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A Novel Lysosome-Associated Membrane Glycoprotein, DC-LAMP, Induced upon DC Maturation, Is Transiently Expressed in MHC Class II Compartment

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Summary

We have identified a novel lysosome-associated membrane glycoprotein localized on chromosome 3q26.3-q27, DC-LAMP, which is homologous to CD68. *DC-LAMP* mRNA is present only in lymphoid organs and DC. A specific MAb detects the protein exclusively in interdigitating dendritic cells. Expression of DC-LAMP increases progressively during in vitro DC differentiation, but sharply upon activation with LPS, TNFα, or CD40L. Confocal microscopy confirmed the lysosomal distribution of the protein. Furthermore, DC-LAMP was found in the MHC class II compartment immediately before the translocation of MHC class II molecules to the cell surface, after which it concentrates into perinuclear lysosomes. This suggests that DC-LAMP might change the lysosome function after the transfer of peptide–MHC class II molecules to the surface of DC.

Introduction

Dendritic cells (DC), which are widely distributed throughout the body, are the most potent antigen-presenting cells (APC) particularly involved in the initiation of Ag-specific immune responses (Steinman, 1991; Banchereau and Steinman, 1998). Immature DC, such as the epidermal Langerhans cells, capture antigens with high efficiency. Upon inflammatory stimuli, DC migrate from the periphery to the lymphoid organs, home to the T-dependent areas, and activate naive T cells. Along their migration, DC mature from an antigen-uptake/processing phenotype, typical of immature DC spread in nonlymphoid tissues, to the distinctive antigen-presenting phenotype of interdigitating dendritic cells (IDC) gathered in lymphoid tissues (Steinman, 1991; Austyn, 1996). Upon complete differentiation, IDC efficiently present processed antigens to naive T cells and induce primary T cell response (Inaba et al., 1983; Inaba and Steinman, 1985). The lack of IDC-specific marker has hampered their characterization. They are phenotypically distinguished by the expression of CD83 (Zhou et al., 1992); p55 (Mosialos et al., 1996); costimulatory molecules such as CD40, CD80, and CD86 (Björck et al., 1997); and by a strong expression of MHC class II molecules (Steinman, 1991). The MHC class II molecules that present antigen-derived peptides to $CD4^+$ T cells consist of $(\alpha/\beta/\text{invariant (Ii) chain})_3$ heteronanomer in the endoplasmic reticulum (Roche et al., 1991; Cresswell, 1996). After transport to the Golgi apparatus, the MHC class II–Ii complexes are targeted to the endosomal-lysosomal pathway (Wolf and Ploegh, 1995) where the Ii chain is proteolytically degraded, leaving a small fragment CLIP (the class II–associated Ii chain peptide) bound to the released αβ dimers. Another molecule, HLA-DM, which is predominantly located in the MHC class II compartment (MIIC) in human B cells and immature DC (Peters et al., 1991; Karlsson et al., 1994; Rudensky et al., 1994; Sanderson et al., 1994; Tulp et al., 1994; West et al., 1994; Nijman et al., 1995), facilitates the exchange of the residual CLIP with antigen-derived peptides to be loaded on class II molecules (Martin et al., 1996; Miyazaki et al., 1996). Class II–peptide complexes are then transported to the cell surface, where they can be recognized by the TCR-CD4 complexes on T cells. Thus, multiple subcellular compartments that could be correlated to different stages of DC maturation are involved in the loading of peptide on class II molecules (Pierre et al., 1997).

In the search for novel molecules restricted to DC, we identified a member of the lysosome-associated membrane glycoprotein family (LAMP), which we named DC-LAMP. This molecule is most homologous to CD68 (Holness and Simmons, 1993), a lysosomal glycoprotein mainly expressed by macrophages and platelets, which might be involved in endocytosis and/or lysosomal traffic. As DC-LAMP could only be detected in IDC, it represents the first marker specific for these mature DC. We demonstrate here that DC-LAMP, which appears during DC maturation, localizes in the MIIC, where peptides associate with class II molecules. According to the cellular and subcellular distribution of DC-LAMP, we propose a role for this novel lysosomal protein in the remodeling of specialized antigen-processing compartments and in MHC class II–restricted Ag presentation.

Results

Identification of a Novel Member of the LAMP Family in Dendritic Cells

A novel 0.7 kb partial cDNA (E02B02) was isolated by screening a $CD1a^+$-derived DC subtraction library. Northern blots probed with the E02B02 clone showed a 3.2 kb mRNA transcript strongly expressed in dendritic cells but not in CHA, the driver epithelial cell line used for

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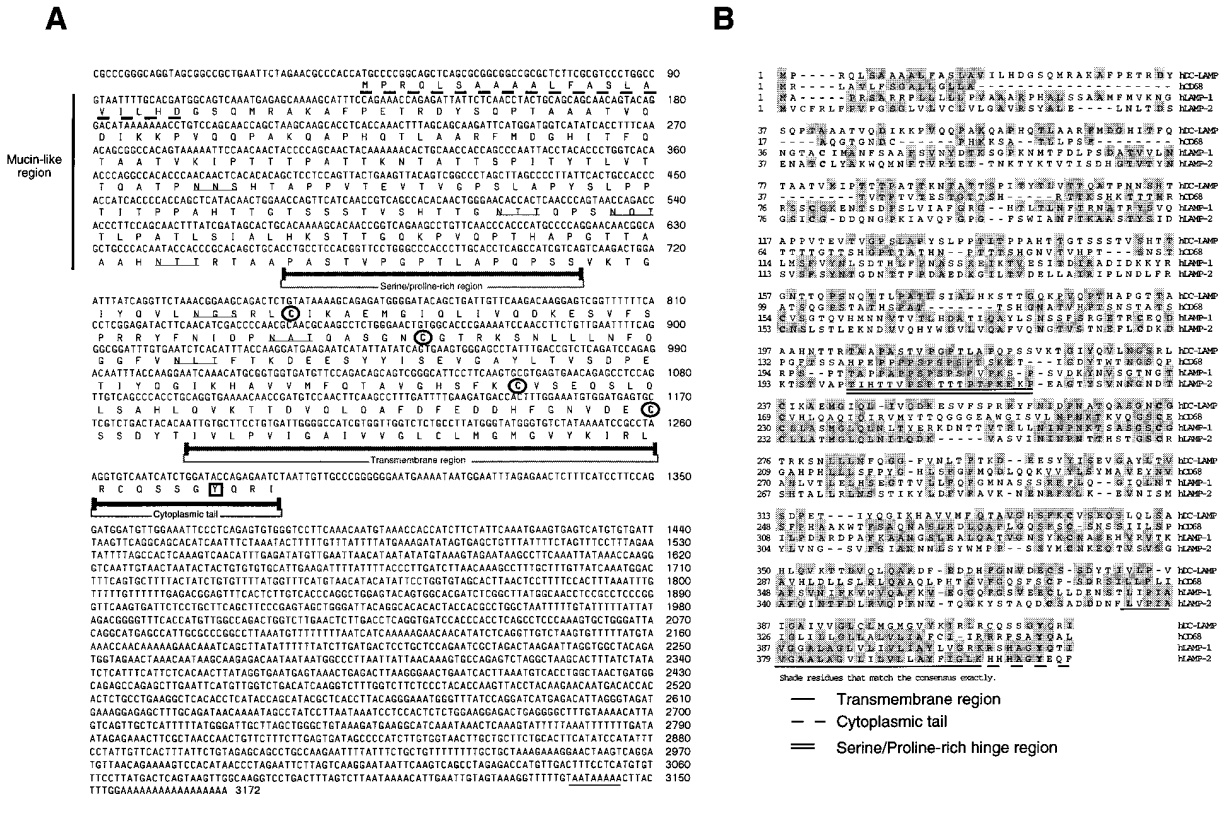

Figure 1. Nucleotide and Amino Acid Sequences of DC-LAMP

(A) Human *DC-LAMP* cDNA nucleotide sequence and deduced amino acid sequence. The N-terminal signal peptide is underlined by a dotted line. Sequences corresponding to the serine/proline hinge region, the transmembrane region, and the cytoplasmic tail are underlined in bold. Potential N-linked glycosylation sites (N-X-S/T) and the polyadenylation signal are underlined.

