The Cytoplasmic Tail of CD1d Contains Two Overlapping Basolateral Sorting Signals*

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Dmitrii G. Rodionov, Tommy W. Nordeng, Thomas L. Kongsvik, and Oddmund Bakke†
From the Division of Molecular Cell Biology, Department of Biology, University of Oslo, 0316 Oslo, Norway

CD1d is a member of the CD1 polypeptide family that represents a new arm of host defense against invading pathogens. In our previous work (Rodionov, D. G., Nordeng, T. W., Pedersen, K., Balk, S. P., and Bakke, O. (1999) J. Immunol. 162, 1488–1495) we have shown that CD1d contained a classic tyrosine-based internalization signal (YQGV) in its short cytoplasmic tail. CD1d is expressed in polarized epithelial cells, and we found that the cytoplasmic tail of CD1d also contained information for basolateral sorting. Interestingly, a mutation of the critical tyrosine residue of the endosomal sorting signal did not result in the loss of basolateral targeting of the mutant CD1d. To search for a basolateral sorting signal we have constructed a full set of alanine mutants, but no single alanine substitution inactivated the signal. However, deletions or mutations of either the C-terminal valine/leucine pair or the critical tyrosine residue from the internalization signal and either residue from the C-terminal valine/leucine pair inactivated basolateral sorting. Our data thus suggest that the cytoplasmic tail contains two overlapping basolateral signals, one tyrosine- and the other leucine-based, each being sufficient to direct CD1d to the basolateral membrane of polarized Madin-Darby canine kidney cells.

CD1 polypeptides, evolutionarily related to the major histocompatibility complex class I molecules, represent a new class of antigen-presenting molecules that bind and present lipids and glycolipids rather than peptide antigens and are implicated in host defense against invading pathogens (for review, see Refs. 1–4). CD1d has been reported to present glycolipid antigens such as α-galactosylceramide to the CD1d-restricted natural killer T cells (5–8), but recent data demonstrate that CD1d may interact with a broader array of T cells (9). We have recently studied the mechanisms of intracellular trafficking of CD1d in MDCK1 cells and found that the short cytoplasmic tail of CD1d was important for its internalization and basolateral sorting (10). Our results showed that CD1d contained a classical tyrosine-based internalization signal in its cytoplasmic tail. Replacing either the tyrosine or the hydrophobic valine residue in the +3 position from the tyrosine residue with alanine resulted in a loss of active internalization. However, alanine substitution of neither the critical tyrosine nor the valine disrupted the basolateral distribution of CD1d. Nonetheless, basolateral sorting information in the cytoplasmic tail of CD1d was sufficient to redirect the otherwise apically distributed protein CD6 to the basolateral surface, indicating that the tail contained sufficient information for basolateral sorting (10).

Although the mechanisms for apical sorting remain largely undefined, a number of basolateral sorting signals have been identified. Basolateral sorting signals are currently subdivided into two major classes: signals that are either co-linear or not co-linear with the signals for coated pit localization. Signals that are co-linear with the signals for coated pit localization can be further subdivided into the tyrosine-based basolateral sorting signals, such as those of lysosomal associated membrane protein-1 (11), lysosomal acid phosphatase (12), and TGN38 (13) and leucine-based signals found, for example, in the invariant chain (14), Fc receptor II (15), and furin (16). Signals that are not co-linear with the signals for the coated pit internalization may be either tyrosine-dependent, such as signals in the vesicular stomatitis virus G protein (17) and the low density lipoprotein receptor proximal signal (18), or tyrosine-independent such as in polyimmunoglobulin receptor (19) and the transferrin receptor (20). Tyrosine- and leucine-based sorting signals are believed to interact with one or more of the adaptor complexes, AP1 at the TGN, AP2 at the plasma membrane, and AP3 intracellularly (reviewed in Refs. 21–24), and it has recently been reported that interaction with AP1B may be a part of the polarization machinery (25, 26).

