a tetraspanin (transmembrane 4 superfamily) domain (2,3). Although the function of LAMPs is unknown, LAMP-3 is a reliable activation marker for basophils (4–7) because, after stimulation of cells via the FcεRI receptor, LAMP-3 is translocated from its intracellular localisation to the plasma membrane (4,8). This process can be quantified by flow cytometry (1). The translocation of LAMP-3 has been shown to be accompanied by the release of histamine and leukotriene C₄ (LTC₄) in these cells (4,8). In peripheral blood leukocytes, expressions of LAMP-1 and LAMP-2 were identified as activation-dependent processes, and the involvement of these molecules in the adhesion of cells to the vascular endothelium has been discussed (9).

Thus far, expression of LAMP-3 has been reported in human bone marrow mast cells and in the leukemic human mast cell line 1 (HMC-1) (10,11). Interestingly, expression of LAMP-3 was increased in mast cells of patients with indolent mastocytosis (10). Reasons for this are unclear, but a thorough, systematic analysis of LAMPs regarding their expression and turnover after appropriate stimulation of human mast cells, as done here, might shed light on this question.

In the present study, we have compared the intracellular and extracellular expressions of LAMPs in mast cells isolated from human skin and in HMC-1 cells before and

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after cell activation. As a secondary goal, we hoped to identify a reliable activation marker for these cells, as established for basophils. Such a marker might also be helpful in studies of mast cell activation in general because mast cells from different donors and origins differ markedly in their responsiveness to different stimuli with respect to the release of their various prestored and newly generated mediators (12,13). As a final goal, the present investigations were to provide initial insights into the intracellular distribution of LAMPs in mast cells. This is all the more of interest because mast cell mediators differ widely in their chemical nature, and some of them, such as histamine, heparin, and tryptase, are released from stores within specific electron-dense mast cells granules, whereas others, such as leukotrienes and prostaglandins, are rapidly generated by enzymatic cleavage from membrane phospholipids, and other mediators, the so-called cytokines, are prestored or newly synthesised by peptide assembly in the Golgi apparatus of the cells (12–14). Coexpression of LAMPs with acidic hydrolases was investigated in these granules to distinguish a lysosomal location of LAMPs from that in other organelles (15).

The data showed a distinct intracellular location and distribution of LAMPs in resting versus activated mast cells, with differences between the two types of mast cells studied here.

MATERIALS AND METHODS

HMC-1 Cell Culture and Isolation of Human Skin Mast Cells

HMC-1 cells, an immature human leukemic mast cell line (kindly provided by Dr. Butterfield, Rochester, MN, USA) that variably expresses mostly nonfunctional high-affinity immunoglobulin (Ig)E receptors (16–18), were cultured in Iscove's medium (Biochrom, Berlin, Germany) supplemented with 10% foetal calf serum (Biochrom), 10 μ M monothioglycerol (Sigma, Deisenhofen, Germany), and antibiotics (streptomycin and penicillin; Biochrom).

Human skin mast cells were isolated from juvenile foreskin obtained after circumcision, as previously described (19). In general, unless stated otherwise, mast cells were isolated from four to eight pooled foreskins. On average, $2.3 \pm 1.2 \times 10^5$ ($n = 10$) mast cells were isolated from 1 g of tissue. Purity of mast cells was $95 \pm 4\%$, as assessed by flow cytometry using staining for the c-Kit SCF receptor CD117 (clone 95C3; Immunotech, Hamburg, Germany), the α chain of the high-affinity IgE receptor, monoclonal antibody 29C6 (kindly provided by Dr. Hakimi, Hoffmann La Roche, Nutley, NJ, USA), and toluidine blue staining at pH 0.5 as mast cell markers.

Viability, as determined by staining with trypan blue, was always greater than 90%.

