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A Critical Tyrosine Residue in the Cytoplasmic Tail Is Important for CD1d Internalization But Not for Its Basolateral Sorting in MDCK Cells

Dmitrii G. Rodionov,* Tommy W. Nordeng,* Ketil Pedersen,* Steven P. Balk,† and Oddmund Bakke‡

The CD1 family of polypeptides is divided into two groups, the CD1b and CD1d group. Both groups are involved in stimulation of T cell response. Molecules of the CD1b group can present Ag derived from bacterial cell walls to T cells; the process of Ag acquisition is thought to take place in endosomes. Little is known about Ag presentation by CD1d. We therefore studied the intracellular trafficking of human CD1d in Madin-Darby canine kidney (MDCK) and COS cells. CD1d was found in endosomal compartments after its internalization from the plasma membrane. It is therefore possible that CD1d acquires its yet unidentified exogenous ligand in the same compartments as the MHC class II and CD1b molecules. CD1d contains a tyrosine-based sorting signal in its cytoplasmic tail that is necessary for internalization. Furthermore, the cytoplasmic tail of CD1d also contains a signal for basolateral sorting that is, however, different from the internalization signal. The Journal of Immunology, 1999, 162: 1488–1495.

CD1 polypeptides appear as a new arm of host defense against invading pathogens (reviewed in Ref. 1; Refs. 2–4). The human CD1 family consists of five nonpolymorphic non-MHC-encoded genes (CD1a, -b, -c, -d, and -e) (the protein product of CD1e has not been identified yet) that have a unique tissue distribution. CD1a, -b, and -c have been found on immature thymocytes, B lymphocytes, Langerhans cells, and activated monocytes whereas CD1d has been primarily located to epithelial cells and hepatocytes in addition to B lymphocytes and thymocytes (2). Genomic structure and sequence comparison of extracellular domains have also revealed the existence of two groups of CD1 molecules: the CD1b group (includes CD1a, -b, and -c) and the CD1d group. Taken together, these facts raise the possibility of different function of the CD1b and the CD1d groups. CD1b and -c molecules have been found to present mycobacterial cell wall-derived Ags to T cells (3), and CD1a has been implicated in the presentation of bacterial Ags (5). No human CD1d-restricted Ags have been identified yet; however, mouse CD1d (a human CD1d homologue) has been shown to bind 22-amino acid-long hydrophobic peptides (6). Furthermore, T cells restricted to mouse CD1d in the context of these peptides have been generated (6). A recent crystal structure of mouse CD1d has revealed the presence of a large hydrophobic groove capable of accommodating very hydrophobic peptide, lipid, or glycolipid Ags (7). Mouse CD1d has also been found to control the function of a subset of T lymphocytes expressing receptors for NK cells (NKT cells) in a ligand-independent fashion that could be, however, augmented by the presence of glycosphingolipids (8–10). Finally, cellular glycosylphosphatidylinositol (GPI) has been identified as a major ligand for mouse CD1d in human T2 cells transfected with mouse CD1d (11). It has been further proposed that GPI-CD1d binding occurred in the endoplasmic reticulum and that the complex was further transported to endosomes for displacement of GPI and loading of exogenous ligands reminiscent of the MHC class II-invariant chain pathway (11).

The CD1 Ag presentation pathways are TAP independent, and this has led to suggestion that CD1 molecules may acquire Ags in the same compartments as do the MHC class II molecules (12). Indeed, CD1b molecules have been found in late endosomal and lysosomal structures (12). Furthermore, localization of CD1b to these compartments is dependent on its cytoplasmic tail (12), which contains a putative tyrosine-dependent internalization signal YXXZ (where Z stands for a bulky hydrophobic residue and X for any residue) (4, 13). Internalization of CD1b constructs was greatly compromised by the deletion of its cytoplasmic tail containing the YXXZ motif (12) and by an alanine substitution of the critical tyrosine residue (14). Moreover, the loss of the cytoplasmic tail of CD1b and alanine substitution of the critical tyrosine have led to a reduced efficiency of Ag presentation (14), hinting that the proper endosomal transport of CD1b could be important for its function in Ag presentation. CD1d also contains a YXXZ sequence in its cytoplasmic tail (15). We were interested in whether CD1d was transported along a route similar to CD1b. In this report, we localized CD1d molecules to the endocytic pathway in canine epithelial MDCK cells and simian COS cells and demonstrated that the YXXZ motif indeed acted as the internalization signal in those cell lines. Therefore, it is conceivable that the CD1d