(B) Alignment of DC-LAMP amino acid sequence with other members of the LAMP family. A multiple alignment of members of the LAMP family was created using the Megalign program with Clustal method on the LASERGENE Navigator (DNASTAR, Madison, WI).

subtraction. The full-length 3155 bp cDNA sequence was obtained by RACE: it contains a 5′ untranslated sequence (nt 1–42), a 1248 bp open reading frame (nt 43–1290) starting with a methionine codon situated in a consensus Kozak sequence, a 3′ untranslated sequence of 1865 nucleotides (nt 1291–3155), and a polyadenylation signal AATAAA at position 3138 followed by a poly(A) tail. The predicted polypeptide sequence of 416 amino acids has the typical features of a type I integral membrane protein. It has a presumptive amino-terminal signal peptide of 21 residues, followed by a long extracellular domain (381 residues), a hydrophobic transmembrane domain (25 residues), and a short cytoplasmic domain (10 residues). The sequence indicates that there are seven potential N-glycosylation sites (Asn-X-Ser/Thr) and several putative O-glycosylation sites (stretches of proline, serine, and threonine) (Figure 1A). The *DC-LAMP* gene was mapped by in situ hybridization, using a fluorescent probe, on the 3q26.3-q27 bands of the long arm of chromosome 3 (Mattei et al., 1990). Sequence comparison with the EMBL databases revealed homology with lysosomal glycoproteins from the LAMP family and, therefore, E02B02 was named DC-LAMP. The predicted amino acid sequence showed the closest homology (29%) with human CD68 (Holness and Simmons, 1993), a DC/macrophages marker, followed by the human LAMP-1 (23%) and LAMP-2 (21%) (Fukuda et al., 1988) (Figure 1B). A phylogenic tree (Figure 2A) illustrates the conservation of amino acid sequences of the different LAMP proteins between different species. The intraluminal portion of the LAMP proteins is made of two domains separated by a serine/proline-rich hinge region. Each domain of LAMP-1 and LAMP-2 contains four cysteines that form two disulfide bridges. In contrast, only the membrane-proximal domains (domain II) of CD68 and DC-LAMP contain four cysteines and probably two disulfide bounds (Figures 1B and 2B). Separate amino acid alignments of domain I (membrane distal domain) or domain II showed that DC-LAMP shares 28% and 31% identity, respectively, with CD68, 18% and 27% with LAMP-1, and 18% and 25% with LAMP-2. Therefore, LAMP domain II regions are more conserved among LAMP family members than domain I regions. Domain I of DC-LAMP and CD68 has several serine, threonine, and proline residues and therefore could be considered as a mucin-like protein covered with O-linked glycans (Holness and Simmons, 1993). Furthermore, DC-LAMP has the short cytoplasmic tail with a glycine-tyrosine motif, which characterizes all known LAMP family members.

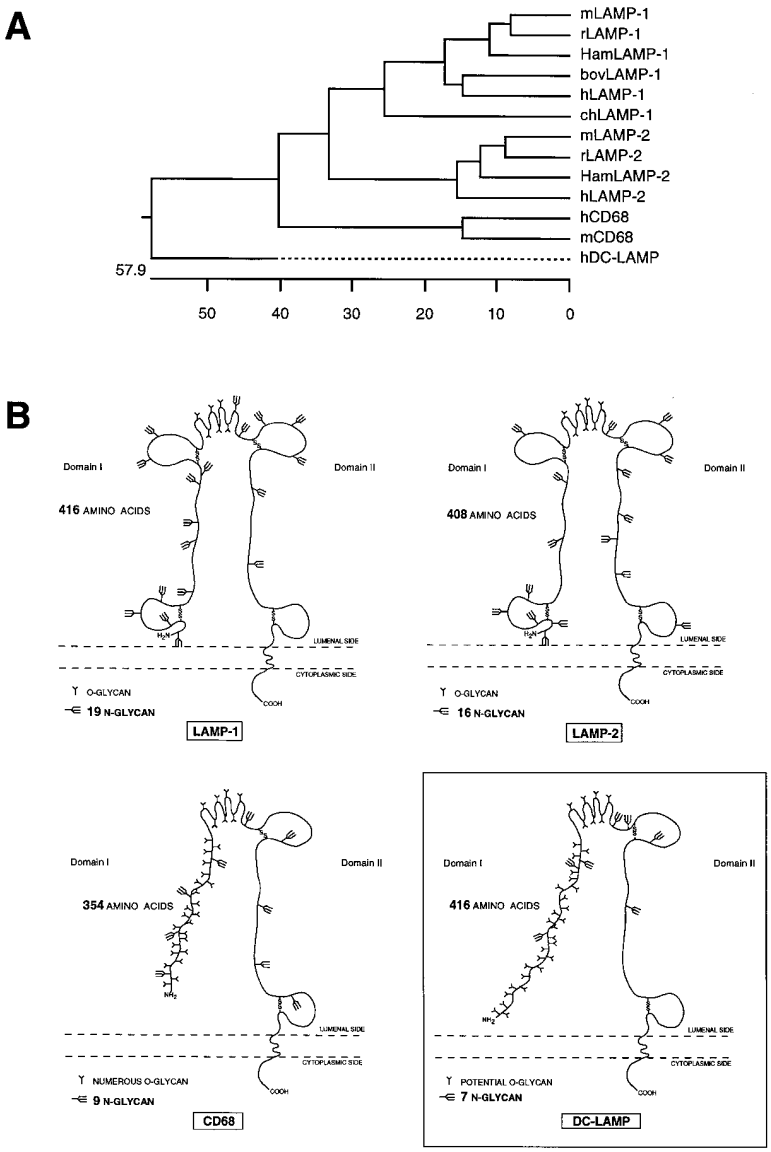

Figure 2. Phylogenic and Structural Relationships between the LAMP Family Members

(A) Phylogenic relationship of LAMP proteins from different species. Amino acid sequences were compared using the Megalign program and then displayed graphically in a phylogenetic tree. Units indicate the number of substitution events. Abbreviations: h, human; r, rat; Ham, hamster; bov, bovine; m, mouse; ch, chicken.

(B) Domain organization of hLAMP-1, hLAMP-2, hCD68, and hDC-LAMP. N-glycans and O-glycans are indicated by tridents and Ys, respectively. Each loop is made by a disulfide bond. The majority of the molecule resides in the luminal side of lysosomes. The structure depicted is adapted from Fukuda, 1991.

Thus, by the search for novel DC molecules, we identified a member of the lysosome-associated membrane glycoprotein similar to CD68.