Cell Stimulation

Stimulation of HMC-1 cells was performed in 24-multiwell plates (Falcon, Lincoln Park, NJ, USA), as previously described (20). Before stimulation, cells were cultured for 24 h in medium without foetal calf serum but with 1%

bovine serum albumin (BSA; Sigma). Cells, 2×10^6 , were incubated in medium containing 1% BSA, 25 ng/ml PMA (Sigma), and 250 nM of the calcium ionophore A23187 (Sigma). Isolated skin mast cells were stimulated in Tyrode's buffer (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, and 0.1% BSA) containing anti-IgE at a dilution of 1:20,000 (Calbiochem-Novabiochem, Bad Soden, Germany) or substance P (10 μ M; Sigma), with cells at a density of 5×10^5 /ml in 24-well plates (Greiner, Germany) (14,21). Plates were incubated for 30 min at 37°C in 5% CO_2 /95% air.

After stimulation, cells were centrifuged and analysed by flow cytometry (see below). Aliquots of cell-free supernatants were stored at -80°C for determination of histamine, LTC_4 and prostaglandin D_2 (PGD_2).

Flow Cytometry

HMC-1 cells and primary skin mast cells were analysed by direct immunofluorescence staining using the following fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies: CD63 (clone CLBGran/12; Immunotech), CD107a (clone H4A3; BD Pharmingen, Heidelberg, Germany), and CD107b (clone H4B4; BD Pharmingen). Specificity of staining was controlled by an FITC-conjugated isotype-matched control antibody (BD Pharmingen). Surface expression was analysed as follows: 1 to 5×10^5 cells were used for each staining experiment. Briefly, HMC-1 cells were washed and incubated with 50 μ l human AB serum (Biotest AG, Dreieich, Germany) for 15 min at 4°C. To block the binding capacities of the MACS-conjugated antibody still attached to the skin mast cells from the isolation procedure, these cells were incubated instead with AB serum containing 100 μ g/ml mouse Fab fragments (Dianova, Hamburg, Germany). Thereafter, cells were incubated with the FITC-labeled antibodies (5 μ g/ml) for 30 min at 4°C. Intracellular staining was performed as described previously (19). Briefly, cells were fixed for 30 min at 4°C with 4% freshly prepared paraformaldehyde containing 0.1% glutaraldehyde. After washing, cells were permeabilised with 0.03% saponin, blocked as described above, and then incubated with saturating concentrations of the LAMP-specific antibodies. At least 5,000 cells were analyzed by an Epics XL flow cytometer (Coulter, Krefeld, Germany).

Immunofluorescence Microscopy

For these experiments, the following antibodies were used: CD63 (clone CLBGran/12; Immunotech) pure (1:500) or conjugated to phycoerythrin (1:50), polyclonal rabbit antibody LAMP-1 (1:50; H-228, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-cathepsin D antibody (1:500; a kind gift from Dr. Hoening, Institute for Biochemistry, University of Göttingen, Göttingen, Germany) (22). The secondary goat anti-rabbit Cy2- and Cy3-conjugated antibodies and the Cy5-conjugated goat anti-mouse antibody were kindly provided by Dr. Jahn of the Max Planck Institute for Biophysical Chemistry (Göttingen, Germany). HMC-1 cells, 2×10^4 , were processed for

