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Langerin, a Novel C-Type Lectin Specific to Langerhans Cells, Is an Endocytic Receptor that Induces the Formation of Birbeck Granules

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Summary

We have identified a type II Ca^{2+}-dependent lectin displaying mannose-binding specificity, exclusively expressed by Langerhans cells (LC), and named Langerin. LC are uniquely characterized by Birbeck granules (BG), which are organelles consisting of superimposed and zippered membranes. Here, we have shown that Langerin is constitutively associated with BG and that antibody to Langerin is internalized into these structures. Remarkably, transfection of Langerin cDNA into fibroblasts created a compact network of membrane structures with typical features of BG. Langerin is thus a potent inducer of membrane superimposition and zippering leading to BG formation. Our data suggest that induction of BG is a consequence of the antigen-capture function of Langerin, allowing routing into these organelles and providing access to a nonclassical antigen-processing pathway.

Introduction

Dendritic cells (DC) are the antigen-presenting cells required to initiate specific T cell immunity (Banchereau and Steinman, 1998). DC residing in nonlymphoid tissue are immature cells that capture antigen via specialized endocytic receptors or by fluid-phase macropinocytosis (Steinman and Swanson, 1995; Watts, 1997). DC process exogenous antigen within MHC class II-rich endosome/lysosome compartments to allow presentation to $CD4^+$ T cells. DC also have a remarkable capacity for cross-priming, a mechanism by which exogenous antigen is alternatively routed into the MHC class I pathway for presentation to $CD8^+$ cytotoxic T cells (CTL) (Nair et al., 1992; Paglia et al., 1996; Albert et al., 1998). Recently, cross-priming was demonstrated in mouse DC following internalization of immune complexes via Fcγ-receptors (Regnault et al., 1999).

Langerhans cells (LC) are immature DC of the epidermis and mucosal tissues. LC express several receptors for antigen uptake, including the Fcγ and Fcϵ receptors (de la Salle et al., 1997) and the DEC205 multilectin (Jiang et al., 1995). However, LC do not express the macrophage mannose-receptor multilectin (Linehan et al., 1999; Mommaas et al., 1999), used for antigen capture by other DC subsets (Sallusto et al., 1995). Expression of different sets of antigen receptors may thus confer antigen-type and intracellular-processing specificity to the different DC subsets.

Ultrastructurally, LC are characterized by a unique pentalamellar cytoplasmic organelle, the Birbeck granule (BG) (Birbeck et al., 1961). BG are disks with a "tennis-racket" appearance consisting of two superimposed membranes separated by leaflets with periodic "zipper-like" striations (Wolff, 1967). Although first identified in 1961, BG remain enigmatic. Thus, the molecular signals leading to their formation are unknown, and insight into their function is limited. Two subcellular origins of BG have been described. An origin from the Golgi apparatus, associated with a secretory function, has been suggested by the intracellular localization of BG (Zelickson, 1966; Wolff, 1967). In contrast, BG have been shown to arise from the cell membrane by an endocytic pathway (Hashimoto and Tarnowski, 1968; Takigawa et al., 1985; Bartosik, 1992). The latter origin is supported by the ability of the LC cytomembrane to superimpose upon itself, resulting in BG-like morphology connected with the extracellular space and known as cytomembrane sandwiching structures (CMS) (Bartosik et al., 1985). The endocytic pathway of BG formation predicts that, by engulfing portions of the cytomembrane, BG could play a role in the capture and subsequent intracellular routing of material, either soluble or bound to receptors, that becomes trapped in these organelles. Indeed, it has been demonstrated that antibody to CD1a (Hanau et al., 1987), ferritin-conjugated concanavalin A (Takigawa et al., 1985), or horseradish peroxidase (Bartosik, 1992) may be internalized into BG, presumably as a result of passive engulfment. By extension, the existence of an active mechanism by which antigen specifically gains access to the BG can be hypothesized.

We previously described monoclonal antibody (mAb) DCGM4, which recognizes an epitope specific to LC (Valladeau et al., 1999). We have now characterized the molecule recognized by DCGM4, which we have termed Langerin. Langerin is a novel transmembrane C-type lectin involved in ligand internalization and associated with CMS and BG. Introduction of Langerin cDNA into fibroblasts strikingly resulted in BG accumulation. Our results identify Langerin as an LC-specific endocytic

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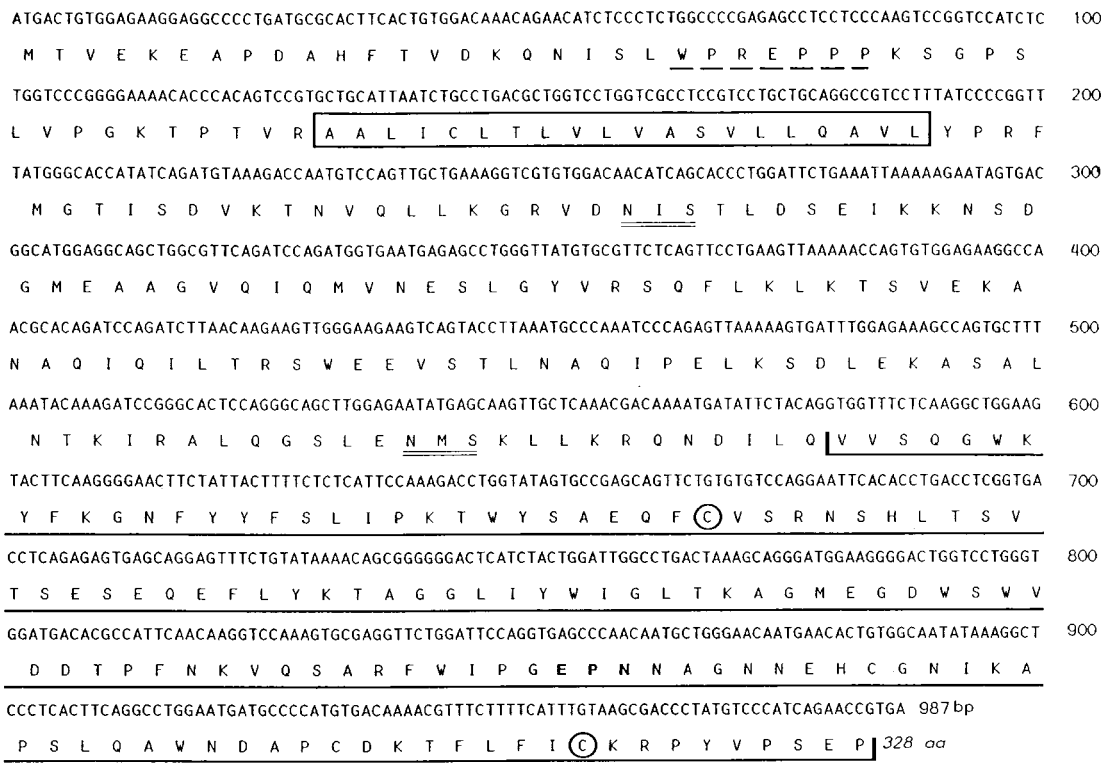