DC-LAMP mRNA Is Specifically Expressed in DC and Is Up-Regulated after DC Activation or Maturation

When expression of *DC-LAMP* mRNA was studied in normal human tissues, a strong single band of 3.2 kb was detected by Northern blot in appendix, thymus, lymph node, and lung (adult and fetal), and a much weaker signal was found in spleen (Figure 3C; data not shown). No messenger was observed in bone marrow, pancreas, placenta, brain, heart, or peripheral blood leukocytes. When cell lines were analyzed, *DC-LAMP* was abundant in DC, scarce in PMA-ionomycin activated Jurkat T cells and JY lymphoblastoid B cells, and absent in myelo-erythrocytic TF1 cells, kidney carcinoma CHA cells, and lung fibroblast MRC5 cells (Figure 3A). Among freshly isolated cells, *DC-LAMP* was only present in resting or PMA-ionomycin activated DC, but not in activated monocytes, T cells, granulocytes, PBL and B cells (Figure 3B). Identical results were obtained by RT–PCR with the same cell lines and the cells isolated ex vivo (data not shown), therefore confirming the restriction of cellular distribution to DC. Semiquantitative RT–PCR was next used to follow *DC-LAMP* gene transcription. During the culture of $CD34^+$ human cord blood progenitors in the presence of GM-CSF and TNFα, *DC-LAMP* mRNA was hardly detected at day 6, but abundant at day 12. Further up-regulation was observed after triggering final maturation of the DC by 4 days of coculture with hCD40L-transfected L cells (Figure 3D). Similarly, while monocytes do not express a detectable amount of *DC-LAMP* mRNA, a faint band could be amplified after 6 days of culture in the presence of GM-CSF and IL-4. Again, following activation of these monocyte-derived DC through CD40, the level of mRNA sharply increased

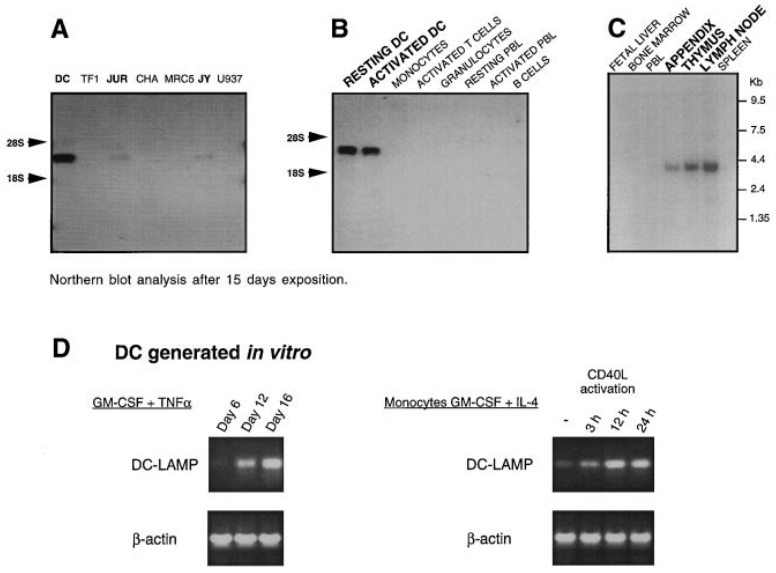

Figure 3. Analysis of *DC-LAMP* mRNA Expression

(A) Northern blot analysis of *DC-LAMP* expression in 20 μg RNA prepared from various PMA-ionomycin-activated human cell lines and $CD34^+$-derived DC.

(B) Northern blot analysis of *DC-LAMP* expression in 20 μg RNA prepared from freshly isolated cells. Monocytes, granulocytes, and B cells were a pool of nonactivated, 1, and 6 hr PMA-ionomycin-activated cells. DC, T cells, and PBL were activated with PMA-ionomycin for 1 and 6 hr.

(C) Commercially available poly(A)$^+$ Northern blots of a variety of human lymphoid tissues (Clontech 7754–1) probed with *DC-LAMP*.

(D) RT–PCR analysis of *DC-LAMP* expression on different types of DC generated in vitro. RT–PCR was carried under standard conditions starting with 5 ng cDNA for 35 cycles. Control β-actin RT–PCR was performed with the same cDNA. cDNA was purified from DC generated in vitro from $CD34^+$ progenitors cultured in the presence of GM-CSF and TNFα for 6 and 12 days and for an additional 4 days with CD40L L cells (Day 16), or from monocytes cultured in the presence of GM-CSF and IL-4 for 6 days with or without 3, 12, and 24 hr activation with CD40L L cells.

within 3 hr and reached a maximum after 12 hr (Figure 3D). Weak transcription of *DC-LAMP* was found in $CD4^+CD11c^+CD3^-$ germinal center dendritic cells (GCDC) freshly isolated from tonsils, which could be enhanced by either PMA-ionomycin or CD40L activation, and in freshly isolated Langerhans cells (data not shown). PMA-ionomycin-activated macrophages generated in vitro, LPS-activated monocytes, and CD40L-activated B cells also weakly express *DC-LAMP* mRNA.

In conclusion, *DC-LAMP* appears to be increasingly transcribed upon maturation/activation signals in DC.

DC-LAMP MAb Stains Exclusively IDC on Human Lymphoid Tissues

We next directly determined which cells express DC-LAMP protein in vivo by staining human lymphoid tissue sections with a mouse anti-DC-LAMP MAb raised against the recombinant DC-LAMP-Fc chimera. Double staining of tonsil sections with anti-CD3 MAb (red) showed that the DC-LAMP$^+$ cells (blue) are large, interdigitated cells exclusively localized in the T cell areas, but not in B cell follicles (Figure 4Aa). Likewise, on spleen, lymph node, and medulla of thymus tissue sections, DC-LAMP$^+$ cells are only found in T cell areas (Figures 4Ab, 4Ac, and 4Ad). In small intestine, DC-LAMP$^+$ cells are observed in T cell areas corresponding to Peyer's patches (Figure 4Ae). In contrast, neither epidermal $CD1a^+$ (red) Langerhans cells nor $CD1a^-$ dermal DC express DC-LAMP (Figure 4Af). On tonsil sections, the large DC-LAMP$^+$ cells present in T cell areas (Figure 4Ba) expressed high levels of CD40 (DC-LAMP, blue; CD40, red) (Figure 4Bb), and most of them were also $CD83^+$ (CD83, red; DC-LAMP, blue), demonstrating that they most likely represent IDC (Björck et al., 1997) (Figure 4Bc). Interestingly, none of the $CD68^+$ cells (red), including the tingible body macrophages and the GCDC present in germinal centers, as well as the few macrophages found in the T cell areas coexpressed DC-LAMP (blue). Indeed, the staining with DC-LAMP and CD68 appeared mutually exclusive (Figure 4Bd).

Thus, the staining pattern in a range of human lymphoid tissues is consistent with DC-LAMP being expressed solely in IDC.

DC-LAMP Protein Expression Increases Progressively during DC Differentiation and Is Sharply Up-Regulated during DC Maturation

To extend RT–PCR data regarding the transcription of *DC-LAMP*, we next used our specific MAb to analyze by FACS the presence of the protein during DC development and maturation. In agreement with the predicted lysosomal localization of the protein, DC-LAMP was barely detectable at the cell surface, while intracellular staining was observed.