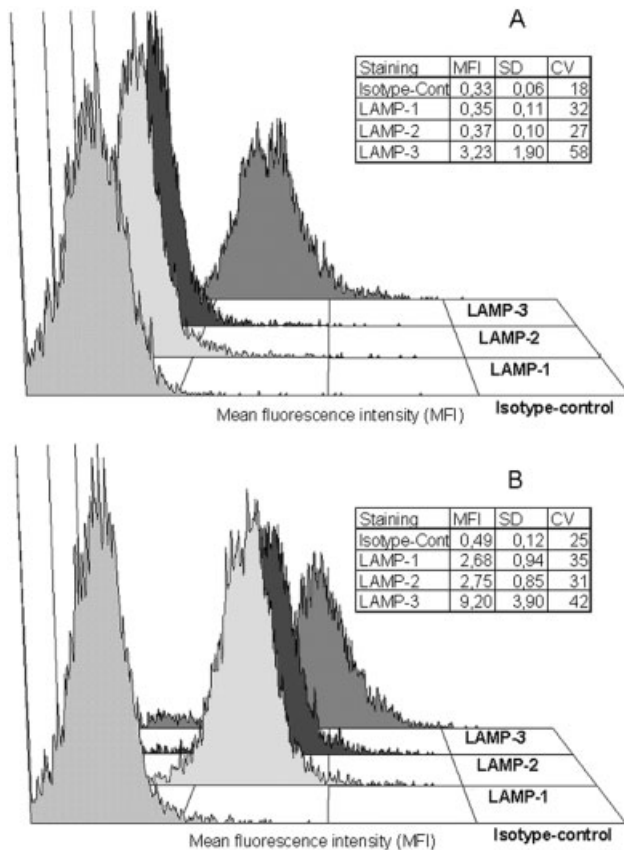


FIG. 1. Flow cytometric detection of LAMP-1, LAMP-2, and LAMP-3 in unpermeabilised (A) and permeabilised (B) HMC-1 cells. **A:** Histogram overlays show that only LAMP-3 is detectable on the surface of HMC-1 cells. **B:** After fixation and permeabilisation of cells, all three LAMPs are detectable, indicating their intracellular localisation. Tables show values for mean fluorescence intensity (MFI) and the corresponding standard deviation (SD) and coefficient of variation (CV).

immunofluorescence staining, as previously described (23), using Triton X-100 as detergent.

Image analysis was performed with Metamorph software (Universal Imaging Corp., Downingtown, PA, USA).

Mediator Release Assays (Histamine, LTC₄, PGD₂)

Histamine was measured with a histamine-specific EIA kit (Coulter-Immunotech, Krefeld, Germany) or with a fluorescence-based Histamine Autoanalyzer II (Technicon, Dublin, Ireland). LTC₄ and PGD₂ were determined with specific EIA kits (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions.

RESULTS

Cytometric Analysis of LAMP Expression

On the surface of unfixed and unstimulated HMC-1 cells, almost no LAMP-1 or LAMP-2 proteins were detectable, whereas LAMP-3 was expressed on practically all cells (Fig. 1A). This expression profile did not change after short-term (1 h) or long-term (24 h) stimulation of cells with 40 nM PMA, 250 nM calcium ionophore, or a com-

bination of both (data not shown). After fixation and permeabilisation to allow for the detection of intracellularly expressed proteins, all three LAMPs were found (Fig. 1B). LAMP-3 was expressed not only on the surface but also in an intracellular pool. The mean fluorescence intensities for LAMP-1 and LAMP-2 showed comparable levels, whereas the total signal for LAMP-3 exhibited much higher values.

On the surface of freshly isolated human skin mast cells, LAMP-1 and LAMP-2 were measurable in up to 10% of cells, whereas LAMP-3 was expressed by practically 100% of cells (Fig. 2A). In permeabilised cells, a strong fluorescence signal for LAMP-3 and intermediate signals for LAMP-1 and LAMP-2 were obtained (Fig. 2B).

Stimulation of skin mast cells with an anti-IgE-specific IgM monoclonal antibody or with substance P induced a rapid upregulation of all three LAMPs, with a larger number of reactive cells to LAMP-1 and LAMP-2 and a more intense reactivity for LAMP-3 (Figs. 3 and 4). As soon as 1 min after stimulation at 37°C, a maximal response to the IgE cross-linking antibody was ascertained that started to

