Structural Requirements for Major Histocompatibility Complex
Class II Invariant Chain Trafficking in Polarized Madin-Darby
Canine Kidney Cells*

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The invariant chain (ii) targets major histocompatibility complex class ii molecules to an endocytic processing compartment where they encounter antigenic peptides. Analysis of ii-transferrin receptor chimeras expressed in polarized Madin-Darby canine kidney (MDCK) cells shows that the ii cytoplasmic tail contains a dihydrophobic basolateral sorting signal, Met<sup>16</sup>-Leu<sup>17</sup>, which is recognized in both the biosynthetic and endocytic pathways. Pro<sup>15</sup>-Met<sup>16</sup>-Leu<sup>17</sup> has previously been identified as one of two dihydrophobic ii internalization signals active in non-polarized cells. Pro<sup>15</sup> is also required for endocytosis in MDCK cells but not for basolateral sorting, indicating that the internalization signal recognized at the plasma membrane is distinct from the sorting signal recognized by basolateral sorting machinery. Another dihydrophobic sequence, Leu<sup>2</sup>-Ile<sup>8</sup>, is required for rapid internalization of the chimeric receptors in MDCK cells but not for basolateral sorting, providing further evidence that the structural requirements for basolateral sorting and internalization differ. Deletion analysis suggests that basolateral sorting of newly synthesized ii-tr$	ext{R}$ chimeras is also mediated by the membrane-proximal region of the ii cytoplasmic tail. However, this region does not promote polarized basolateral recycling, indicating that the structural requirements for polarized sorting in the biosynthetic and endocytic pathways are not identical.

Polarized epithelial cells have structurally and functionally distinct plasma membrane domains with distinct protein and lipid compositions (reviewed in Refs. 1 and 2). In simple epithelia, the apical membrane faces the external environment and is specialized for secretion and nutrient absorption, while the basolateral membrane is in contact with the underlying tissue and performs fundamental cellular functions, such as intercellular adhesion and nutrient uptake from the blood supply. To generate and maintain polarity, membrane proteins which function at either the apical or basolateral border must be delivered to the appropriate cell-surface domain.

In Madin-Darby canine kidney (MDCK)$^1$ cells, most newly synthesized apical and basolateral membrane proteins comigrate through the Golgi complex and are selectively delivered to either the apical or basolateral surface (3, 4). Studies during the past few years have established that polarized biosynthetic delivery to the basolateral surface requires distinct sorting signals located within the cytoplasmic domains of trafficking membrane proteins (5–8). Basolateral sorting signals which have been identified within the cytoplasmic domains of trafficking membrane proteins can be distinguished based upon whether or not they are colinear with internalization signals (reviewed in Ref. 9). Most basolateral sorting signals which overlap with internalization signals are dependent for activity upon the same tyrosine residue found to be important for endocytosis. In some cases, however, the structural requirements for basolateral sorting and internalization differ (10, 11), demonstrating that the signal recognized by the basolateral sorting machinery is not identical to the signal recognized within plasma membrane clathrin-coated pits. Members of the other class of basolateral sorting signal, which are spatially separate from endocytic motifs, include the membrane-distal basolateral sorting signal of the low density lipoprotein receptor (10) and the basolateral sorting signal of vesicular stomatitis virus glycoprotein G (12), both of which are tyrosine-dependent, as well as the basolateral sorting signal of the polymeric immunoglobulin receptor, which does not depend upon tyrosine for activity (13).

Two basolateral sorting signals have been identified which are similar to dihydrophobic sorting signals that are known to mediate delivery to the endocytic pathway directly from the biosynthetic pathway or from the plasma membrane via endocytosis (reviewed in Ref. 14). A dileucine sequence in the cytoplasmic tail of the Fc receptor ii B2 isoform (FcR) is required for both basolateral sorting and endocytosis (15, 16), demonstrating that this signal belongs to the class of basolateral sorting signals which are linear with internalization signals. In contrast, a Leu-Val sequence targets newly synthesized CD44 to the basolateral surface but does not promote internalization (17). However, endocytosis of CD44 may be inhibited by direct submembranous interactions between its cytoplasmic domain and the cytoskeleton, preventing clustering in plasma membrane clathrin-coated pits (18).

The trans-Golgi network is generally considered to be the primary site for polarized basolateral sorting in MDCK cells (2, 19), although direct evidence for this is lacking. However, to maintain their polarized cell-surface distribution, basolateral

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1 The abbreviations used are: MDCK, Madin-Darby canine kidney; II, human invariant chain; TR, human transferrin receptor; FcR, Fc receptor II B2 isoform; CEF, chicken embryo fibroblasts; RSV(A), Rous sarcoma virus subtype A; mAb, monoclonal antibody; TT, human transferrin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; H<sub>2</sub>T, II cytoplasmic tail; DMEM, Dulbecco’s modified Eagle’s medium; MHC, major histocompatibility complex.