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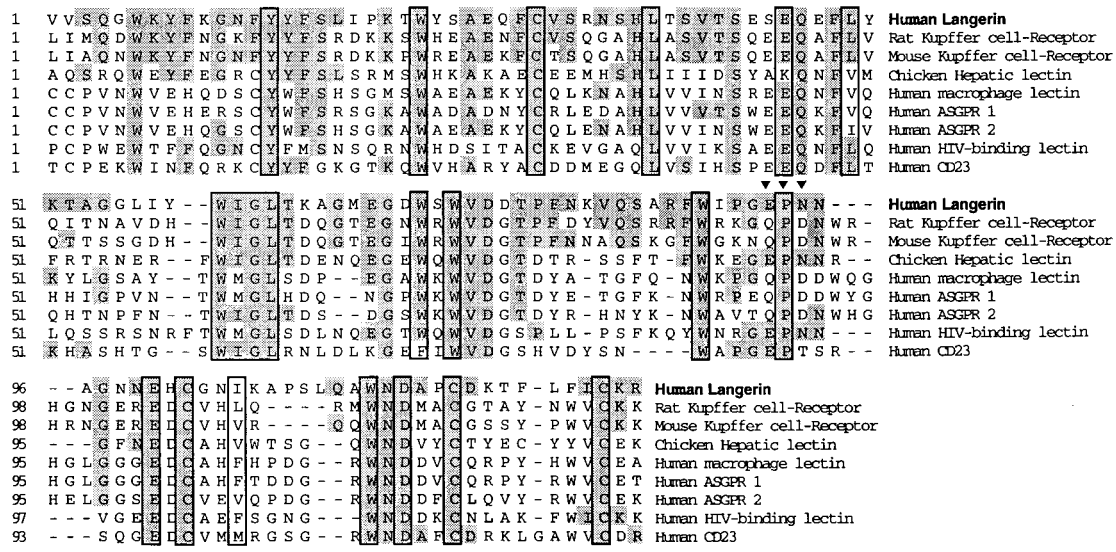

Figure 1. Sequence Analysis of Human Langerin

(A) Nucleotide and amino acid sequences. Sequences shown were obtained from Langerin cDNA clone M4T24C6 (accession number AJ242859). The boxed sequence (hydrophobic amino acids 44–63) forms a signal anchor expected to represent the transmembrane domain. The C-type lectin domain with two cysteines forming a disulfide bridge (rounded) is shown in single underline. Broken and double underlines (positions

receptor that induces BG formation, and thus as a potentially important component of a nonconventional pathway for antigen capture.

Results

Langerin Is a Type II C-Type Lectin with an Intracytoplasmic Proline-Rich Motif

We used transient expression cloning in murine fibroblastic COP5 cells to identify Langerin, the molecule recognized by the LC-specific mAb DCGM4 (Valladeau et al., 1999). A full-length 1999 bp cDNA sequence was isolated (M4T24C6) following transfection of COP5 with a cDNA library prepared from CD34-derived DC supplemented with TGFβ. Clone M4T24C6 contains a 208 bp 5′ UTR, a 987 bp open reading frame, a 3′ UTR of 833 nucleotides, and a polyadenylation signal AATAAA at position 1836 bp followed by a poly(A) tail of 132 bp (data not shown). The open reading frame predicts a novel polypeptide of 328 amino acids (aa) (Figure 1A) with a molecular mass of 37.5 kDa, in accordance with the 40 kDa observed by immunoprecipitation of the natural protein (Valladeau et al., 1999). The presence of a hydrophobic signal anchor (residues 44–63) indicates a type II transmembrane protein. Langerin belongs to the Ca^{2+}-dependent (C-type) lectin family with a single carbohydrate recognition domain (CRD) (Figure 1A) featuring a glutamate-proline-asparagine (EPN) motif (position 285–287) predicting mannose-type specificity (Weis et al., 1998). In accordance with the predicted Ca^{2+} dependency, treatment of LC with the chelating agent EDTA resulted in loss of reactivity with mAb DCGM4 (data not shown). Two potential N-glycosylation sites are present at positions 87–89 and 180–182 aa. Finally, Langerin contains an intracellular domain of 43 aa with a proline-rich motif (WPREPPP) as a potential signal transduction site (Ren et al., 1993; Cohen et al., 1995).

Amino acid sequence comparison in the EMBL database revealed marked homology between Langerin and several type II lectins, mainly in their CRD domain. Langerin CRD displays the highest homology with the rodent Kupffer cell receptors (43.5% aa identity) (Hoyle and Hill, 1988), followed by the chicken hepatic lectin (32.8% aa identity) (Bezouska et al., 1991), the human macrophage lectin (Suzuki et al., 1996), and the hepatic asialoglycoprotein receptors (Spiess and Lodish, 1985; Spiess et al., 1985) (Figure 1B).

Finally, using the Stanford G3 radiation hybrid panel, the human *Langerin* gene was mapped to chromosome 2p13 (data not shown).

Langerin mRNA Is Specifically Expressed by Langerhans Cells

As judged by semiquantitative RT–PCR, Langerin mRNA is abundant in freshly isolated LC and is detected at lower levels in resting DC derived from $CD34^+$ progenitors cultured with GM-CSF and TNFα (Caux et al., 1992). Notably, expression was downregulated following DC activation through CD40-ligation or PMA/ionomycin (Figures 2A and 2B). In contrast, no Langerin mRNA expression could be detected in monocytes, T lymphocytes, granulocytes, PBL, B cells, or skin basal cells (Figures 2A and 2B). Furthermore, analysis of various cell lines indicated that Langerin was absent in myeloerythrocytic TF1 cells, CHA kidney carcinoma, Jurkat T cells, MRC5 lung fibroblasts, JY lymphoblastoid B cells, and U937 monocytic cells (Figure 2A). In normal human tissues, a strong band at the expected size of 2.0 kb was detected by Northern blot in lung but not in pancreas, kidney, skeletal muscle, liver (adult and fetal), placenta, brain, heart, bone marrow, or peripheral blood (Figure 2C; data not shown). An additional faint band was observed at about 3.0 kb, but its identity is currently unknown. In line with the protein distribution previously reported (Valladeau et al., 1999), these results indicate that Langerin mRNA is restricted to LC.