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3 Abbreviations used in this paper: GPI, glycosylphosphatidylinositol; AP, adaptor protein complex; GST, glutathione S-transferase; LAMP, lysosome-associated membrane protein; LDL, low density lipoprotein; MDCK cells, Madin-Darby canine kidney cells; PFA, paraformaldehyde; TIR, transferrin receptor; BL, basolateral; TGN, trans-Golgi network.
and CD1b groups acquire their respective Ags in similar endocytic compartments.

CD1d is expressed in polarized intestinal epithelial cells in nature, and we were therefore interested in the sorting of CD1d in polarized epithelial MDCK cells. We show here that the distribution of CD1d in MDCK cells is predominantly basolateral. The basolateral surface of tissue epithelial cells faces the vascular space and, like MHC class II (16–21), the CD1d molecules thus have the possibility to encounter and present Ag to emigrating T cells. So far, two classes of basolateral sorting motifs have been described. The first class includes signals that are related to the clathrin-coated pit localization and internalization signals and depend on either a key tyrosine residue (22, 23) or a leucine-based motif (21, 24). The second class includes tyrosine-dependent or independent signals that are not colinear with the coated pit localization signals (22, 25). We found that basolateral sorting information was present in the cytoplasmic tail of CD1d. However, since the mutation of the tyrosine internalization signal did not significantly alter CD1d targeting, we conclude that a basolateral sorting signal of unknown nature is located within the cytoplasmic tail of CD1d.

Materials and Methods

Antibodies

Preparation of D5 Ab will be described in detail elsewhere (S. P. Balk, manuscript in preparation). Briefly, mouse monoclonal D5 Ab were generated against CD1d-GST fusion proteins. A series of CD1d-GST fusions containing the α1 domain alone, the α1 and α2 domains, or the α1–α3 domains were constructed in pGEX2tk vector using convenient restriction sites in the CD1d cDNA (15) or PCR to introduce restriction sites. These proteins were expressed as GST fusion proteins and purified on glutathione agarose beads (Pharmacia Biotech, Piscataway, NJ). The purified proteins were eluted sequentially with glutathione and SDS and then pooled.

Mouse mAbs against the pooled CD1d-GST fusion proteins were generated by fusing hyperimmun BALB/c spleen cells to murine myeloma (NS-1) cells. Wells were screened by ELISA against the pooled fusion proteins, and positives were subsequently screened against GST to eliminate contaminants. Three to four positive clones with different expression levels of the protein of interest were selected in each case.

Idination of Abs

D5 and SC2 Abs were labeled with Na125I using chloramine T (Sigma, St. Louis, MO) or Iodo-Beads (Pierce, Rockford, IL), respectively, according to the manufacturers’ recommendations. Briefly, Abs (100 μg) were incubated with 25 mM CdCl2 overnight to induce expression of the protein of interest. Clones expressing constructs of interest were identified by screening with either D5 (for CD1d constructs) or SC2 (for CD8 constructs) Ab. Three to four positive clones with different expression levels of the protein of interest were selected in each case.

Immunofluorescence microscopy

Transfected MDCK cells expressing various proteins were grown on cover slips and fixed in 35-mm wells the day preceding the transfection to yield less than 30% confluent layers. Plasmid DNA (0.5 μg) was dissolved in 0.1 ml DMEM containing 10% NuSerum and mixed with 1 ml DMEM containing 10% NuSerum, 400 μg/ml DEAE-dextran, and 0.1 mM chloroquine (per 35-mm well). The cells were washed twice with PBS (pH 7.4) before the addition of the DNA solution. Cells were incubated with the DNA for 3–4 h at 37°C. 5% CO2 shocked with ice-cold 10% DMSO (2–3 min), and grown in full growth medium for 2 days for the expression of proteins.