Kinetic analysis during $CD34^+$-derived DC culture showed that DC-LAMP is present from day 12 and progressively increases up to day 14 (Figure 5A). Of note, DC-LAMP up-regulation coincides with the highest level of surface HLA class I and II, CD83, CD80, and CD86 that characterizes mature DC (Figure 5A; data not shown). Furthermore, signals inducing DC maturation such as TNFα, CD40L, or LPS (data not shown) significantly increase both the percentage of DC-LAMP$^+$ DC (45%–70%) and the intensity of the staining (MFI, 693–1022) (Figure 5B). However, even after a prolonged maturation signal, a small fraction of $CD34^+$-derived DC does not express DC-LAMP, most likely reflecting a lack of synchronization in their stage of differentiation. Unactivated monocyte-derived DC (day 6) do not contain DC-LAMP, whereas 96 hr TNFα, LPS, or CD40L activation induces a strong DC-LAMP staining in 60%–100% of the cells (Figures 5B and 5C). As shown in Figure 5C, DC-LAMP appears rapidly after LPS activation of monocyte-derived

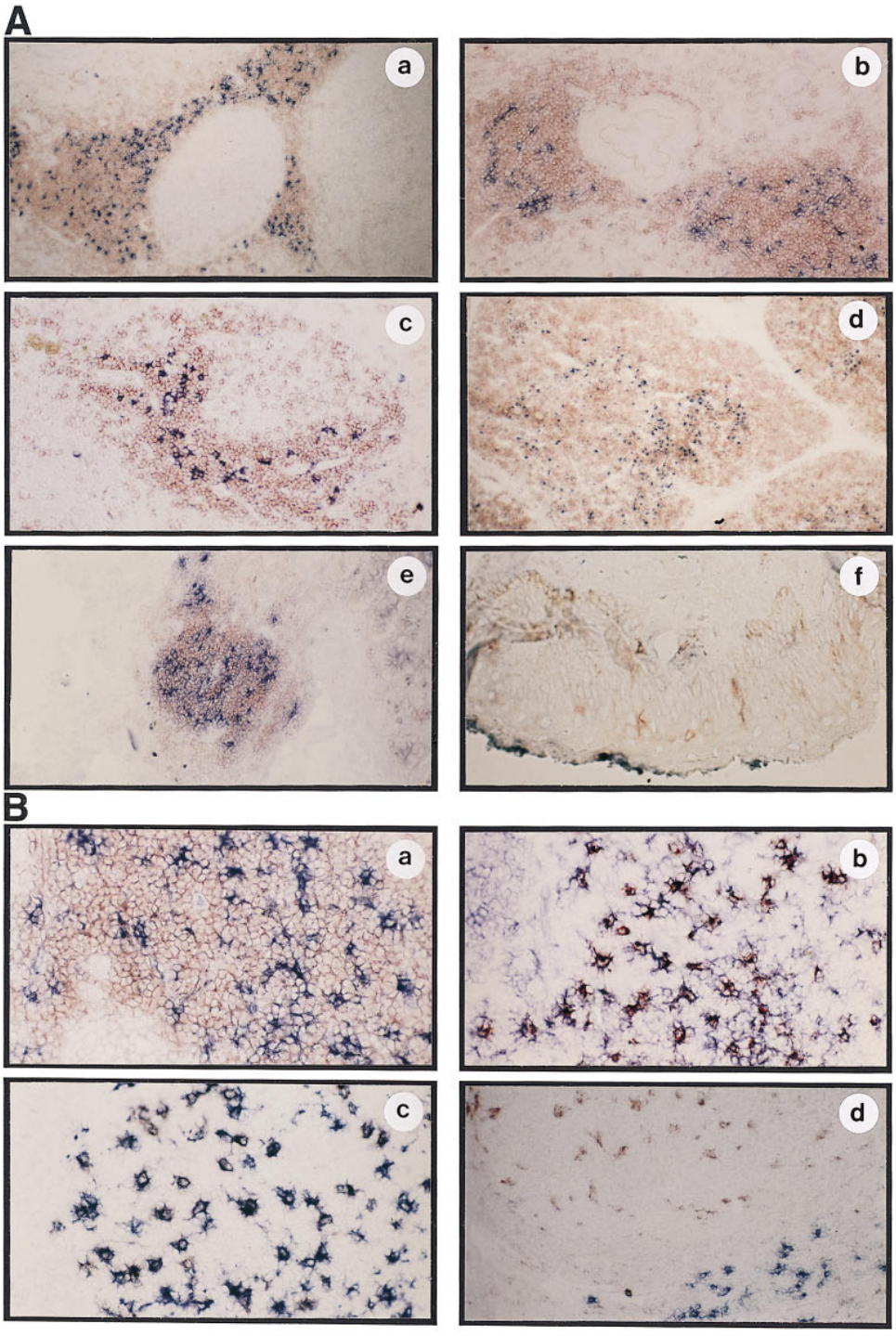

Figure 4. Immunohistological Characterization of DC-LAMP Expression on Human Lymphoid Organs and Skin Tissue Section

(A) Localization of DC-LAMP$^+$ cells on different human tissue sections: tonsil (a), spleen (b), lymph node (c), medulla of thymus (d), small intestine (e), and skin (f). Anti-CD3 (red) and anti-DC-LAMP (blue) double staining identifies DC-LAMP$^+$ cells localized in T cell areas of the lymphoid organs (a–e) (a and d: original magnification, 100×; b, c, and e: 200×). In skin section (f), anti-CD1a (red) and anti-DC-LAMP (blue) double staining identifies CD1a$^+$ Langerhans cells that do not express DC-LAMP (original magnification 400×).

(B) Phenotypic characterization of DC-LAMP$^+$ cells on human tonsil sections. Double staining identifies large DC-LAMP$^+$ cells (blue) localized in T cell areas (CD3, red) (a). DC-LAMP$^+$ cells are CD40$^+$ (red) (b), and most of them coexpressing CD83 (red) are purple, (c) but they do not express CD68 (red) (d) (magnification 400×). CD68$^+$ DC-LAMP$^-$ cells in germinal centers are probably tingible body macrophages (d).

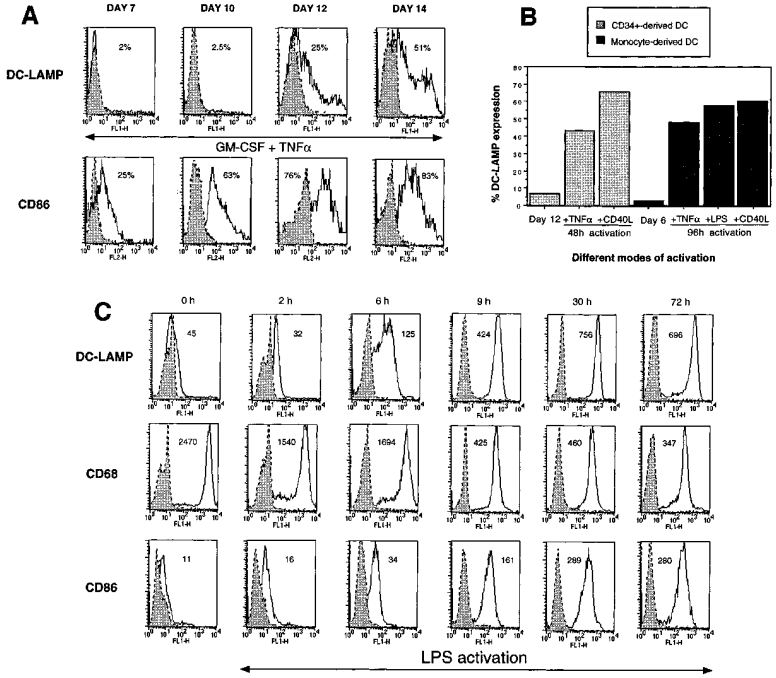

Figure 5. Regulation of DC-LAMP Expression during DC Maturation

DC-LAMP expression was analyzed by cytofluorimetry. DC-LAMP phenotype was studied after DC permeabilization in saponin, as described in Experimental Procedures, whereas CD86 was stained on the surface. Results are representative of four experiments.

(A) Kinetic of intracytoplasmic DC-LAMP expression during $CD34^+$-derived DC maturation. DC maturation was followed by CD86-PE cell surface staining. Gray overlap histograms show negative fluorescence controls with an isotype-matched MAb of unrelated specificity.

(B) Different modes of activation induce DC-LAMP expression on both monocyte- and $CD34^+$-derived DC. Flow cytometric analysis was performed on day 12 $CD34^+$-derived DC and after 48 hr TNFα or CD40L L cell activation. Monocyte-derived DC were analyzed at day 6 and after 96 hr of TNFα, LPS, or CD40L activation. Results are indicated in percent of DC-$LAMP^+$ cells.