Anti-Lag Antibody Recognizes an Intracellular Epitope of Langerin

The mAb anti-Lag ("Langerhans-associated granule") described by Kashihara and colleagues recognizes an intracellular epitope associated with BG (Kashihara et al., 1986). However, the identity of the 40 kDa antigen detected with anti-Lag has not been determined. We examined the relationship between Langerin and Lag. As shown in Figure 3A, immunoprecipitation of Langerin from CD34-derived DC supplemented with TGFβ revealed a 40 kDa band by Western blotting either with anti-Langerin or anti-Lag mAbs, indicating that the two antibodies recognize the same molecule. Furthermore, reactivity with anti-Lag was specifically abrogated on COP5 fibroblasts transfected with a truncated Langerin construct (D1) lacking the N-terminal portion of the cytoplasmic domain (Figure 3B), demonstrating that anti-Lag recognizes an intracellular epitope of Langerin.

Langerin Is Constitutively Associated with CMS and BG

The constitutive subcellular localization of Langerin was further characterized. DC were obtained in vitro from cord blood $CD34^+$ progenitors cultured with GM-CSF and TNFα and complemented with TGFβ to increase Langerin expression and the proportion of LC (Valladeau et al., 1999). Using confocal laser microscopy, Langerin was readily detected at the cell surface (Figure 4A). Furthermore, intracellular staining after cytomembrane permeabilization indicated colocalization with anti-Lag reactivity (Figure 4B), confirming association of the two epitopes in LC.

As anti-Lag recognizes BG, and as Langerin is downregulated during LC maturation (Figure 2B; Valladeau et al., 1999) in coincidence with the loss of BG (Romani et al., 1989), we further examined the relationship between Langerin and BG. Immunoelectron microscopy on fixed

87–89 and 180–182) indicate the proline-rich domain and potential N-glycosylation sites, respectively.
(B) Analysis of the C-type lectin domain (CRD) of human Langerin. Alignments of the lectin domain of Langerin and other C-type lectins are indicated as follows: conserved amino acids are shaded and residues of the consensus motif of this family are boxed. Open arrows show the part of the Ca^{2+} binding site involved in sugar specificity.

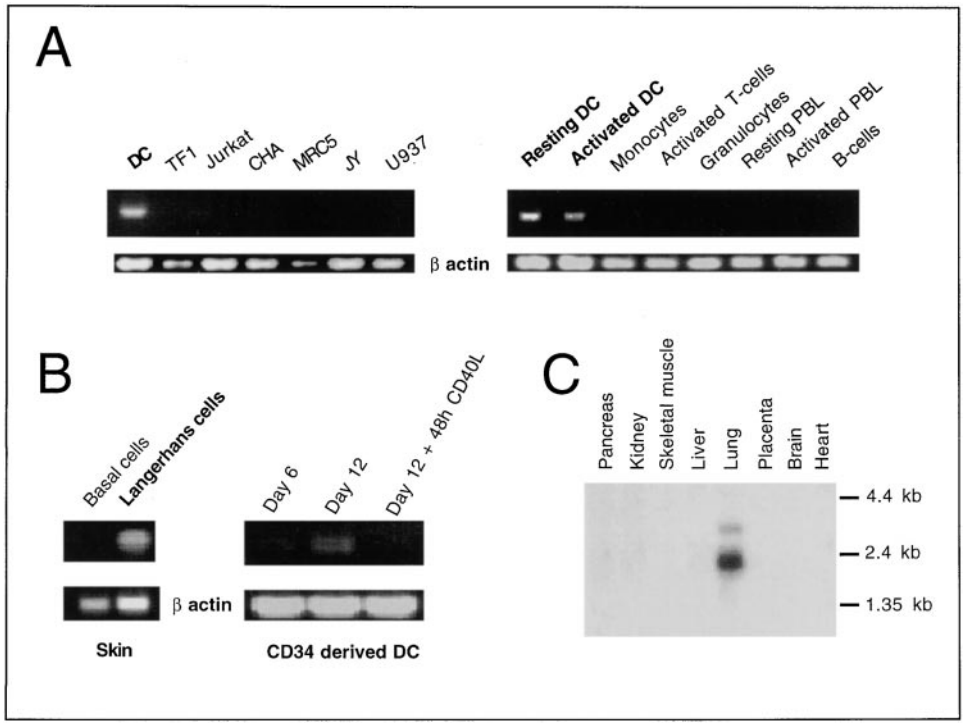

Figure 2. Analysis of Human Langerin mRNA Expression

(A and B) RT–PCR analysis showing restriction of expression of Langerin to freshly isolated LC and DC derived from $CD34^+$ progenitors. Northern blot analysis demonstrating the prominent presence of a 2.0 kb band in lung tissue is shown in (C).

DC demonstrated that in contrast to anti-CD1a antibody, which uniformly stains the LC cytomembrane (data not shown), cell surface Langerin was not randomly distributed at the plasma membrane but instead accumulates in deep striated invaginations open to the outside of the cell (Figures 4C and 4D). Such structures represent the CMS that lead to BG formation (Hashimoto and Tarnowski, 1968; Bartosik et al., 1985). Finally, Langerin was readily detected constitutively inside intracellular BG but not in MHC class II–rich compartments (Figure 4E). These findings demonstrate the preferential localization of Langerin in BG and in their precursor structures at the cytomembrane.

Engagement of the Lectin Domain of Langerin Induces Rapid Internalization into BG

In order to study trafficking mediated via cell surface Langerin, we examined freshly isolated LC before fixation.

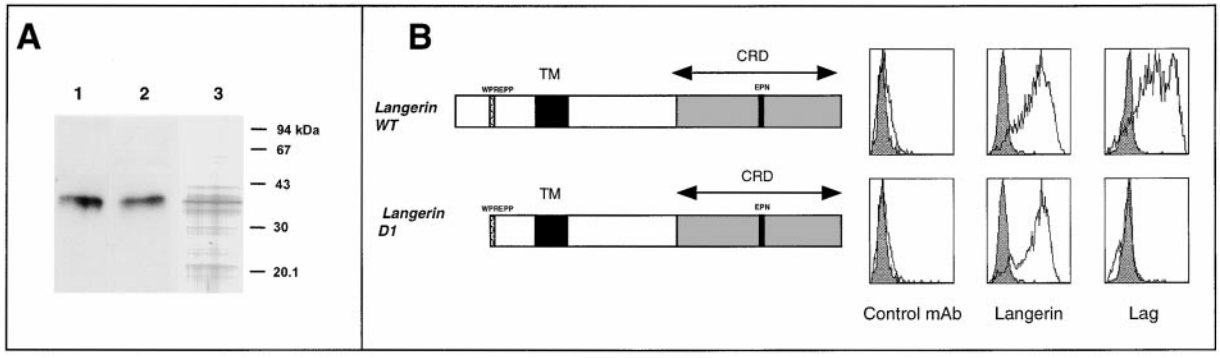

Figure 3. Monoclonal Antibody Anti-Lag Recognizes an Intracellular Epitope of Langerin

(A) Immunopurified Langerin is detected both by DCGM4 (lane 1) and anti-Lag (lane 2) mAbs revealed with ECL Western blotting system. Silver staining of Langerin is shown in lane 3.