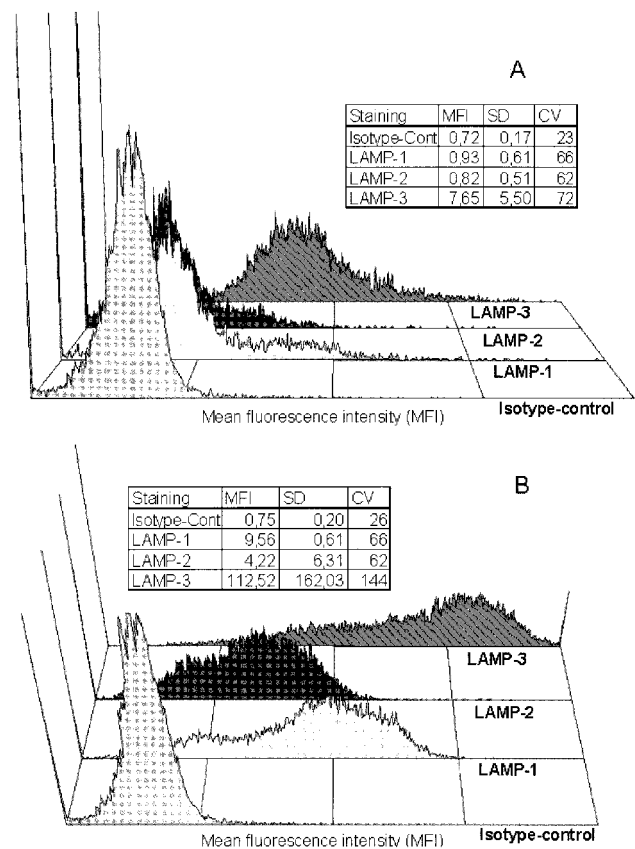


FIG. 2. Flow cytometric detection of LAMP-1, LAMP-2, and LAMP-3 in unpermeabilised (A) and permeabilised (B) skin mast cells. **A:** Histogram overlays show that LAMP-3 is detectable on the surface in up to 100% of cells, whereas LAMP-1 and LAMP-2 are expressed in only about 10% of cells. **B:** After fixation and permeabilisation, a strong intracellular signal for all three LAMPs was detected. Tables show values for mean fluorescence intensity (MFI) and the corresponding standard deviation (SD) and coefficient of variation (CV).

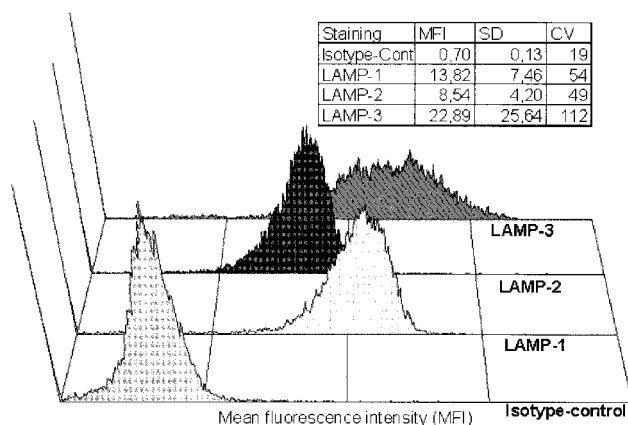


Fig. 3. Flow cytometric detection of LAMP-1, LAMP-2, and LAMP-3 in anti-IgE-stimulated skin mast cells. After stimulation for 15 min with a cross-linking anti-IgE antibody, the translocation of all three LAMPs was observed. Tables show values for mean fluorescence intensity (MFI) and the corresponding standard deviation (SD) and coefficient of variation (CV).

decline continuously after 10 min of stimulation (Fig. 4). Stimulation with substance P induced a weaker effect that reached its maximum only 3 min after stimulation. Independent of the stimulus used, 50% to 60% of translocated LAMP-2 disappeared during a prolonged incubation time of 100 min, indicating its rapid internalisation. Anti-IgE- and substance P-induced translocations were completely blocked when the cells were stimulated at 4°C (Fig. 4).

Intracellular Colocalisation of LAMP-1, LAMP-3, and Cathepsin D

Figure 5 shows the immunofluorescent localisation of LAMP-1, LAMP-3, and cathepsin D in paraformaldehyde-fixed and Triton X-100-permeabilised HMC-1 cells after using double immunofluorescent staining, as described above. For each pair of images, identical microscopic parameters were used. In Figure 5a, the immunofluorescence signal for LAMP-3 is seen as coarse punctate granules distributed at the cell periphery and throughout the cytoplasm, in contrast to the fine granular immunoreactivity of LAMP-1 mainly within the cell interior (Fig. 5b). Overlapping fluorescence intensity profiles for both LAMPs were seen to only a minor extent (Fig. 5c). In contrast, a strong colocalisation of immunofluorescence staining was found for LAMP-3 and cathepsin D (Fig. 5d-f).