(C) Kinetic of intracytoplasmic DC-LAMP and CD68 expressions during monocyte-derived DC maturation with LPS (2–72 hr). DC maturation was followed by CD86-FITC cell surface staining. Gray overlap histograms show negative fluorescence controls with a MAb of unrelated specificity. Results are indicated in MFI (mean fluorescence intensity).

DC; maximal staining is observed within 6 hr of LPS activation and correlates with up-regulation of CD86, a good marker of DC maturation. Interestingly, we found that CD68, the gene most homologous to *DC-LAMP*, is expressed at an early stage of DC differentiation and is down-regulated upon maturation on either monocyte- and $CD34^+$-derived DC (Figure 5C; data not shown).

Taken together, these results show that DC-LAMP protein is rapidly up-regulated upon DC maturation.

DC-LAMP Is Transiently Expressed in the MIIC

To analyze the subcellular localization of DC-LAMP, we have studied different stages of DC maturation by confocal microscopy. The DC-LAMP signal is absent or very weak at the immature stage of $CD34^+$-derived DC maturation (day 10–11), when most of the MHC class II molecules are still intracellular and when the homologous lysosomal marker, CD68, is highly expressed (Figure 6; data not shown). DC-LAMP appears first during "spontaneous" maturation of DC (day 12) within vesicles spread throughout the whole cell that contain MHC class II molecules, LAMP-1, LAMP-2, CD68, cathepsin D, and HLA-DM (Figure 6; data not shown). These vesicles correspond to the previously described MIIC (Peters et al., 1991). After CD40L activation, DC-LAMP accumulates into more central and larger vesicles, which are class II^-, LAMP-1^+ and LAMP-2^+. Meanwhile, CD68 progressively disappears. At the final stage of DC maturation, while all MHC class II molecules are exported to the cell surface, DC-LAMP is only concentrated with LAMP-1 and LAMP-2 in perinuclear lysosomes (Figure 6; data not shown). Kinetic analysis in monocyte-derived DC is facilitated by a tighter synchronization of DC maturation upon activation. DC-LAMP, which is absent at immature stages (Figure 7A), appears in the MIIC at an intermediate stage of DC maturation (5–6 hr LPS) (Figure 7B). Again, after full maturation (24–96 hr LPS), all intracellular MHC class II molecules are translocated to the cell surface, and DC-LAMP is condensed into perinuclear lysosomes coexpressing high levels of LAMP-1 (Figure 7C). At both stages of maturation (intermediate-mature), DC-LAMP colocalizes with LAMP-1 (Figures 7E and 7F). Soluble antigens captured by DC transit through the MIIC (Nijman et al., 1995). We therefore performed a FITC-Dextran uptake for 1 hr at 37°C on nonactivated or for 6 hr on LPS-activated monocyte-derived DC. As expected, immature DC capture high levels of FITC-Dextran but do not express DC-LAMP, while class II molecules are still intracellular (Figures 8A and 8D). After 6 hr of LPS activation, DC-LAMP appears and colocalizes with FITC-Dextran, establishing that DC-$LAMP^+$ vesicles intersect with the endosomal compartment. At this intermediate stage of maturation, DC-LAMP is present in MHC class II^+ vesicles (Figure 7B), which also contain HLA-DM (Figure 8C) and display an acidic pH revealed by the accumulation of the lysotracker molecules (Figure 8F). Those observations are in agreement with the FACS data reported above.

Collectively, these results revealed that DC-LAMP appears transiently in the MIIC just before the translocation of class II molecules to the cell surface and next concentrates into perinuclear lysosomes.

Discussion

Dendritic cells constitute an heterogenous population of cells, present at trace level in all tissues. Few markers

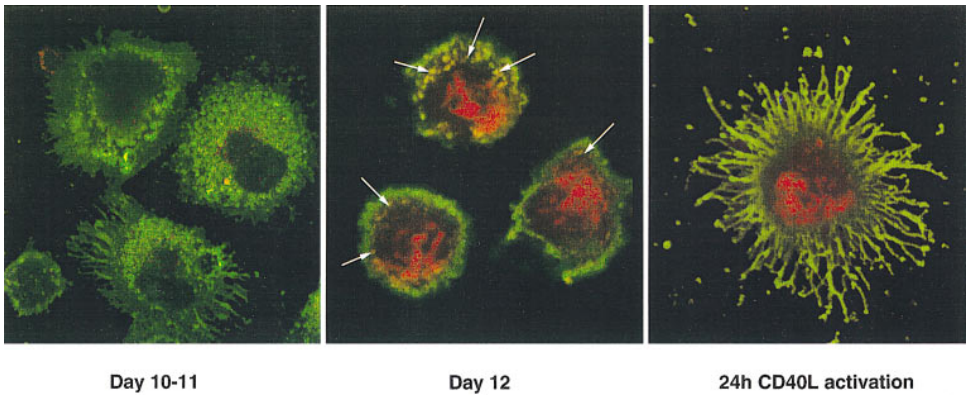

Figure 6. Analysis by Confocal Microscopy of the Subcellular Localization of DC-LAMP during the Maturation of $CD34^+$ Derived DC

Double-color confocal microscopy analysis of HLA-DR (green) and DC-LAMP (red) was performed on immature (day 10–11), intermediate (day 12), and fully mature (24 hr CD40L activation) $CD34^+$-derived DC. The superposition between the two fluorochromes (yellow color with arrows) is indicative of the colocalization of class II molecules and DC-LAMP in the same vesicles.

exist for the characterization of DC subtypes. We report here the identification of a novel member of the LAMP family, called DC-LAMP, which is specifically expressed by a subset of DC, the interdigitating dendritic cells (IDC). On the basis of sequence and domain homologies, DC-LAMP is clearly a member of the LAMP family of lysosomal associated glycoproteins. Common structural features of this family include a molecular mass of 90–120 kDa, a polypeptide core of ~40 kDa, a high number (7–20) of N-linked glycosylation sites in their luminal domain, a single transmembrane region, and a short cytoplasmic tail of 10–11 amino acids (Fukuda, 1991). As for the other LAMP members, 90% of DC-LAMP protein should be located in the lumen of lysosomes, and the intraluminal portion can be divided in two domains by a serine/proline-rich hinge region (Fukuda et al., 1988). DC-LAMP and CD68 share very similar predicted structure and are the only two members of this LAMP family with a single membrane-proximal (domain II) lamp-like domain, where the highly conserved