Stable transfection of MDCK cells and clonal selection

MDCK cells were stably transfected by the calcium phosphate procedure of Wigler et al. (26). Clones expressing the DNA constructs in pMP4 vector under control of metallothionein promoter were selected in the presence of hygromycin B (0.3 mg/ml). Resistant clones were selected and incubated with 25 mM CdCl2 overnight to induce expression of the protein of interest. Clones expressing constructs of interest were identified by screening with either D5 (for CD1d constructs) or SC2 (for CD8 constructs) Ab. Three to four positive clones with different expression levels of the protein of interest were selected in each case.

Internalization assays

For internalization assays involving uptake of nonradioactive D5 Ab, transfected MDCK cells were grown on cover slips. Expression of transfected proteins was induced by overnight incubation with 5–25 μM CdCl2. Cover slips were incubated with D5 Ab in full growth medium on ice for 1 h. The unbound Ab was washed out, and cover slips were further incubated in full growth medium at 37°C (5% CO2) for various time intervals. Cells were then fixed with 3% PFA in PBS for 10 min at room temperature and

![FIGURE 1. Cytoplasmic tails of different constructs used in this study](http://www.jimmunol.org/).
eventually incubated with another primary Ab diluted in PBS, 0.1% saponin for additional 30 min. Cells were next labeled with a secondary Ab conjugate or a mixture of secondary Abs. Fluorescence was detected as described above.

For assays involving internalization of $^{125}$I-D5, MDCK cells expressing different CD1d constructs were grown in 35-mm wells in a nonpolarized fashion. Expression of CD1d constructs was induced with 5–25 μM CdCl$_2$. Cells were incubated with $^{125}$I-D5 (1–2 μg/ml) on ice for 1 h, washed six times in ice-cold PBS containing 0.2% FCS, and chased in full growth medium for different periods of time at 37°C bathwater. The cells were then cooled on ice, and the surface Ab was stripped with a low pH wash (0.15 M NaCl, 0.5 M acetic acid). The cells were subsequently lysed in 1 M NaOH to release internalized Ab. Radioactivity present in the low pH wash and in cell lysate was determined with a Cobra Auto-Gamma counter. Values were corrected for nonspecific binding and uptake of $^{125}$I-D5 that was determined in parallel experiments with nontransfected MDCK cells.

The amount of internalized Ab (%) was calculated as the ratio of the radioactivity present in cell lysate to the sum of the radioactivity present in the low pH wash and in the lysate.

**Surface labeling of polarized cells**

Stably transfected MDCK cells were grown on Transwell polycarbonate filter units (10$^5$ cells/filter; Costar, Cambridge, MA) for 4 days before experiments. Expression of the constructs of interest was induced by overnight incubation with 0–25 μM CdCl$_2$. The results depended on the expression level; the proteins of interest were sorted basolaterally only at moderate levels of expression. At high expression levels the proteins were mis sorted in a nonpolarized fashion, a phenomenon that has been noted previously for other proteins (Refs. 23, 26, and 27; T. W. Nordeng and O. Bakke, submitted for publication). For the experiments involving the use of a confocal microscope, cell were labeled with a primary Ab added from either the basal or the apical side for 1 h on ice. Cells were washed with PBS (pH 7.4) containing 0.9 mM CaCl$_2$ and 0.5 mM MgCl$_2$ (PBS + ), and fixed with 3% PFA. The filters were then excised and stained with a secondary Ab. Immunofluorescence images were acquired as described above.

For the experiments involving the use of iodinated Abs, cells grown on Transwell filters were cooled on ice and incubated with either $^{125}$I-D5 or $^{125}$I-SC2 (2–10 μg/ml) added apically or basolaterally. The integrity of tight junctions was followed by monitoring the leakage of radioactivity from the basolateral to the apical side. Unbound Abs were removed by extensive washing with PBS + , and filters were excised and counted with a Cobra Auto-Gamma counter. Nonspecific binding was determined in parallel experiments with nontransfected MDCK cells and corrected for.