(B) Anti-Lag reactivity is specifically abrogated in COP5 fibroblasts expressing NH_2-truncated Langerin (clone D1). COP5 cells were transfected with wild-type Langerin or with clone D1 cDNA, permeabilized with saponin, and stained with DCGM4, anti-Lag, or an isotype control mAb for FACS analysis (open histograms). Control fibroblasts were analyzed after transfection of unrelated cDNA (asialoglycoprotein receptor) (gray histograms).

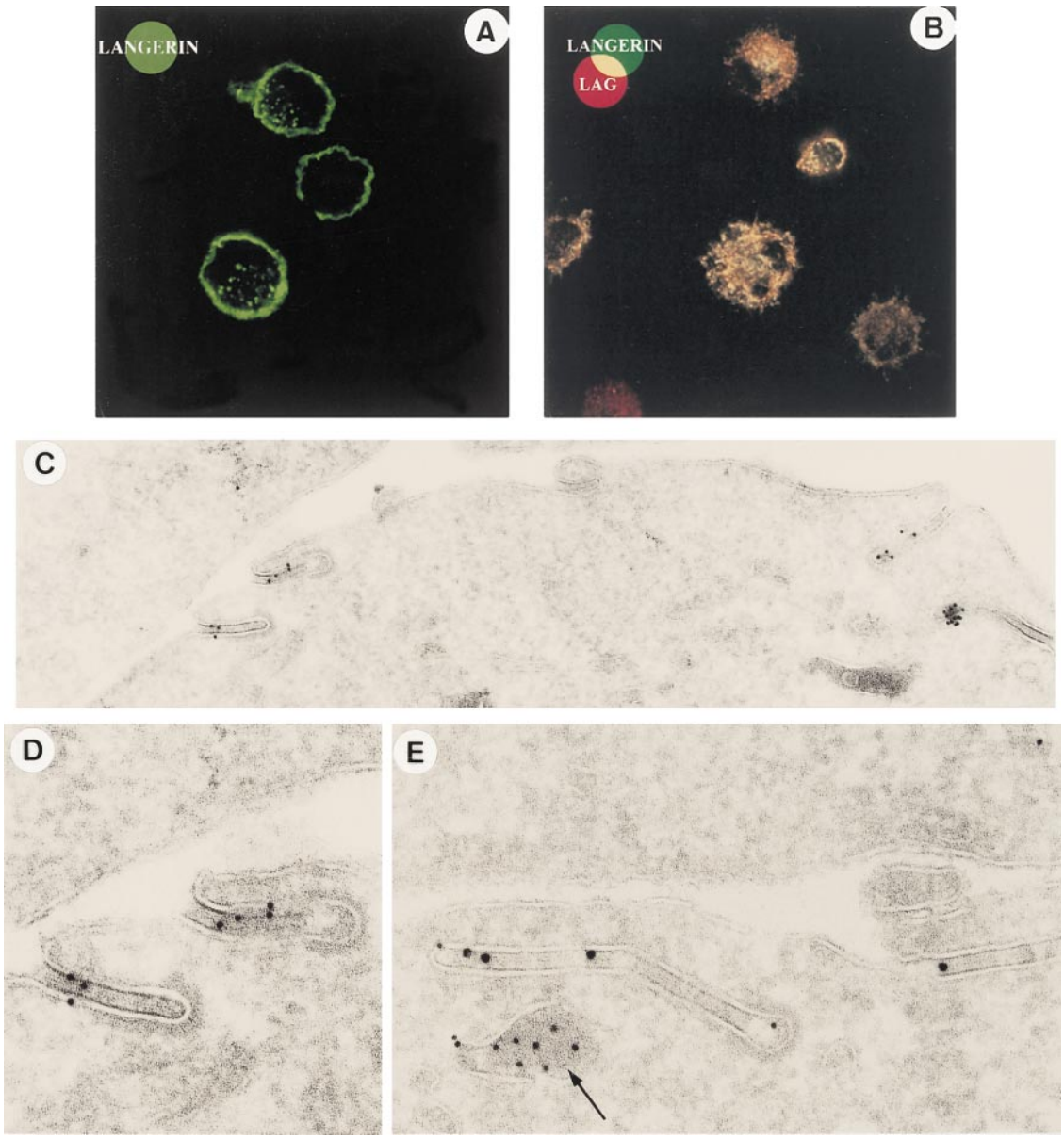

Figure 4. Langerin Is Constitutively Associated with CMS and BG

TGFβ-derived LC were fixed and subsequently stained and processed for confocal laser microscopy (A and B) or electron microscopy (C–E). (A) Langerin (green) is detected at the LC surface, whereas anti-Langerin and anti-Lag (red) epitopes colocalize intracellularly (yellow) in permeabilized cells (B). Langerin (10 nm gold) is associated with cytomembrane sandwiching structures (CMS) and with intracellular Birbeck granules (BG) (C–D). By contrast, double immunolabeling with DCGM4 (15 nm gold) and anti-MHC class II (10 nm gold) reveals that Langerin is absent from MIIC (arrow) (E).

The process of vital staining with mAb DCGM4 significantly increased the number of invaginated and superimposed cytomembrane structures (CMS) (Figure 5A) as compared to anti-CD1a antibody (Figure 5B). In addition, the CMS were stained with the anti-Langerin mAb (Figure 5A). When cells were warmed up to 37°C, mAb DCGM4 staining was rapidly observed inside BG and sometimes in coated vesicles (CV) (Figures 5C and 5D). We previously described that internalization of anti-Langerin for 20 min at 37°C did not result in colocalization with MHC class II molecules (Valladeau et al., 1999), in striking contrast to the above BG routing. This result was confirmed here using a number of time points from 5 min to 2 hr (data not shown). In agreement with the microscopy data, internalization of anti-Langerin mAb is extremely rapid even in the absence of antibody cross-linking, with kinetics similar to receptor-mediated endocytosis (Figure 5E).