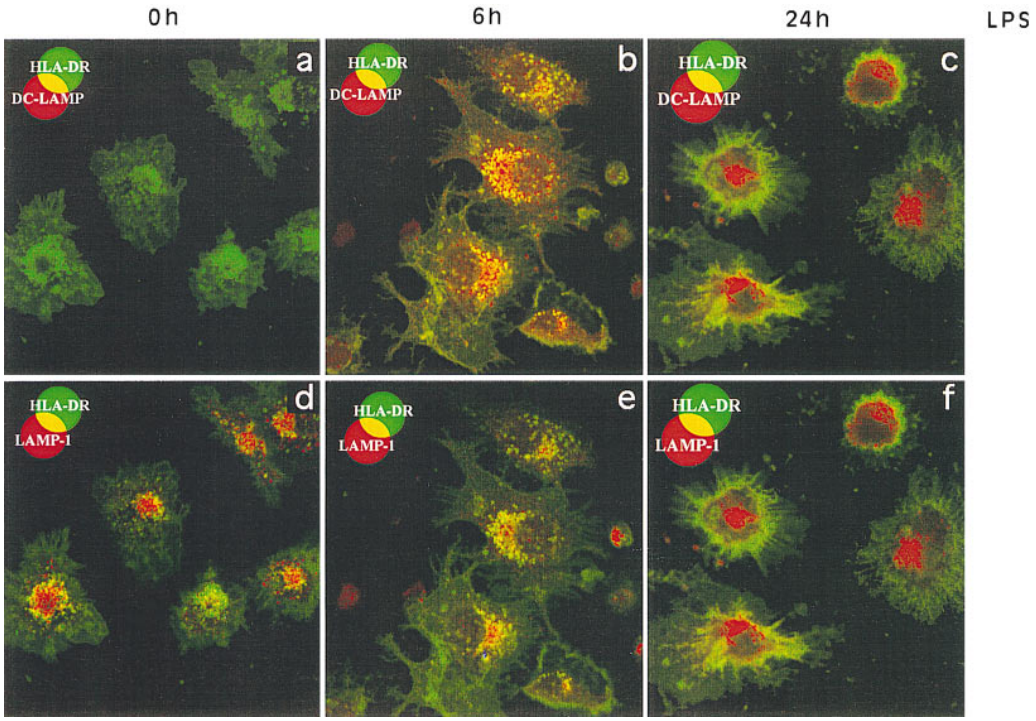

Figure 7. Analysis by Confocal Microscopy of the Subcellular Localization of DC-LAMP during Maturation of Monocyte-Derived DC

Triple-color confocal microscopy analysis of HLA-DR (green), DC-LAMP (red) (A, B, and C) and LAMP-1 (red) (D, E, and F) was performed on unactivated (0 hr LPS) monocyte-derived DC or after 6 and 24 hr LPS activation. The superposition between two fluorochromes (yellow color) is indicative of the colocalization of class II molecules and DC-LAMP or LAMP-1 in 6 hr activated monocyte-derived DC.

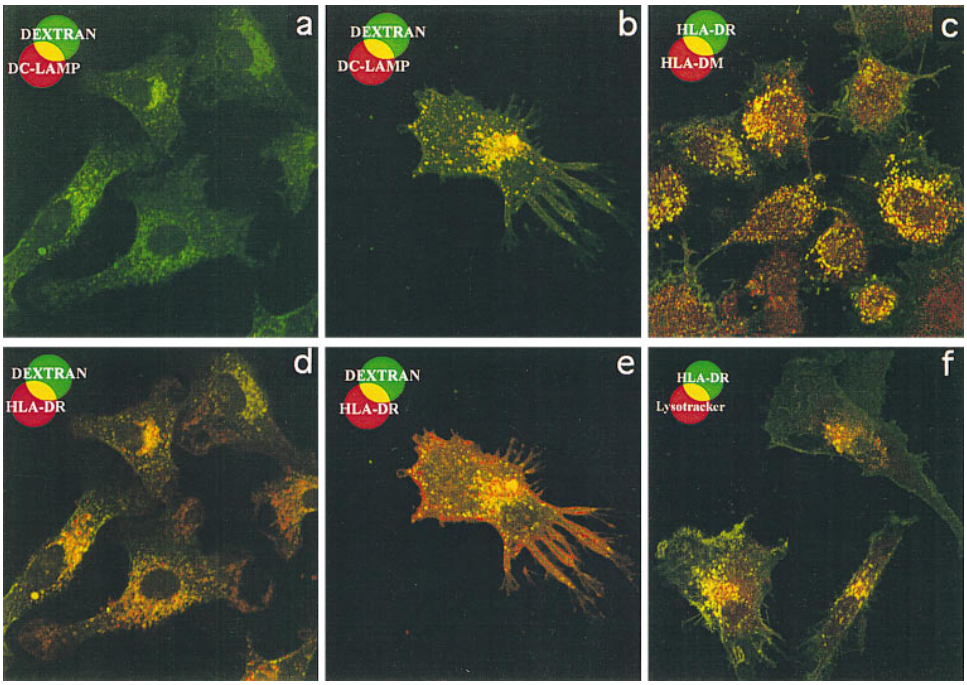

Figure 8. Characterization by Confocal Microscopy of the DC-LAMP$^+$ Compartment on LPS-Activated Monocyte-Derived DC

FITC-Dextran uptake for 1 hr at 37°C was performed with 0 or 6 hr LPS-activated monocyte-derived DC. Double staining of these cells was realized with DC-LAMP (red) (A and B) and HLA-DR (red) (D and E). The superposition between the two fluorochromes (yellow color) indicates that the DC-LAMP$^+$ HLA-DR$^+$ compartment intersects with FITC-Dextran loaded vesicles in 6 hr LPS-activated monocyte-derived DC.

6 hr LPS-activated monocyte-derived DC (see Figure 7B for HLA-DR and DC-LAMP on the same cells) were stained with HLA-DR (green) and HLA-DM (red) (C) or lysotracker (red) (F). The superposition between the two fluorochromes (yellow color) is indicative of a colocalization of class II molecules and HLA-DM in the acidic MIIC.

cysteines form disulfide bounds that probably stabilize the protein (Fukuda, 1991). In their N-terminal portion (domain I), CD68 and DC-LAMP contain a region rich in serines and threonines, which may bear O-linked glycan chains and is therefore considered as a mucin-like structure (Holness and Simmons, 1993). Preliminary immunoprecipitation studies revealed that, like the other members of the family, DC-LAMP is highly glycosylated: the observed mass of the mature glycoprotein on SDS-PAGE is around 70–90 kDa (data not shown), whereas the predicted mass for the polypeptide core is 44 kDa. The extensive glycosylation of LAMP molecules is thought to be important for protecting the polypeptide cores from proteolytic degradation within the lysosome (Barriocanal et al., 1986). Another conserved feature of LAMP molecules, a short cytoplasmic tail with a GY motif, essential for adressing the LAMP molecules to lysosomes, is also present in DC-LAMP (Williams and Fukuda, 1990; Harter and Mellman, 1992). Despite their structural homology and the high degree of conservation across different species, members of the LAMP family are located on different chromosomes: human LAMP-1, LAMP-2, CD68, and *DC-LAMP* are mapped on chromosomes 13q34, Xq24–25 (Mattei et al., 1990), 17p13, and 3q26–27, (D.R. Greaves, personal communication), respectively. This suggests that these proteins diverged relatively early in the evolution, probably acquiring distinct functions. Of note, to date no monogenic human disease has been linked to any of the LAMP member loci.

DC express a repertoire of molecules common to other leukocytes and a high level of MHC molecules, but, up to now, very few MAbs are truly DC specific. Immature, epithelial Langerhans cells are characterized by expression of CD1a (Fithian et al., 1981), Lag (Kashihara et al., 1986), and Langerin, a protein recently identified in our laboratory (unpublished data), but they lack DC-LAMP. In contrast, immunohistological analysis shows DC-LAMP to be an IDC-specific marker. IDC are mature DC located in T cell areas of lymphoid tissues, characterized by a strong expression of MHC class II molecules, a low Ag uptake capacity, and a potent ability to stimulate naive T cells (Steinman, 1991). They express a set of markers shared by other cells, including a high level of MHC class II, CD80, CD86, and CD40, as well as CD83 (Zhou et al., 1992; Björck et al., 1997), and p55 (Mosialos et al., 1996). The correlation between DC-LAMP expression and DC maturation was directly established by in vitro studies. While DC-LAMP is absent in immature DC (day 8 CD34$^+$-derived DC, monocyte-derived DC), it is rapidly up-regulated (3–9 hr in monocyte-derived DC) upon maturation induced by TNFα, LPS, or CD40L. Moreover, detection of DC-LAMP correlates with strong expression of CD86 and surface MHC class II molecules, which are both features of the end stage of DC maturation. While DC-LAMP MAb stains exclusively IDC on tissue sections, a low level of DC-LAMP mRNA expression could be induced upon activation in vitro in other APC types (monocytes, macrophages, and B cells), further suggesting a role for this novel lysosomal protein in antigen presentation.