**Electron microscopy**

To identify compartments within the endocytic pathway, MDCK cells expressing CD1d were incubated with 5-nm and 10-nm BSA-coated colloidal gold particles prepared as described by Slot and Geuze (28). Cells were first labeled with 5-nm gold particles for 3 h followed by overnight chase to mark late endosomes and lysosomes. Cell were subsequently labeled with 10-nm gold particles for 1 h to visualize the earlier endosomal structures. After washing in PBS the cells were pelleted by centrifugation at 2000 rpm in Sigma 202 M centrifuge, and the supernatant was removed. Then the cells were resuspended in Sorensen’s phosphate buffer (pH 7.4) containing 4% PFA and 0.1% glutaraldehyde followed by centrifugation at 10,000 rpm. The pelleted cells were fixed for 1 h at room temperature. After fixation, the cells were incubated in 2.3 M sucrose for 1 h at room temperature and mounted on silver pins. Mounted cells were frozen and stored in liquid nitrogen. The specimens were sectioned on a Reichert-Jung ultramicrotome with a Reichert FCS cryo attachment using a Drukker International diamond knife. Immunocytochemical labeling of thawed cryosections was performed mainly as described in Ref. 29.

**Materials**

DMEM and FCS were obtained from BioWhittaker (Walkersville, MD). Oligonucleotides for PCR mutagenesis were synthesized by Medprobe (Oslo, Norway). Materials for PCR amplification, restriction, ligation, and sequencing were from New England Biolabs (Beverly, MA). The rest of the materials (unless specified otherwise) was purchased from Sigma.

**Results**

**Localization of CD1d in the endocytic pathway**

In this study, we used mouse monoclonal D5 and rabbit polyclonal RPD1 Abs to detect human CD1d in stably transfected MDCK cells. As shown in Fig. 2, D5 labels the surface of MDCK cells stably transfected with CD1d but not that of mock-transfected cells. Similar results were obtained using D5 in transiently transfected COS cells (data not shown).

To analyze the intracellular transport of CD1d, we attempted to colocalize CD1d with different markers of the endocytic pathway: the TIR is an early endosomal marker; HLA-DR1 is localized in both early and late endosomes; and LAMP-1 is a marker for late endosomes and lysosomes (for a review see Ref. 4; Ref. 30). We created a double-transfected MDCK cell line expressing CD1d and TIR, labeled its surface with D5 Ab, and allowed it to internalize at 37°C for 10 or 20 min. These cells were fixed, and TIR was detected with the 66IG10 mAb. As shown in Fig. 3, CD1d-D5 complexes were detected in TIR-positive vesicles after 10 min of chase. We also observed colocalization of CD1d and TIR after 20 min of chase, but more CD1d-D5 complexes had reached a TIR-negative compartment at that time. Next, we performed a similar pulse-chase experiment with the D5 Ab on MDCK cells transfected with CD1d and HLA-DR1. Colocalization between CD1d-D5 complexes and the anti HLA-DR1 Ab L243 was observed after both 10 and 20 min of chase. These data are in line with previous results showing that MHC class II molecules are present in both early and late endosomes (31–33). Finally, we performed a similar chase experiment with cells transfected with CD1d alone. The cells were then double labeled with an Ab directed against endogenous LAMP-1. D5-CD1d complexes were not found to colocalize extensively with the anti-LAMP-1 Ab after 10 min of chase, whereas the degree of colocalization was significantly increased after 20 min of chase. Similar results were obtained in transiently transfected COS cells (data not shown).

We used cryo immuno electron microscopy to corroborate the data obtained with fluorescence microscopy. To visualize the endocytic pathway, MDCK cells stably expressing CD1d were incubated with 5-nm gold particles for 3 h, followed by overnight chase to mark late endosomes and lysosomes. Cells were subsequently incubated with 10-nm gold particles for 1 h to visualize early and late endosomal structures. As shown in Fig. 4A, CD1d (revealed by 15-nm gold particles) colocalized with the 10-nm gold particles in early endosomal compartments. In addition, CD1d could be detected on the endoplasmic reticulum membranes (Fig. 4A). Localization of CD1d to late multivesicular endosomal structures containing both 5- and 10-nm gold particles was also observed (Fig. 4B). Taken together, these results demonstrate that CD1d molecules are present throughout the endocytic pathway in MDCK cells.