Mediator Release From Activated Mast Cells

To compare the translocation of LAMPs with that of mediator release in anti-IgE-stimulated skin mast cells on a quantitative basis, cells from different foreskins were prepared and stimulated separately. In this way, it was possible to recognise interindividual variations in the responsiveness of mast cells from different skin preparations. Figure 6 shows representative results from three different mast cell preparations. As shown in Figure 6A, only small variations in the LAMP-1 and LAMP-3 transloca-

tions were ascertained (coefficients of variation: 7% for LAMP-1 and 19% for LAMP-3). The activation-induced LAMP translocation was always accompanied by the release of histamine (Fig. 6B), LTC₄ (Fig. 6C), and PGD₂ (Fig. 6D), but larger interindividual variations were obvious with respect to these parameters (coefficients of variation: 70% for histamine, 62% for LTC₄, and 42% for PGD₂). After evaluating the absolute quantities of mediator released per cell, histamine ranged from 1 to 6 pg, PGD₂ from 14 to 32 fg, and LTC₄ from 1 to 8 fg.

DISCUSSION

This report represents the first systematic analysis of the expression of intracellular and cell membrane LAMP-1, LAMP-2, and LAMP-3 in human skin mast cells and in HMC-1. Immunofluorescence microscopy showed that LAMP-3 is located at the mast cell cytoplasmic membrane in resting cells and that all three LAMPs are located in mast cell granules, colocalising in part with the lysosomal

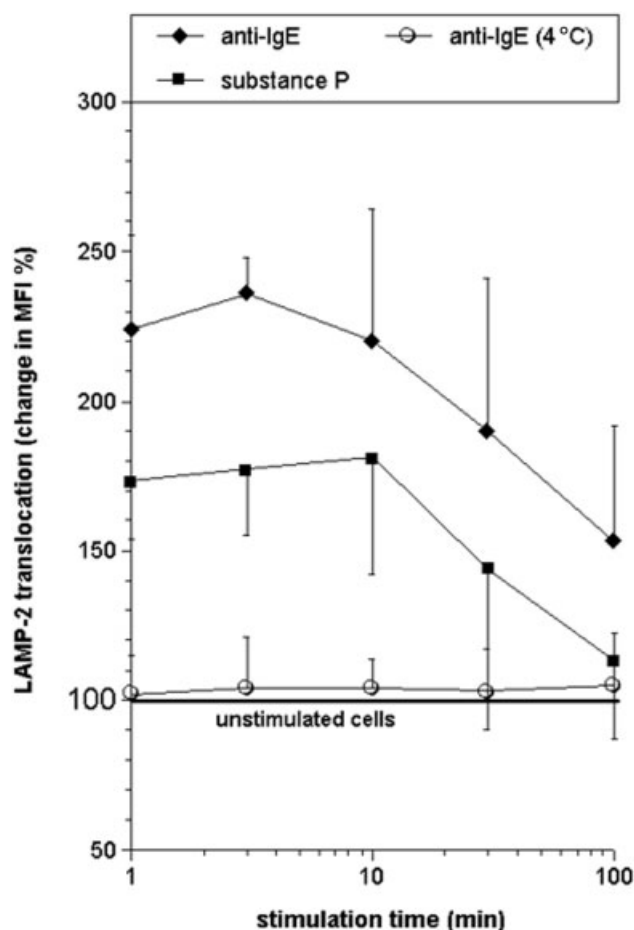


Fig. 4. Time-dependent translocation of LAMP-2 in anti-IgE- and substance P-stimulated mast cells as measured by flow cytometry. The extent of translocation was calculated as percentage of change in mean fluorescence intensity compared with unstimulated cells (unstimulated cells = 100%). The curve with open circles represents stimulation of cells with anti-IgE at 4°C. Data are mean $\pm$ standard deviation of three independent experiments.