Incubation of dendritic cells or Langerin-transfected COP5 cells with mannan at 4°C considerably reduced the binding of anti-Langerin (DCGM4) (Figure 6). This result demonstrates that the mannose-binding domain of Langerin is functional and in close proximity to the epitope recognized by DCGM4. Accordingly, DCGM4

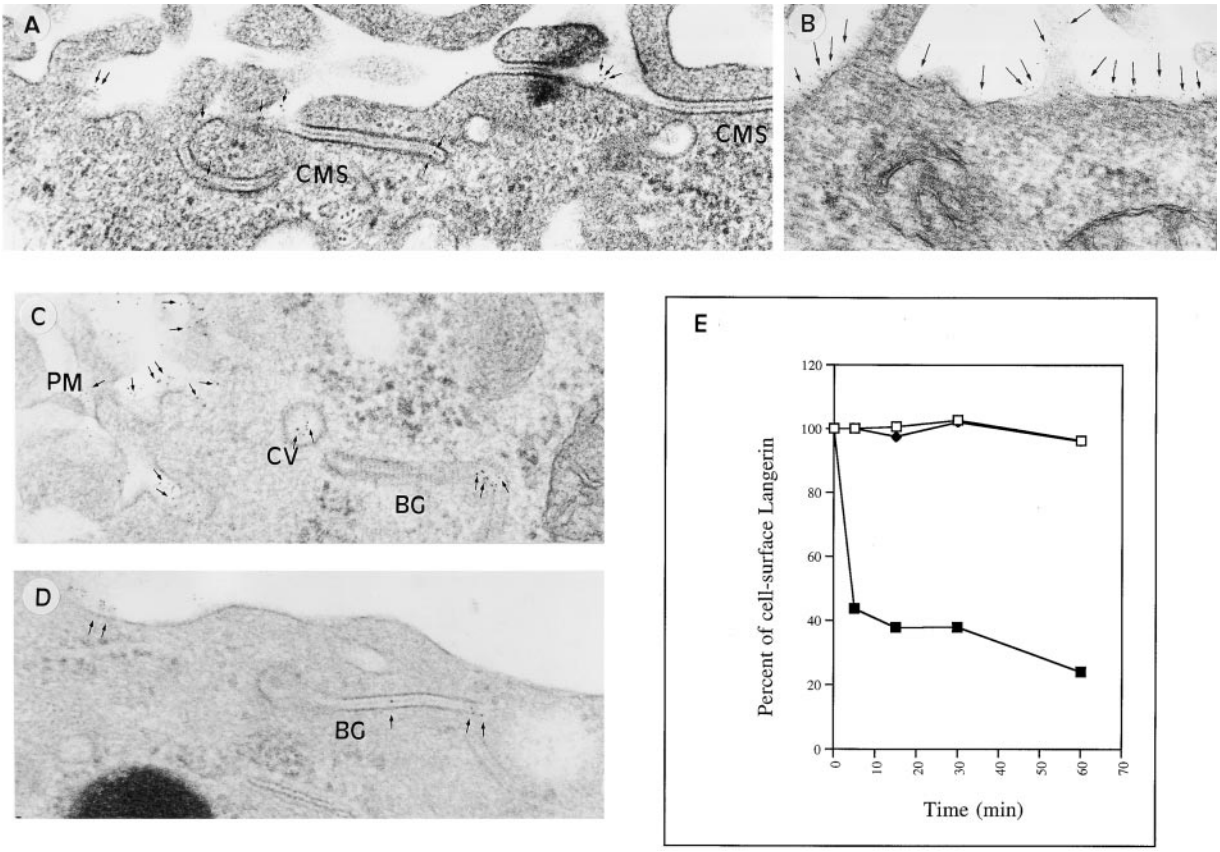

Figure 5. Antibody to Langerin Is Internalized into BG from the Cytomembrane

Vital staining of skin LC with anti-Langerin mAb reveals striated CMS labeled with antibody (A), in marked contrast with the aspect of membranes following anti-CD1a staining (B). After incubation at 37°C for 2 min, anti-Langerin mAb reaches Birbeck granules (BG) and may also access coated vesicles (CV) from the plasma membrane (PM) (C and D). Staining was revealed with anti-mouse Ig coupled to colloidal gold particles (10 nm). (E) In the absence of cross-linking, anti-Langerin mAb DCGM4 displays rapid internalization kinetics at 37°C (closed squares). As controls, no decrease of cell surface fluorescence was detected at 37°C in paraformaldehyde-fixed cells (closed diamonds) or in samples kept at 4°C (open squares).

reactivity was abrogated on a truncated Langerin construct lacking the C-terminal portion of the CRD, including the mannose-binding domain (data not shown). In addition, incubation at 37°C with mannan resulted in disappearance of DCGM4 staining (Figure 6), indicating that recognition of sugar by the lectin domain triggers internalization. In contrast, CD1a staining was not modified by incubation with mannan, and incubation with N-acetyl-galactosamine, a sugar recognized by galactose-type binding lectins with a QPD motif, had no effect on DCGM4 reactivity (data not shown).

Together, these results demonstrate that Langerin contains a functional lectin domain, induces CMS, and functions as an endocytic receptor to translocate ligand from the cell surface into BG.

Transfection of Langerin cDNA Induces BG Formation in Fibroblasts

The above data indicated that engagement of Langerin induces CMS and internalization into BG. To further address the extent of Langerin participation in the membrane superimposition and zippering to produce BG, we transfected Langerin cDNA into murine fibroblastic COP5 cells and performed electronic microscopy. Strikingly, transfection resulted in a massive accumulation of superimposed membranes separated by a central leaflet typical of BG (Figure 7A). The sandwiching process included cytomembrane structures open to the outside of the cell (Figure 7B). The nuclear (Figures 7A and 7C) and endoplasmic reticulum (ER) membranes (Figure 7D) were also zippered, suggesting that Langerin can initiate membrane superimposition immediately after synthesis of the protein. In contrast, mitochondria, which have no membrane continuity with the ER, never showed membrane zippering. As expected, COP5 cells transfected with cDNA encoding another type II lectin (asialoglycoprotein-receptor) did not display BG (Figure 7E). Our findings demonstrate that expression of the LC-restricted Langerin gene is sufficient to drive BG formation in fibroblasts.