In this study, we sought to identify the basolateral sorting signal in the cytoplasmic tail of CD1d. Our data indicate that there are two overlapping signals within the very last five C-terminal amino acids of CD1d, one tyrosine- and one leucine-based, each being sufficient for its basolateral sorting.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs—**DNA composition of the cytoplasmic tails of constructs used in this study is given in Fig. 1. Mutations in the cytoplasmic tail of CD1d were created by PCR using the wild-type CD1d cDNA as a template. All constructs were subcloned into the pMEP4 vector (Invitrogen) and sequenced.

**Cell Growth—**MDCK (strain II) cells were grown in full growth medium (DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 25 units/ml penicillin, and 25 μg/ml streptomycin) in 5% CO2 in a 37 °C incubator.

**Stable Transfection of MDCK Cells and Clonal Selection—**MDCK cells were stably transfected by the calcium phosphate procedure as described elsewhere (27). Clones expressing DNA constructs under control of the inducible metallothionein promoter in the pMEP4 vector were selected in the presence of hygromycin B (0.3 mg/ml). Resistant clones were isolated and incubated with 25 μM CdCl2 overnight to induce expression of the protein of interest. Clones expressing constructs of interest were identified by screening with the D5 antibody (10).

**Induction of Antibodies—**D5 antibody was labeled with Na125I using IODO-BEADEs (Sigma) as described previously (10). Briefly, the antibody (100 μg) was incubated with 1 μCi of Na125I and IODO-BEADEs for 10 min on ice. Iodinated antibody was then separated from free Na125I on Sephadex G-25M columns (Amersham Pharmacia Biotech). The
specific activity of the labeled antibody was determined by trichloroacetic acid precipitation. The amount of soluble radioactivity was generally less than 5% of total radioactivity.

**RESULTS AND DISCUSSION**

In a previous study (10), we reported that a deletion of the last six amino acids from the CD1d cytoplasmic tail (Fig. 1, delta SYQGLV construct) abolished basolateral sorting of CD1d in MDCK cells (Fig. 2). Furthermore, fusing the last eight CD1d cytoplasmic amino acids to the transmembrane and extracellular domains of the CD8 molecule re-routed this otherwise apical protein to the basolateral surface of MDCK cells. Interestingly, mutation in the tyrosine residue critical for internalization signal required an intact tyrosine residue e.g. (Refs. 14 and 15). This was clearly not the case for the VL-based signal of CD1d, as both residues had to be mutated to impair the basolateral sorting. We, however, noticed that the valine was also a part of the tyrosine-based internalization signal in addition to the residues delta VL (Fig. 2). As shown in Fig. 3, these mutants were no longer sorted basolaterally, indicating that valine and leucine residues were in fact involved in basolateral sorting. It is well established that leucine-based endosomal sorting signals are not necessarily recognized for basolateral sorting (11, 28–32), and our results document the reverse: a basolateral sorting signal that is not active in internalization.

Previous studies have shown that single point mutations within a leucine-based sorting motif were sufficient to abolish basolateral sorting (e.g. Refs. 14 and 15). This might in principle indicate the presence of apical sorting information in these mutants as a truncation of the last eight cytoplasmic residues of CD1d led to truly non-polarized sorting (50/50 apical/basolateral distribution, Fig. 2). However, we cannot draw any conclusion until more is known.

As it was required to mutate residues both within the tyrosine-based internalization signal in addition to the residues in the putative leucine-based basolateral sorting signal in order to disrupt basolateral sorting of CD1d, it is tempting to conclude that the cytoplasmic tail of CD1d contains two overlapping basolateral sorting signals. Efficient basolateral sorting by the tyrosine-based signal required an intact tyrosine residue

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**Fig. 1.** Cytoplasmic tails of different CD1d constructs used in this study. WT, wild type.