The tight control of DC-LAMP expression during DC maturation was confirmed by confocal microscopy, and a striking distribution of the molecule was observed.

Early immature DC, which express most of their MHC class II molecules intracellularly, could not be stained with anti-DC-LAMP MAb. DC-LAMP was first detected at an intermediate maturation stage (6 hr LPS) within small intracellular vesicles spread through the cytoplasm as well as within perinuclear lysosomes. At this stage of maturation, the peripheral DC-LAMP$^+$ structures represent acidic vesicles that intersect with the endosomal compartment and contain MHC class II molecules and HLA-DM as well as lysosomal markers LAMP-1, LAMP-2, and CD68, to a weaker extent. Therefore, DC-LAMP first appears in the so-called lysosomal MHC class II$^+$ compartment (MIIC), which has been initially described in human B cells (Peters et al., 1991) and, more recently, in immature DC (Nijman et al., 1995; Pierre et al., 1997). The MIIC is thought to be the site of efficient peptide loading on MHC class II molecules in human B cells (Rudensky et al., 1994; Tulp et al., 1994; West et al., 1994). Thus, the appearance of DC-LAMP parallels the remodeling of the endosome/lysosome compartment observed during human DC maturation, reminiscent of a recently published study on mice by Pierre et al. (1997). Of note, during hourly kinetic study of human monocyte–derived DC maturation (Figure 7; data not shown), we have been unable to detect by confocal microscopy the nonlysosomal class II$^+$ LAMP-1$^-$ vesicles (CIIV), first identified in murine B cells (Amigorena et al., 1994). Whether this corresponds to a true difference between human and mouse DC (Pierre et al., 1997) will need further investigation. Induction of DC maturation by either LPS or TNFα is accompanied by a transient burst of MHC class II gene transcription, an increase of MHC class II protein half-life, an enhanced formation of MHC-class II–peptide complexes, and their prompt transfer to the cell surface (Cella et al., 1997). Increased proteolytic degradation of Ii by cathepsin favors the loading of peptides onto MHC class II (Creswell, 1998) molecules and the translocation of these molecules to the cell surface. Whether the accumulation of DC-LAMP protein in the peripheral MIIC and the progressive disappearance of CD68 also participate in MHC class II remodeling remains to be established. In any case, at later stages of DC maturation, the MIIC vanishes as all the MHC class II molecules move to the cell surface (Cella et al., 1997; Pierre et al., 1997), while DC-LAMP, LAMP-1, and LAMP-2 (Fukuda et al., 1988; Williams and Fukuda, 1990), which all contain the conserved GY lysosomal targeting motif, concentrate into perinuclear lysosomes.

Comparison with other LAMP proteins may provide additional clues regarding the functions of DC-LAMP. LAMP-2 has been described as a receptor for selective uptake and degradation of cytoplasmic proteins within lysosomes (Cuervo and Dice, 1996). As only the GY motif is conserved between the rat LAMP-2 cytosolic 12 amino acid sequence implicated in this uptake and DC-LAMP, it is unlikely that DC-LAMP will have a similar function. LAMP-1, LAMP-2, and CD68 are mostly found in lysosomes, but they can also be detected at the cell surface, especially of tumor cells (Lippincott-Schwartz and Fambrough, 1987; Ramprasad et al., 1996). Their highly glycosylated domain has been implicated in binding to extracellular lectins and in cell migration (Saitoh et al., 1992; Garrigues et al., 1994). It is unknown whether in some circumstances DC-LAMP could play a similar role at the DC surface. The reciprocal expression of CD68 and DC-LAMP by DC suggests that despite their structural homology, these two molecules may serve distinct functions. It has been proposed that through its mucin-like domain, CD68 could favor an efficient presentation by MHC class II molecules (Rabinowitz and Gordon, 1991; Holness and Simmons, 1993). Moreover, surface CD68 binds oxidized low-density lipoprotein (Ramprasad et al., 1996) and shuttles rapidly between the endosomes and the plasma membrane. It also recognizes phosphatidyl-rich liposomes and may facilitate the capture of apoptotic bodies. Indeed, despite a very weak expression at the cell surface, the conserved Y residue is likely to promote an efficient internalization of DC-LAMP (Harter and Mellman, 1992).

In conclusion, we have identified a novel member of the LAMP family, which represents a marker specific to mature human dendritic cells. The presence of DC-LAMP in the MIIC in late mature DC indicates that this may serve an important function during the processing of exogenous antigens. DC-LAMP might also participate in the functional remodeling of the MIIC by facilitating the translocation of MHC class II molecules to the cell surface. However, direct information about the role of DC-LAMP will await the analysis of mice deficient for this gene.

Experimental Procedures

Hematopoietic Factors, Cells, and Cell Lines

rhGM-CSF, rhTNFα, rhSCF, and rhM-CSF were used at optimal concentration as described (Caux et al., 1996). rhG-CSF (ED_{50}, 0.01–0.03 ng/ml; R & D, Abington, UK) was used at an optimal concentration of 25 ng/ml.

PBMC and T cells were purified using standard protocols (Bates et al., 1997). B cells were obtained from human tonsils as described (Liu et al., 1996). Langerhans cells were prepared from normal skin by CD1a positive selection. A purity higher than 95% was systematically achieved. Germinal center dendritic cells (GCDC) were prepared as previously described (Grouard et al., 1996). The purity of GCDC was higher than 97%. GCDC were stimulated either by PMA-ionomycin for 3 hr or by an anti-CD40 MAb (10 μg/ml G28–5 MAb; kindly provided by Ed. Clark, University of Washington, Seattle, WA) for 20 hr.

Granulocytes and macrophages were generated in vitro from CD34$^+$ progenitors in the presence of G-CSF and SCF for 12 days, and M-CSF and SCF for 12 days, respectively. Cells were either nonactivated or activated by PMA-ionomycin for 1 and 6 hr (PMA, 1 ng/ml; Sigma, St Louis, MO) (ionomycin, 1 μg/ml; Calbiochem, La Jolla, CA) and pooled. The TF1, Jurkat, MRC5, JY, and U937 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). CHA is an epithelial kidney carcinoma cell line kindly provided by C. Bain (Centre Léon Bérard, Lyon, France). All cell lines stimulated by PMA-ionomycin for 1 and 6 hr were pooled. Murine fibroblasts transfected with human CD40 ligand (CD40L L cells) were produced in the laboratory. All cell types were cultured in complete RPMI 1640 (GIBCO BRL, Gaithersburg, MD) (Caux et al., 1996).

Generation of DC from CD34$^+$ Cells and from Monocytes

Isolation of CD34$^+$ progenitors from umbilical cord blood samples was achieved using Minimacs separation columns (Miltenyi Biotec GmbH, Bergish Gladbach, Germany). Cultures of CD34$^+$ cells were established in the presence of SCF, GM-CS,F and TNFα as described (Caux et al., 1996). At day 12–17, 70%–90% of cells were CD1a$^+$ DC. Monocytes were purified by immunomagnetic depletion (Dynal, Oslo, Norway) after preparation of PBMC, followed by a 52% Percoll gradient. Monocyte-derived DC were produced by culturing

purified monocytes for 6 days in the presence of GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). Cells were activated with LPS (Sigma) at the concentration of 25 ng/ml for 1–72 hr.

cDNA Libraries and Isolation of the E02B02 cDNA Clone

Total RNA was isolated from PMA-ionomycin activated $CD1a^+$ $CD14^-$-derived DC (at day 12 of the culture) (Caux et al., 1996) and from PMA-ionomycin-activated CHA cell line following standard procedures. RNA was treated with DNase I before mRNA purification using the Oligotex-dT kit (Qiagen GmbH, Hilden, Germany). $PolyA^+$ RNA (2 μg) was used to make a cDNA library in the pSport vector (Superscript Plasmid System Kit, GIBCO BRL). A library subtraction cDNA was performed as described (Bates et al., 1997). In this protocol, the tracer (subtracted) was the $CD1a^+$-derived DC cDNA, and the driver (subtractive) was CHA cDNA. A 0.7 kb cDNA containing a poly(A) tail was isolated from the $CD1a^+$-derived DC subtraction library.