Discussion

In the present study, we report the molecular cloning and characterization of Langerin, a novel lectin specifically expressed by Langerhans cells. Remarkably, Langerin

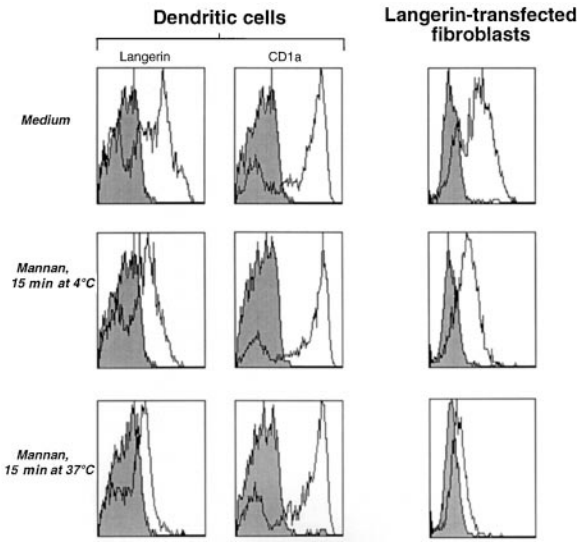

Figure 6. Langerin Displays a Functional Lectin Domain

In vitro–derived DC supplemented with TGFβ were incubated for 15 min with mannan (1 mg/ml) at 4°C or 37°C. Cells were subsequently labeled with mAbs anti-Langerin or anti-CD1a (Becton Dickinson), and cell surface fluorescence was analyzed by FACS. Gray histograms correspond to an Ig isotype control mAb.

is an endocytic receptor that functions as a potent inducer of BG formation by superimposition and zippering of cell membranes.

DC represent a heterogenous population of cells with different ontogenic pathways and maturation states. While several molecules, including the E-cadherin adhesion receptor and the T cell costimulatory molecules CD80 and CD86, are differentially regulated during DC maturation (Romani et al., 1989), their expression is not restricted to DC. Molecular markers restricted to DC subsets, such as Langerin in LC, will be highly valuable tools for cell monitoring in normal tissues and disease states. The mAb anti-Lag has been widely used to stain LC as an intracellular marker (Kashihara et al., 1986). We have demonstrated that anti-Lag detects an intracellular epitope of Langerin, establishing the identity of the glycoprotein recognized by this antibody. In line with our previous localization of Langerin protein (Valladeau et al., 1999), we demonstrated the restriction of mRNA expression to LC-type DC. A particularly intense signal was detected in lung, likely representing anti-Langerin reactive LC lining the pulmonary epithelium (Valladeau et al., 1999).

DC are found to express a growing number of transmembrane lectins that appear to serve distinct functions. Endocytic activity, key to receptor-mediated uptake of antigen, is shared between type I multi-CRD lectins represented by the macrophage-mannose receptor (MMR) (Sallusto et al., 1995) and DEC205 (Jiang et al., 1995) and type II single-CRD lectins homologous to Langerin and exemplified by the CD23 low-affinity IgE receptor (Bieber, 1992). In addition, DC express other lectins expected to modulate their function, such as NKRP1A (Poggi et al., 1997) or DCIR, a newly described type II lectin displaying a tyrosine-based inhibitory motif and encoded within the NK-receptor gene complex on chromosome 12p13 (Bates et al., 1999). The gene encoding human Langerin maps to chromosome 2p13, a locus that has not been linked to any known immunological disorder. Lastly, Langerin displays the highest degree of sequence similarity with the mouse and rat liver Kupffer cell receptors (Hoyle and Hill, 1988). However, Langerin mRNA is not detected in liver, and we have recently identified an EST encoding a mouse Langerin homolog (data not shown), therefore establishing that Langerin is not the human counterpart of the murine Kupffer cell receptor.

DC have highly specialized endocytic structures, which are developmentally regulated upon maturation (Cella et al., 1997a; Pierre et al., 1997). These compartments express specific molecules such as Langerin in BG of immature LC and DC-LAMP in lysosomes of mature DC (de Saint Vis et al., 1998). Several features of Langerin make it an attractive candidate receptor for nonconventional antigen routing in LC. First, Langerin is a highly efficient endocytic receptor that induces BG, into which ligand is internalized. Indeed, Langerin activity is engaged by binding of ligand, as mimicked by DCGM4 antibody or mannan. Since the lectin domain of Langerin recognizes mannose, antigens bearing this sugar, which is common on microorganisms, may be rapidly internalized into concomitantly formed BG following their binding to Langerin. By analogy, mannose-containing microorganisms enter the clathrin-mediated endocytic pathway following binding to the macrophage mannose-receptor (MR) multilectin (Stahl, 1992). Second, in contrast to the MR, which mediates internalization into the MHC class II pathway (Sallusto et al., 1995), we did not detect colocalization of internalized anti-Langerin antibody with HLA-DR molecules. Third, engagement of Langerin must subsequently recruit downstream adaptor molecules in order to mediate ligand internalization and cytoskeleton movement for membrane superimposition. While Langerin lacks a conventional intracellular tyrosine-based motif used to mobilize the clathrin endocytic pathway by the MR or DEC205 endocytic lectins (Stahl, 1992; Jiang et al., 1995), it displays a proline-rich sequence (WPREPPP) (amino acids 23–28). Such a motif is known to interact with SH3 (src homology 3) domain proteins (Ren et al., 1993) found in adaptors that thereby regulate vesicular trafficking and cytoskeleton movement (Cohen et al., 1995), as described for Ras-related proteins (Hall, 1993), for mechanochemical GTPase such as dynamin (Herskovits et al., 1993), and for actin-binding protein. In view of the above nonclassical properties of Langerin and of the cross-priming capacity of DC, it will be of interest to evaluate whether material endocytosed through Langerin gains access to the MHC class I pathway.

Our demonstration that Langerin is a potent inducer of BG formation provides molecular insight into the mechanism by which these organelles develop. BG thus result from transformation of preexisting components upon engagement of Langerin with itself or its ligand. Our results may therefore reconcile the two different subcellular origins of BG, from the Golgi and from the cell surface, by showing that membranes have the potential to be superimposed by Langerin irrespective of their localization. High local concentration of Langerin is likely to represent an important parameter for membrane