**Fig. 2.** Alanine substitution of a single amino acid in the cytoplasmic tail of CD1d does not impair its basolateral sorting information. Cells grown on Transwell polycarbonate filters were incubated with I-D5 antibody on either the apical (AP) or basolateral (BL) side for 1 h on ice, and the bound radioactivity was counted. Bars represent the standard deviation from six independent experiments on cells with varying levels of CD1d expression. WT, wild type.
and one of the bulky hydrophobic residues at the position +3 or +4. A basolateral tyrosine sorting signal with a hydrophobic residue in position +4 has to our knowledge not been reported before, but in most studies the context of tyrosine signals was not investigated in detail. Our data thus suggest that we have identified a new context for a tyrosine-based basolateral signal. It is noteworthy that endosomal sorting mediated by the same tyrosine-based signal was abrogated by single mutation of the +3 valine only (10). This strongly suggests that the context requirements for internalization and basolateral sorting by the tyrosine-dependent signal are different.

The leucine-based basolateral signal is also special, as this type of sorting signal has so far not been found at the very end of naturally occurring molecules. However, it is reported that internalization of certain molecules was still efficient and dependent on a leucine-based signal when all residues C-terminal to the signal were deleted. This has been described, for example, for the dileucine signal in CD3 (33) and in the interleukin-6 signal transducer gp130 (34). It is therefore not surprising that the leucine-based basolateral sorting signal at the very C-terminal end of the CD1d molecule is functional.

We chose to investigate the steady-state distribution of CD1d constructs because our previous studies have shown that newly synthesized CD1d molecules use several hours to reach the cell surface (10), and detectable levels of metabolic labeling were achieved only after 2–3 h of labeling. Therefore, measurements of surface appearance of the newly synthesized CD1d molecules would be difficult to perform and interpret. Internalization and possible recycling of CD1d molecules at the cell surface might also be relatively rapid adding to the problem. We nonetheless believe that CD1d molecules are sorted directly from the TGN to the cell surface like most newly synthesized proteins in the MDCK cells (35). Indirect evidence for this is that CD1dYA and CD1dVA mutants have lost their internalization signal (10) but were still sorted basolaterally (Fig. 2). Had the newly synthesized CD1d molecules been initially delivered to the apical membrane and subsequently internalized and transported to the basolateral membrane, a mutation in the internalization signal should have led to a predominantly apical or non-polarized distribution of the CD1dYA and CD1dVA mutants, which is not the case.

Endosomal tyrosine- and leucine-based sorting signals have been shown to interact with adaptor molecules (e.g., Refs. 36–42). It is generally accepted that tyrosine-based signals may interact with the medium chain of adaptor complexes (36, 37, 40) whereas the leucine signals have been reported to bind to the β chain of AP2 (41) and/or the medium chains of AP1 and AP2 (42, 43). A study by Ohno et al. (26) has identified a novel medium chain (μ1B) that is only expressed in polarized cells. It was recently shown that this chain (which is able to replace μ1A in AP1) may reconstitute polarized sorting in a cell line lacking this molecule (25). Furthermore, the AP4 adaptor may be involved in basolateral sorting of molecules containing both tyrosine- and leucine-based sorting signals. This leads to the suggestion that internalization/endosomal and basolateral sorting signals may be recognized by different adaptors. Our finding that the context of the CD1d tyrosine signal is different for internalization and basolateral sorting and that its leucine-based signal is only functional for polarized sorting lends support to the existence of separate adaptor machineries for internalization and basolateral sorting.

At this point, we can only speculate why there are two basolateral signals within the CD1d molecule. This redundancy is not unique as other molecules also contain more than one polarization signal. For instance, two basolateral signals have been found in the low density lipoprotein receptor (29), and the complex consisting of major histocompatibility complex class II and invariant chain contains no less than four different basolateral signals (14). Separate sorting signals in these molecules may in principle bind more than one adaptor at the same time. In contrast, the two basolateral sorting signals in CD1d are overlapping, and steric hindrance will most likely not allow that they function simultaneously. Because only the tyrosine, but not the leucine-based signal, is involved in internalization of CD1d, it is clear that the signals are able to interact with different components of the intracellular machinery, but the precise mechanisms of such interactions remain to be elucidated.

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