**The YXXV motif in the cytoplasmic tail of CD1d is required for its internalization**

The short cytoplasmic domain of CD1d contains a putative tyrosine-based internalization signal YXXV (Fig. 1). We decided to...
investigate the role of this motif on CD1d internalization from the plasma membrane.

We studied internalization of the CD1d constructs by fluorescence microscopy. Cells grown on cover slips were labeled with D5 Ab on ice, unbound Ab was washed out, and cells were further incubated in full growth medium before fixation with 3% PFA. Total TIR distribution was detected with 66g10, HLA-DR1 was detected with L243, and endogenous LAMP-1 was detected with the α-LAMP-1 Ab. Colocalization between internalized Cd1d (red channel) and the different endocytic markers (green channel) occurs as yellow staining. Bar, 10 μm.

and studied its effect on internalization of CD1d. Internalization of the CD1d VA mutant resembled that of the tyrosine mutant (data not shown). We also mutated the serine residue at the +1 position, which is presumably unrelated to the tyrosine-based internalization signal. This mutation had no effect on internalization, and the pattern of internalization of the CD1d SA construct was indistinguishable from that of the wild-type CD1d (data not shown). Furthermore, similar results were obtained when the wild-type CD1d and its mutants were transiently expressed in COS cells (data not shown).

Internalization of various CD1d constructs was quantified by measuring the uptake of iodinated D5 Ab. MDCK cells expressing the CD1d constructs were grown in a nonpolarized fashion and labeled on ice with 125I-D5 before incubation at 37°C waterbath. As shown in Fig. 6, the wild-type CD1d (filled circles) was internalized rapidly (about 50% during first 10 min), whereas the internalization of the tyrosine mutant (open circles) was almost
threefold lower. The valine to alanine substitution (filled squares) decreased the CD1d internalization by a factor of two, whereas the serine to alanine substitution (open squares) had no effect on internalization, in agreement with the fluorescent microscopy results. The cytoplasmic tail of CD1d, therefore, contains a classic YXXZ-type internalization signal.

We then investigated whether the tyrosine-based motif was sufficient for endocytosis. To this end, we constructed a chimeric protein containing the cytoplasmic tail of CD1d fused to the extracellular and transmembrane domains of CD8 molecule to yield a chimeric CD8CD1d construct (Fig. 1) and studied its internalization in MDCK cells in a pulse-chase experiment. It has been shown previously that a tailless CD8 molecule (CD8Δ, Fig. 1) does not internalize readily (M. Røe, K. Pedersen, A. Simonsen, A. Kelly and O. Bakke, manuscript in preparation). Our data confirms this observation; there is almost no internalization of CD8Δ-specific 3C9 Ab in MDCK cells transfected with the CD8Δ construct after 30 min of chase (Fig. 7C). However, the chimeric construct containing the internalization motif from CD1d internalized rapidly, and most of the surface 3C9-chimera complexes were found in the intracellular compartments after the 30-min chase (Fig. 7D). Therefore, the tyrosine-based motif in the cytoplasmic tail of CD1d is necessary and sufficient for the endocytosis.

The tyrosine-based internalization motif of CD1d is not required for basolateral sorting

A number of molecules contain overlapping signals for internalization and basolateral sorting in their cytoplasmic tails. We therefore investigated the distribution of CD1d and its mutants in polarized MDCK cells. Newly synthesized proteins are normally internalized in MDCK cells in a pulse-chase experiment. It has been shown previously that a tailless CD8 molecule (CD8Δ, Fig. 1) does not internalize readily (M. Røe, K. Pedersen, A. Simonsen, A. Kelly and O. Bakke, manuscript in preparation). Our data confirms this observation; there is almost no internalization of CD8Δ-specific 3C9 Ab in MDCK cells transfected with the CD8Δ construct after 30 min of chase (Fig. 7C). However, the chimeric construct containing the internalization motif from CD1d internalized rapidly, and most of the surface 3C9-chimera complexes were found in the intracellular compartments after the 30-min chase (Fig. 7D). Therefore, the tyrosine-based motif in the cytoplasmic tail of CD1d is necessary and sufficient for the endocytosis.