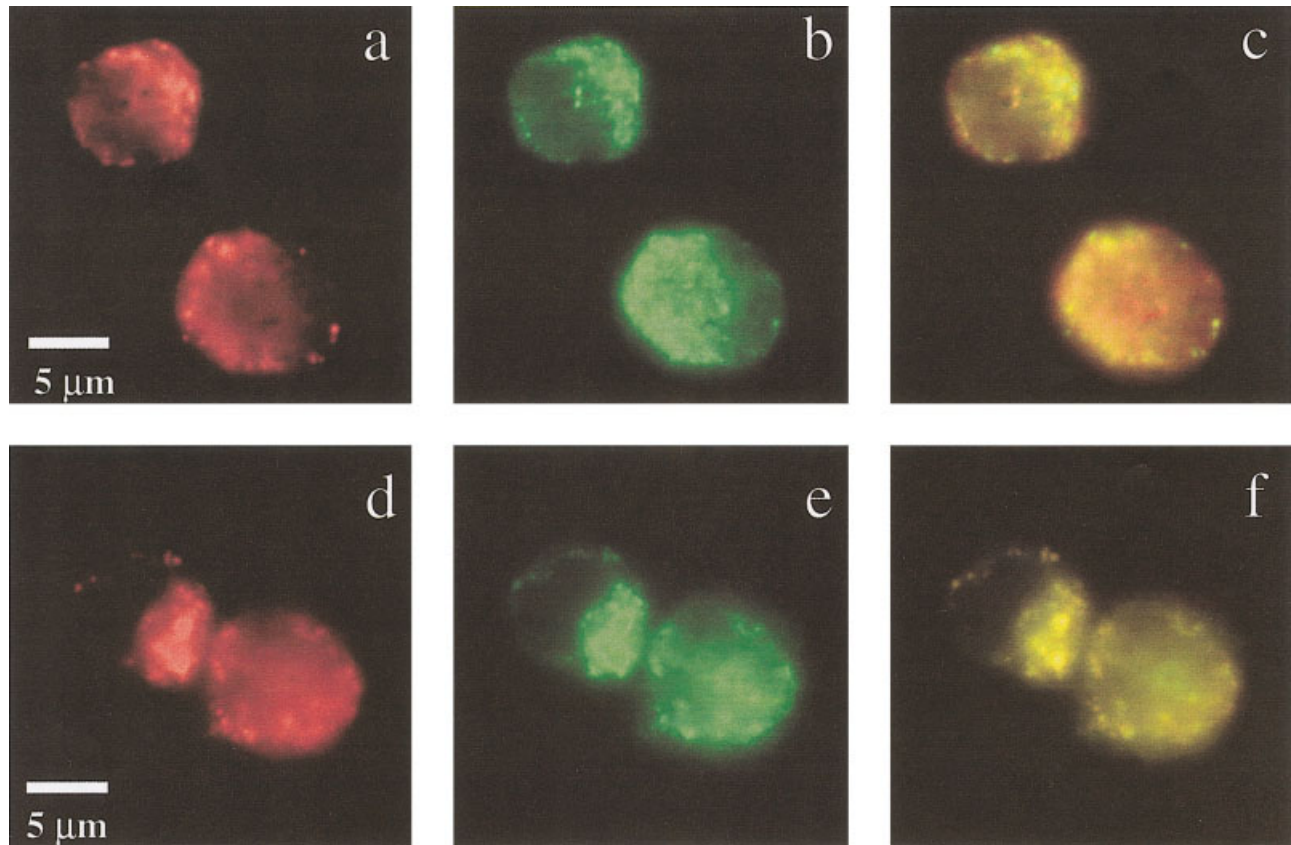


FIG. 5. **a:** Immunofluorescence microscopy of HMC-1 cells stained with a monoclonal antibody against LAMP-3 detected with a secondary antibody conjugated with Cy5 (red). **b:** Staining pattern of the polyclonal rabbit antibody to LAMP-1 and a secondary goat anti-rabbit Cy3-conjugated antibody (green). **c:** Partial colocalisation of both parameters (yellow). Immunofluorescence staining of HMC-1 cells with a **(d)** phycoerythrin-conjugated anti-LAMP-3 antibody (red) and a **(e)** polyclonal rabbit antibody to cathepsin D detected with a secondary Cy2-conjugated secondary antibody (green). **f:** Marked colocalisation of both markers (yellow).

marker cathepsin D, as shown for LAMP-3 in skin mast cells. During activation of the latter, but not of HMC-1 cells, all three LAMPs are translocated in an energy-dependent process from intracellular compartments to the plasma membrane.