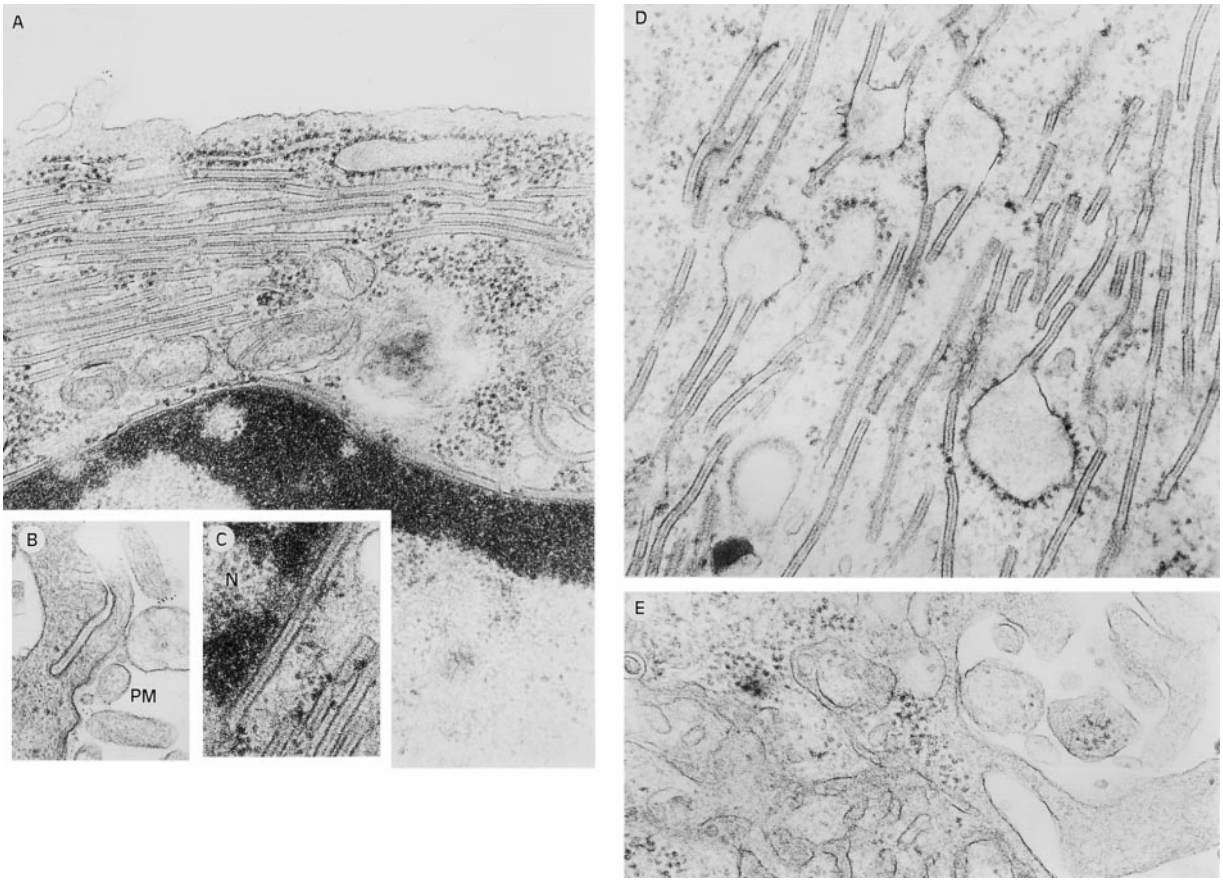

Figure 7. Expression of Recombinant Langerin Induces BG Formation in Fibroblasts

Electron microscopy on murine COP5 fibroblasts transfected with Langerin cDNA demonstrating massive membrane superimposition and zippering (A–D). This process is observed from the plasma membrane (PM) (B), the nuclear membrane (A and C), and the endoplasmic reticulum (D). Absence of BG in COP5 cells transfected with asialoglycoprotein-receptor cDNA as control, as shown in (E). Abbreviations: PM, plasma membrane; N, nucleus.

superimposition. In keeping with this, Langerin expression in COP5 transfectants, under the strong SRα promoter, is likely higher than that in LC; hence the massive network of BG-like membranes observed throughout the cell. Although Langerin itself mediates BG formation, other stimuli including sensitizing compounds (Kolde and Knop, 1987; Hanau et al., 1989) and cytomembrane disruption by chemicals (Bartosik et al., 1985; Mikulowska et al., 1988; Kanerva, 1989), enhance BG formation, possibly by influencing membrane plasticity and favoring engagement of Langerin. We previously demonstrated that Langerin expression is upregulated by TGFβ (Valladeau et al., 1999). It should thus be of interest to investigate whether conditions enhancing TGFβ production within the LC microenvironment also favor BG formation.

Extracellular domains of Langerin likely interact to promote membrane superimposition. This interaction could actually be the basis for membrane zippering, leading to the characteristic crystal-like lattice structure of BG (Zelickson, 1966; Wolff, 1967). To allow zippering of superimposed membranes, extracellular Langerin may be bridged, for instance, by multimannosylated compounds binding to the lectin domain of two or more Langerin molecules. Consistent with a role of Ca^{2+}-dependent sugar recognition in membrane zippering, LC deprived of Ca^{2+} display BG vesiculation due to membrane unzippering (Andersson et al., 1988). Directed mutagenesis, in particular of the C-type lectin domain including the EPN motif and the intracellular proline-rich region, is in progress to decipher the molecular mechanism of membrane superimposition and zippering.

Identification of Langerin as an endocytic receptor that induces BG formation strongly suggests that these organelles are connected to a novel pathway for antigen processing. Moreover, BG may allow sequestration of antigen selectively internalized via Langerin for storage within an environment sheltered from degradation until LC receive appropriate activation and migration signals. Finally, sequestration of plasma membranes through BG formation may play a role in the emigration of LC out of the epidermis (Larsen et al., 1990).

In conclusion, the identification of Langerin paves the way to understanding the function of the enigmatic BG, which likely represents a key feature of the DC system.

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, Abingdon, U.K.) was used at a concentration of 25 ng/ml. rhTGFβ_1 (R&D) was used at 1 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). Granulocytes were generated in vitro from $CD34^+$ progenitors in the presence of G-CSF and SCF for 12 days. Cells were either not activated or activated by PMA-ionomycin for 1 hr and 6 hr (PMA, 1 ng/ml [Sigma]; Ionomycin, 1 μg/ml [Calbiochem]) and pooled. Transfections were performed with the murine fibroblastic COP5 cell line (Tyndall et al., 1981). The TF1, Jurkat, MRC5, JY, and U937 cell lines were obtained from 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 hr and 6 hr were pooled. Murine fibroblasts transfected with human CD40 ligand (CD40L L cells) were produced in the laboratory (van Kooten et al., 1994). All cell types were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Flow Laboratories), 10 mM HEPES, 2 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol and gentamicin (80 μg/ml).