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delivered directly from the TGN to the apical or basolateral membrane in MDCK cells (34). Since it takes several hours for the newly synthesized CD1d molecules to reach the cell surface (S.P. Balk, unpublished observation), we decided to investigate the steady-state distribution of CD1d in polarized MDCK cells rather than the appearance of newly synthesized molecules at the cell surface. Cells grown on polycarbonate filters were incubated on ice with D5 Ab added either to their basolateral or apical side, fixed, and studied by confocal microscopy. Cells expressing the wild-type CD1d were labeled only from the basolateral side (Fig. 8, A and B), indicating that CD1d indeed contains a basolateral sorting signal. We then tested the distribution of the CD1d YA mutant. The staining observed was predominantly basolateral (Fig. 8, C and D), suggesting that the tyrosine residue was not a critical part of the basolateral sorting signal. Furthermore, CD1d VA and CD1d SA constructs were also localized mainly to the basolateral domain (data not shown).

The results obtained with confocal microscopy were further supported by surface labeling with 125I-D5 Ab. As shown in Fig. 9, 98% of the wild-type CD1d was present on the basolateral domain. Similarly, almost 90% of the tyrosine mutant and over 80% of CD1d VA and CD1d SA constructs were found at the basolateral surface (Fig. 9). These results indicate that either the internalization and basolateral sorting signals of CD1d are not identical, or CD1d harbors multiple basolateral sorting signals, as has been shown for a number of other molecules (21, 22).

The cytoplasmic tail of CD1d contains basolateral sorting information

Most proteins that are expressed predominantly at the basolateral surface contain the basolateral sorting information in their cytoplasmic domains. We therefore searched for information in the cytoplasmic tail of CD1d. To this end, we constructed a deletion mutant of CD1d that had its last six cytoplasmic residues truncated (Fig. 1). This deletion construct was distributed equally between basolateral and apical domains of MDCK cells (Figs. 10 and 11), indicating that a basolateral sorting information was indeed present in the cytoplasmic tail of CD1d. We then investigated the polarized distribution of the CD8 δ and CD8−CD1d constructs (Fig. 1) in MDCK cells. The full-length CD8 and CD8 δ are sorted to the apical domain of polarized MDCK cells (M. Roe, K. Pedersen, A. Simonsen, A. Kelly, and O. Bakke, manuscript in preparation). As shown in Fig. 11, more than 75% of the CD8 δ construct was sorted apically judging from labeling with 125I-SC2 Ab. On the contrary, the chimeric CD8−CD1d construct was sorted predominantly to the basolateral domain (Fig. 11). The cytoplasmic tail of CD1d, therefore, contains a basolateral sorting signal that is distinct from the tyrosine-based internalization signal. Moreover, this basolateral sorting signal is stronger than the apical sorting signal(s) in the luminal and/or transmembrane domains of CD8.

Discussion

In this study, CD1d molecules were localized to the endocytic pathway in MDCK and COS cells by the use of fluorescence and electron microscopy. CD1d is internalized from the plasma membrane and transported along the endocytic pathway although from our experiments we cannot exclude the possibility of also a direct sorting of CD1d molecules from the TGN to endosomes. Furthermore, CD1d localizes in endocytic compartments that contain the polarized distribution of wild-type CD1d and its tyrosine mutant in MDCK cells. Cells transfected with the wild-type CD1d cDNA (A and B) or the tyrosine mutant (C and D) were grown on Transwell polycarbonate filters and incubated with the D5 Ab on the basolateral (A and C) or the apical (B and D) side for 1 h on ice. The cells were fixed with 3% PFA and stained with an FITC-conjugated goat anti-mouse Ab. Bar, 10 μm.
Human CD1d contains a tyrosine-based internalization signal YXXV in its cytoplasmic tail that is necessary and sufficient for efficient delivery of CD1d1 molecules to endosomes. Alanine substitution of the critical tyrosine residue reduced internalization of CD1d from the plasma membrane of MDCK by threefold. A similar trend has been observed in the studies involving other tyrosine-based internalization signals (13). Furthermore, a relatively conservative substitution of valine in the +3 position by alanine caused a significant decrease in internalization efficiency. This has been also demonstrated for a number of other tyrosine-based sorting signals (13, 35, 36). On the contrary, substitution of the upstream serine residue unrelated to the putative internalization motif did not change the rate of internalization.