Thus far, data from the literature regarding mast cell or basophil LAMP expression are available primarily for LAMP-3. Thus, this molecule has been detected in human bone marrow mast cells (10), HMC-1 cells (11), the rat basophilic cell line RBL-2H3 (24), and various megakaryocytic cell lines (25). In other cells, such as in neutrophils (26), basophils (5), and endothelial cells (27), LAMP-3 is exclusively restricted to intracellular structures of unstimulated cells. The appearance of LAMP-3 at the cell membrane of mast cells, as shown in the present study, seems not to be the consequence of unintentional stimulation during the isolation procedure or during normal cell culture because LAMP-1 and LAMP-2 were almost undetectable in freshly isolated cells and in HMC-1 cells. These results indicate that LAMP-3 exerts lysosome- and endosome-related functions in mast cells and that additional functions might be ascribed to LAMP-3 when it is expressed in the plasma membrane of unstimulated cells. In

this context, the participation of LAMP-3 in adhesion and spreading processes has been discussed (28–30). In particular, the molecular association between the β_1 integrins α_3 , α_4 , α_6 and LAMP-3 may be of interest because these integrins are expressed in mast cells and HMC-1 cells (31,32). Moreover, the cytoplasmic tail of CD63 is associated with tyrosine kinase activity, thus supporting the assumption that this molecule is involved in intracellular signalling events (33).

In skin-derived mast cells, although stimulation with an anti-IgE-specific IgM monoclonal antibody or substance P induced rapid translocation of LAMPs, neither PMA nor the combination of PMA and the calcium ionophore A23187 evoked a comparable response in HMC-1 cells. This is surprising in view of the fact that these stimuli induce production and secretion of, e.g., interleukin-8 (20). The absence of LAMP-1 and LAMP-2 was unexpected, all the more because HMC-1 cells possess a constitutively activated c-kit receptor (34) that seems to be responsible for the more activated phenotype of these cells, compared with primary mast cells. Accordingly, these cells are characterised by a high degree of spontaneous piecemeal degranulation and by high levels of spon-