The full-length cDNA of this gene was amplified using the RACE Marathon kit (Clontech, Palo Alto, CA) and two oligonucleotides, 5′-TATTTGATGCCTTCATCTTTACAGCCCA (NGSP1) and 5′-CACTCAA GAAAGAACAGTTGGTTAGCGA (GSP1), with the recommended cycling program 1. PCR products were cloned in the pCRII plasmid (Invitrogen, San Diego, CA) and sequenced using a Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an automated sequencer (Applied Biosystems).

Northern Blot Analysis and RT–PCR

Northern blots were performed with total RNA (20 μg) hybridized with the original cloned 722 bp fragment labeled by random priming with [^{32}P]dCTP, as described (Bates et al., 1997). Multiple human adult and fetal tissue blots (Clontech) were similarly used. For RT–PCR, total RNA was reverse transcribed using random hexamer primers (Pharmacia, Upsalla, Sweden) and the Superscript RNase-H reverse transcriptase (GIBCO BRL). PCR was performed in a DNA thermal cycler (Perkin Elmer) for 35 cycles (1 min denaturation at 94°C, 1 min annealing at 60°C, and 2 min elongation at 72°C) with Taq polymerase (gene Amp PCR reagents kit; Perkin Elmer Cetus). β-actin RT–PCR was used as control for the efficiency of the reaction. Sense (nucleotides 99–118, 5′-GCACGATGGCAGTCAAATGA) and antisense (nucleotides 825–806, 5′-GAAGTATCTCCGAGGTGA AA) primers were used to amplify E02B02.

Production of Recombinant E02B02 and Generation of MAb

Human recombinant E02B02 was expressed in *E. coli* as Ig fusion and alkaline phosphatase fusion proteins. A HindIII–XhoI fragment was derived from PCR with E02B02 cDNA as template. The fragment was inserted into a modified form of pCDM8 (InVitrogen, Carlsbad, CA). This vector had the pCDM8 XhoI–NotI region replaced with a fragment encoding human immunoglobulin G1, Fc (residues 74–768 of EMBL accession X70421), or mature human placental alkaline phosphatase without its C-terminal membrane attachment domain (residues 301–1749 of EMBL accession U09661). Recombinant plasmids were electroporated in COP5 cells, and after 5–7 days of culture, Ig fusion protein (~50 kDa) was purified from supernatant by HiTrapA chromatography (Pharmacia, Upsalla, Sweden). E02B02-alkaline phosphatase recombinant fusion protein was purified by DEAE anion exchange chromatography on a Zephyr-D silicium column (IBF Sepracor, Villeneuve, France).

Mouse monoclonal antibodies against E02B02 were generated in BALB/c mice as previously described (Fossiez et al., 1996), with three successive intraperitoneal injections of Freund's adjuvant (Sigma) and 1 μg of purified E02B02 Ig fusion protein. Hybridoma supernatants were selected on E02B02-alkaline phosphatase transfected COP5. Antibody binding was revealed with peroxidase-conjugated sheep anti-mouse IgG (Biosys, Compiègne, France).

Immunohistological Localization of $DC\text{-}LAMP^+$ Cells

Double stainings on human lymphoid tissue sections were performed as previously described (Liu et al., 1996). In brief, human tissue sections were stained using mouse IgG2a anti-CD3 (Pharmingen, San Diego, CA), mouse IgG2b anti-CD83 (Immunotech), and mouse IgG3 anti-CD68 (Dako, Glostrup, Denmark) together with mouse IgG1 anti-DC-LAMP (produced in our laboratory). Human tonsil sections were also stained using mouse IgG1 anti-CD40 (MAb 89, produced in our laboratory), saturated for 10 min with 10% mouse serum, and stained again using mouse anti-DC-LAMP-biotin. Skin sections were stained using mouse Ig2b anti-CD1a (Becton Dickinson, Mountain View, CA) together with anti-DC-LAMP. The binding of mouse IgG1 antibodies was revealed by sheep anti-mouse IgG1 (The Binding Site, Birmingham, UK), followed by incubation with alkaline phosphatase coupled to mouse antibodies specific for alkaline phosphatase (APAAP complexes, Dako). The binding of mouse IgG2a, IgG2b, and IgG3 antibodies were respectively revealed by biotinylated sheep anti-mouse IgG2a, IgG2b, and IgG3 (The Binding Site), followed by ExtrAvidin-peroxidase (Sigma). Alkaline phosphatase activity was developed by the Fast Blue substrate (Sigma), whereas peroxydase activity was developed by 3-amino-9-ethylcarbazole (Sigma).

Cytofluorimetric Analysis of Intracellular and Cell Surface Phenotype

For intracellular staining, cells were incubated with mouse IgG1 anti-DC-LAMP (10 μg/ml) for 30 min at 4°C in the presence of permeabilization medium (0.1% saponin and 1% SVF). After two washes, cells were incubated with FITC-goat anti-mouse Ig (Dako). Single-color surface immunofluorescence was performed according to standard techniques using FITC-CD86 or PE-CD86 (Pharmingen). Negative controls were performed with unrelated isotype-matched murine MAbs. Fluorescence was analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Confocal Microscopy

Intracellular immunofluorescence staining was conducted as previously described (Rovere et al., 1998). On polylysin-coated coverslips, cells fixed for 15 min with 4% paraformaldehyde in PBS were washed twice in 10 mM glycine in PBS and twice in PBS and permeabilized with 0.5% saponin-1% BSA-PBS for 30 min. Coverslips were incubated for 30 min at room temperature with 5 μg/ml anti-LAMP-1 (Pharmingen), anti-HLA-DR (Becton Dickinson), anti-DC-LAMP, or anti-HLA-DM in permeabilization medium. Rabbit anti-HLA-DM was raised against a peptide encoding the cytoplasmic tail of the HLA-DM β chain.

After three washes, cells were incubated for 30 min with secondary labeled antibodies (donkey anti-mouse coupled to Texas red or Cyanin 5 [Vector Laboratories, Burlingame, CA]), washed, incubated with mouse preimmune serum for 30 min, washed again, postfixed with 2% paraformaldehyde, incubated for 30 min with a second primary antibody coupled to biotin, rinsed, and labeled with fluorescein-conjugated streptavidin or with a second primary antibody directly coupled to fluorescein. Uptake of FITC-Dextran (Molecular Probes, Eugene, OR) was performed for 1 hr at 37°C before or after 5 hr of LPS treatment. Fixed cells were labeled with HLA-DR and DC-LAMP as above. Visualization of acid pH compartments was performed using the weakly basic amine lysotracker (Molecular Probes) that selectively accumulates in low internal pH cellular compartments. The cells were incubated with the lysotracker for 1 hr at 37°C and fixed. Coverslips were mounted onto glass slides with Fluoromount (Southern Biotechnology Associates, Birmingham, AL). Confocal microscopy was performed using the Confocal Laser Scanning Microscopy TCS 4D (Leica Lasertechnik GmbH, Heidelberg, Germany) (Rovere et al., 1998).

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EMBL Accession Number

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