Like MHC class II (21, 37), human CD1d is sorted basolaterally in polarized MDCK cells. A number of proteins have been characterized in which both internalization and basolateral delivery depend on a common tyrosine residue, e.g., lysosomal membrane glycoprotein 120 (23), lysosomal acid phosphatase (38), and the proximal signal of LDL receptor (22). However, when the tyrosine residue of the CD1d internalization signal was substituted with alanine, the resulting mutant was still sorted primarily to the basolateral domain (about 90%), although less efficiently than the wild-type (98%). The substitution of the upstream (−1) serine residue or the valine residue in the position +3 from the critical tyrosine also resulted in predominantly basolateral sorting (about 80% in each case). Therefore, the intact tyrosine-based internalization signal is not absolutely required for basolateral sorting of CD1d. This suggests the existence of a separate basolateral sorting signal, which does not comprise information for endocytosis, in the cytoplasmic tail of CD1d, similar to what has been found for the poly Ig receptor (39, 40). Alternatively, CD1d may contain two or more basolateral sorting signals (one of which is the tyrosine-based signal) that are individually sufficient for its basolateral sorting, like signals in the invariant chain (21) and the LDL receptor (22). Another possibility could be that the signals for internalization and basolateral sorting of CD1d may not be identical, similar to what has been found for a number of other molecules (22, 23, 38, 41).

Internalization of proteins containing tyrosine or leucine-based endosomal sorting signals is mediated by their interactions at the plasma membrane with the adaptor protein complex AP-2, a component of clathrin-coated vesicles (reviewed in Ref. 42). In particular, the medium chain of AP-2 has been demonstrated to bind tyrosine sorting signals in vitro (43–45), suggesting that these interactions are the first step of recognition of tyrosine signals by the AP-2 complex. Sorting from the TGN presumably involves interactions with the medium chain of the AP-1 adaptor complex (42), which binds some tyrosine-sorting signals in vitro with lesser affinity than the medium chain of AP-2 complex (46–48). Our results therefore suggest that the tyrosine-dependent sorting signal of CD1d probably interacts with the AP-2 complex at the plasma membrane, resulting in CD1d internalization.

With few exceptions, basolateral sorting in MDCK cells is thought to occur directly from the TGN. However, the exact transport pathway is not defined, nor is the sorting machinery involved. Basolateral sorting signals can generally be divided into two main groups: those that are collinear with signals for clathrin-coated pits localization and those that are not. This has led to the speculation that different pathways for basolateral sorting exist and that components also involved in endocytosis are utilized in the former. Our results indicate that the basolateral sorting signal in CD1d belongs to the tyrosine-independent group of signals and suggest that sorting components distinct from the ones involved in endocytosis is required for basolateral distribution of this molecule. However, we cannot exclude that the tyrosine is part of the targeting motif for basolateral sorting as well, and it may be that the alanine substitution is not sufficient to avoid recognition of such a motif by the polarized sorting machinery.

To begin to identify the basolateral sorting signal(s) of CD1d, we generated a truncated version of the molecule lacking the last six amino acid residues. This construct was sorted in a nonpolarized fashion, indicating that a basolateral sorting signal is present in the cytoplasmic tail of CD1d. Furthermore, fusion of the last eight residues from the CD1d cytoplasmic tail to the extracellular and transmembrane domains of otherwise apically sorted CD8 molecules (M. Røe, K. Pedersen, A. Simonsen, A. Kelly, and O. Bakke, manuscript in preparation) was sufficient to redirect the chimeric receptor to the basolateral plasma membrane. Therefore, the basolateral sorting information was located to the last eight residues of the CD1d cytoplasmic tail. This sequence bears no similarity to known tyrosine-independent basolateral signals (27, 37, 39, 40, 49) and may, therefore, represent a novel basolateral sorting motif. Precise mapping of this motif requires further investigations.
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References