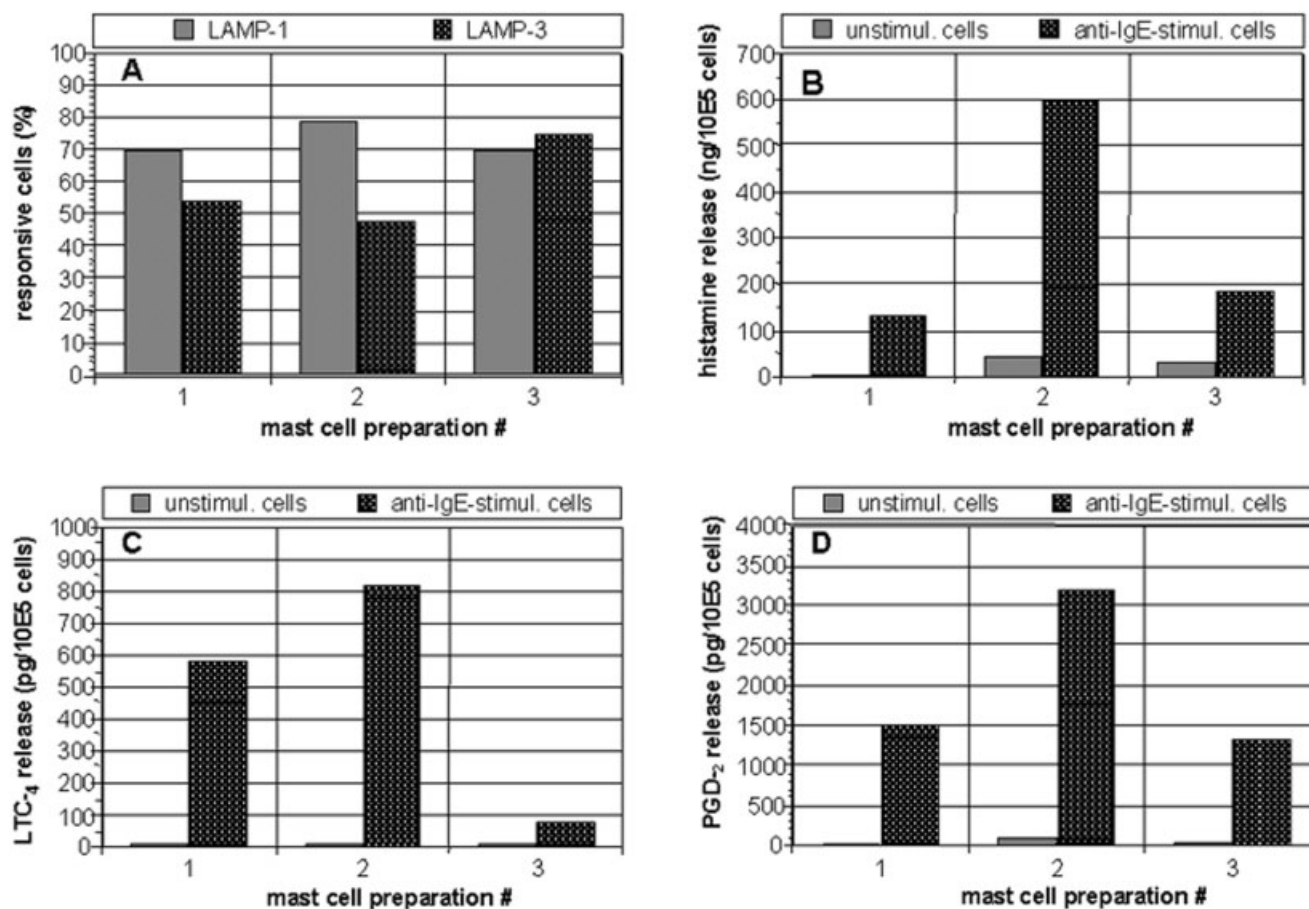


FIG. 6. Interindividual comparison of anti-IgE-induced mediator release and LAMP translocation in mast cell preparations from three different donors. Unstimulated cells and cells stimulated for 15 min with an anti-IgE antibody were analysed for LAMP-1 and LAMP-3 by flow cytometry, and the corresponding supernatants were analysed for their contents of histamine, LTC₄, and PGD₂. **A:** Flow cytometric measurement of LAMP-1 and LAMP-3 translocations, expressed as percentage of responsive cells. **B:** Release of histamine, expressed as nanograms per 10⁵ cells. **C:** Release of LTC₄, expressed as picograms per 10⁵ cells. **D:** Release of PGD₂, expressed as picograms per 10⁵ cells. Mediators were quantified with specific competitive enzyme immunoassays.

taneous histamine and tryptase release (19) (unpublished observation). Because HMC-1 cells are immature cells that are morphologically characterised by the presence of large, immature, electron-lucent granules (35), these results indicate that LAMP-1 and LAMP-2 are expressed primarily in mature granules. Alternatively, a defect in the secretory response of the cells might be associated with the malignant nature of HMC-1 cells. Further studies examining normal mast cells during the process of maturation might shed light on this question.

The present data also suggest that, in contrast to basophils for which LAMP-3 is advocated as a reliable activation marker (4–7), LAMP-3 is not useful in this regard because, in skin mast cells, the molecule is already present on the membrane of practically all unstimulated cells. Instead, LAMP-1 or LAMP-2 might be used to discriminate between resting and activated mast cells, as shown in this study during anti-IgE- or substance P-induced stimulation and in a previous study during modulation of this response in the presence of ultraviolet light (36). Nevertheless, a

strict quantitative correlation between LAMP expression and mediator secretion cannot be made. This is not surprising in view of the more complex cell machinery in the context of the secretory process and the heterogeneous chemical nature of the diverse mast cell mediators. The decline with time of LAMP-2 surface expression after stimulation with anti-IgE and substance P, as observed in this study, suggests a high turnover rate of translocated LAMPs, probably due to internalisation by endocytosis via coated pits, as described for RBL cells (37), although shedding into the cell microenvironment needs to be excluded by additional experiments. In view of the well-known donor-dependent variations in activation-induced mediator release (13) (current data), flow cytometric measurement of LAMPs seems a more reliable activation marker for release of mediators such as histamine, leukotrienes, and prostaglandins.

In conclusion, the present work provides initial evidence for a distinct expression of LAMPs in different types of resting and activated mast cells. Further, LAMP-1 and

LAMP-2 are proposed as activation markers of normal skin mast cells, although it must be taken into consideration that the activation-induced translocation of these molecules to the plasma membrane is a process that is restricted in time. On the basis of the present findings with activated skin mast cells, the increased expression of LAMP-3 in indolent mastocytosis bone marrow mast cells (10) suggests that these cells are activated in the context of the disease.

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