Generation and Isolation of Dendritic Cells

Fresh Langerhans cells were prepared from normal skin by CD1a positive selection. A purity higher than 95% was systematically achieved. In vitro–derived DC were prepared from $CD34^+$ progenitor cells as described (Caux et al., 1992), except that cells were washed and replated at day 7 in fresh medium containing GM-CSF and $TGF\beta_1$ to increase the percentage of LC (Valladeau et al., 1999). Briefly, isolation of $CD34^+$ progenitors from umbilical cord blood samples was achieved using Minimacs separation columns (Miltenyi Biotec GmbH, Bergish Gladbach, Germany) and cultures of $CD34^+$ cells were established in the presence of SCF, GM-CSF, and $TNF\alpha$. Between days 12 and 17, 70%–90% of the cells were $CD1a^+$ DC. Aliquots of cells were activated with 1 ng/ml PMA and 1 μg/ml Ionomycin for 1 hr and 6 hr, pooled, and lysed for RNA extraction.

cDNA Library and Expression Cloning

Total RNA was isolated from CD34-derived DC stimulated with $TGF\beta_1$ (at day 12 of the culture) (Caux et al., 1996). RNA was treated with DNase I before mRNA purification using the Oligotex-dT kit (Qiagen GmbH, Hilden, Germany). Poly(A)$^+$ RNA (2 μg) was used to make a cDNA library in the pCEV4-M plasmid, a derivative of $SR\alpha$ expression vector (Takebe et al., 1988), produced in the laboratory of K. Moore. Transient expression was carried out in COP5 cells. After FACS-sorting with anti-Langerin mAb DCGM4 (Valladeau et al., 1999), plasmid DNA of positive clone M4T24C6 was purified and sequenced using a Taq Dye Deoxy Terminator kit (Applied Biosystems) and an automated sequencer (Applied Biosystems). Homology searches were performed in GenBank and EMBL nucleotide databases. Alignments with the rat Kupffer cell receptor (accession number P10716), mouse Kupffer cell receptor (accession number P70194), chicken hepatic lectin (accession number P02707), human macrophage lectin (accession number D50532), human asialoglycoprotein receptor 1 (accession number P07306), human asialoglycoprotein receptor 2 (accession number P07307), human HIV binding lectin (accession number A46274), and human CD23 (accession number P06734) were analyzed using the MegAlign function of Lasergene (DNAstar). A SalI–NotI fragment containing an NH_2-truncated Langerin cDNA was derived by PCR using sense (5′-TATGTC GACACCATGTGGCCCCGAGAGCCTCCTCCCA-3′) and antisense (5′-ATAGCGGCCGCTCACGGTTCTGATGGGACATA-3′) oligonucleotides and M4T24C6 as template. Using standard procedures, the fragment was inserted in pCEV4 vector, and one clone (D1) was isolated and sequenced.

Northern Blot Analysis and RT–PCR

Northern blot analysis was performed using a human mRNA adult tissue blot (Clontech). The cDNA clone M4T24C6 was cut with the enzymes SalI and NotI under standard conditions and one fragment of 1308 bp was labeled with ^{32}P-dCTP using the High Prime Kit (Boehringer Mannheim, Meylan, France). Unincorporated nucleotides were removed by spin column chromatography (Chromaspin-100 [Clontech]). Membranes were prehybridized under standard conditions. Low and high stringency washes were at 2× SSC, 0.2% SDS and 0.2× SSC, 0.2% SDS, respectively, for 30 min each. Membranes were incubated with Biomax MR film (Kodak) for 15 days. For RT–PCR, total RNA was reverse transcribed using random hexamer primers (Pharmacia, Uppsala, Sweden) and the Superscript RNase-H reverse transcriptase (GIBCO–BRL). PCR were performed in a DNA thermal cycler (Perkin Elmer Cetus) 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 71–88: 5′-TGATGCGCAC TTCACTGT-3′) and antisense (nucleotides 912–896: 5′-CATTGTTG GGCTCACCT-3′) primers were used to amplify M4T24C6.

Biochemical Analysis

Langerin extraction and SDS-PAGE analysis were performed as described (Valladeau et al., 1999). Part of the gel was silver stained and the remaining part was transferred onto Hybond C super membranes. Transferred proteins were detected using anti-Langerin (DCGM4) or anti-Lag mAbs revealed with the ECL Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Cytofluorimetric Analysis

Dendritic cells or COP5 cells were incubated with anti-Langerin (DCGM4) or with anti-Lag (Kashihara et al., 1986) mAbs and revealed with goat anti-mouse Ig-FITC (Becton Dickinson). In some experiments, DC and transfected COP5 cells were preincubated with mannan (1 mg/ml; Sigma) for 15 min at 4°C or 37°C before staining. Intracellular staining was performed in the presence of permeabilization medium (0.3% saponin, 2% BSA). Negative controls were performed with unrelated cDNA (asialoglycoprotein receptor) transfected cells or with an isotype Ig control. Fluorescence was analyzed with a FACScan flow cytometer (Becton Dickinson).

Confocal Microscopy

Cell surface and intracellular immunofluorescence staining was performed as previously described (Rovere et al., 1998). Cells fixed for 15 min with 4% paraformaldehyde in PBS on polylysine-coated coverslips were washed twice in 10 mM glycine in PBS, twice in PBS, and permeabilized or not with 0.5% saponin, 1% BSA-PBS for 30 min. Coverslips were either stained with DCGM4-FITC or sequentially stained with mAb DCGM4-FITC (5 μg/ml), saturated with 10% mouse serum, and stained with anti-Lag (Kashihara et al., 1986). After three washes, cells were incubated for 30 min with secondary labeled antibodies (Texas red donkey anti-mouse Ig [Vector Laboratories]). Coverslips were washed and mounted onto glass slides with Fluoromount (Southern Biotechnology Associates). Confocal microscopy was performed using the Confocal Laser Scanning Microscope TCS 4D (Leica Lasertechnik GmbH, Heidelberg, Germany).

Electron microscopy

Fixed Cells

Staining was performed as described (Valladeau et al., 1999), subsequent to fixation in 2% paraformaldehyde, 0.2% glutaraldehyde. After washing, cells were embedded in 10% gelatin, cut in small blocks, and infiltrated with 2.3 M sucrose for 4 hr at 4°C. Blocks were mounted and frozen in liquid nitrogen. Ultrathin cryosections were indirectly immunolabeled with mAbs DCGM4 and anti-HLA-DR (kind gift of Dr H. L. Ploegh, Dept. of Pathology, Harvard Medical School) and visualized with 15 and/or 10 nm gold particles. Sections were then embedded in a mixture of 2% methyl cellulose and 0.4% uranyl.

Vital Cells

Staining was performed prior to fixation, as described (Valladeau et al., 1999). LC were stained with mAbs DCGM4 or anti-CD1a (mAb DMC1, produced in the laboratory of C. Dezutter-Dambuyant) followed by goat anti-mouse IgG conjugated with colloidal gold particles (Amersham) and either fixed immediately after staining or warmed up to 37°C for 2 min before fixation.

Internalization Assay

CD34-derived DC supplemented with $TGF\beta_1$ were generated as detailed above, and internalization of mAb DCGM4 was performed as described (Cella et al., 1997b). One aliquot of cells was fixed in 1% paraformaldehyde before internalization as measure of the off-rate

of the antibody at 37°C. Samples were stained with mAb DCGM4 coupled to biotin. Cells were then placed in a 37°C water bath for various time periods, cooled on ice, and stained with PE-conjugated streptavidin (Becton Dickinson). After washing, cells were analyzed by FACS. Internalization is given by percentage decrease of cell surface median fluorescence intensity as compared to control samples at 4°C.

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