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membrane proteins which are rapidly internalized via clathrin-coated pits must be efficiently recycled back to this surface. Recent studies have shown that mutations which impair the polarized basolateral delivery of newly synthesized low density lipoprotein receptor and polymeric immunoglobulin receptor also lead to an increase in basolateral-to-apical transcytosis of these proteins, suggesting that in each protein, the same basolateral sorting signal is recognized in both the biosynthetic and endocytic pathways (20, 21).

The MHC class II-associated invariant chain (Ii) assembles with newly synthesized class II αβ dimers in the endoplasmic reticulum and targets them to an endocytic processing compartment in which antigenic peptides are encountered (22). Ii contains within its cytoplasmic domain two independent sorting signals, Leu7-Ile8 and Pro15-Met16-Leu17, related to dihydrophobic signals. Each sorting signal in Ii promotes rapid endocytosis (23–25), and the same or closely related signals are required for intracellular sorting and direct delivery of Ii to the endocytic pathway, which is the predominant route along which class II αβ-Ii complexes are transported (25, 26).

Ii and MHC class II molecules are normally restricted to professional antigen-presenting cells, such as B lymphocytes, macrophages, and dendritic cells. However, expression of both Ii and class II molecules in non-immune epithelial cells can be induced by interferon-γ (27, 28). We have investigated the sorting of Ii in epithelial cells by analyzing the trafficking of Ii-transferrin receptor (TR) chimeras expressed in MDCK cells. We find that the cytoplasmic domain of Ii targets newly synthesized chimeric receptors to the basolateral surface, where they are rapidly internalized. Endocytosed chimeras which recycle to the surface are selectively delivered to the basolateral border, indicating that the cytoplasmic tail of Ii is also recognized by the basolateral sorting machinery in the endocytic pathway. By analysis of mutant Ii-TR chimeras, we demonstrate that a dihydrophobic sorting signal consisting of Met16-Leu17 functions as a basolateral sorting signal which is required for polarized sorting in the endocytic pathway and is involved in basolateral sorting in the biosynthetic pathway. Deletion analysis suggests additional basolateral sorting information resides in the membrane-proximal region of the Ii cytoplasmic tail that is recognized within the biosynthetic pathway but not the endocytic pathway. Both dihydrophobic sequences Leu7-Ile8 and Pro15-Met16-Leu17 that independently mediate efficient endocytosis in nonpolarized cells (23–25) are required for internalization activity in MDCK cells. However, neither Leu7-Ile8 nor Pro15 are required for basolateral sorting of Ii-TR chimeras in MDCK cells providing clear evidence that there are different structural requirements for basolateral sorting and internalization in these cells. Thus, dihydrophobic sorting signals within the cytoplasmic tail of Ii are differentially recognized by the basolateral sorting machinery located within the biosynthetic and endocytic pathways and by plasma membrane clathrin-coated pits.

EXPERIMENTAL PROCEDURES

Expression of Human Ii-TR Chimeras in MDCK Cells—Construction of human Ii-TR chimeras and expression in chick embryo fibroblasts (CEF) using the retroviral vector RCAS-BPA(A) derived from Rous sarcoma virus subtype A (RSV(A)) (29) has been previously described (25). Since both Ii and TR are type II membrane proteins, the polarity of the polypeptide sequence with respect to the membrane is maintained. Recombinant virus produced by transfected CEF was concentrated by centrifugation of 10 ml of tissue culture supernatant at 23,000 rpm for 2.5 h at 4 °C in a Beckman SW40 Ti rotor. The pelleted virus was resuspended in 1 ml of DMEM, then passed through a 0.45-μm filter. The receptor for RSV(A) (30) has been stably expressed in MDCK II cells, rendering them susceptible to infection by RSV(A) (31). MDCK cells derived from a clone expressing the RSV(A) receptor were plated at 106 cells/well of a 24-well tissue culture dish (Costar Corp., Cambridge, MA) and 12 h later were incubated with concentrated recombinant RCAS-BPA(A) virus for 12 h at 37 °C. Afterward, 1 ml of growth medium was added, and the cells were grown to confluency. Expression of mutant human Ii-TR chimeras was analyzed by immunofluorescence using B3/25, a monoclonal antibody (mAb) against the extracellular domain of the receptor, or the goat anti-mouse antibody conjugated to fluorescein isothiocyanate (Cooper Biomedical, Malvern, PA). Individual clones of MDCK cells expressing human Ii-TR chimeras were isolated by limited dilution.

Steady-state Cell-surface Distributions of Human Ii-TR Chimeras—MDCK cells expressing human Ii-TR chimeras were plated at high density (diameter < 10 mm) onto 24-well Costar tissue culture polystyrene plates (0.4 μm pore size) (Costar Corp., Cambridge, MA) and cultured for 3 days, with the media changed on the second day. 125I-Labeled dipheric human transferrin (Tf) (ICN Biomedicals, Irvine, CA) was prepared by incubating 500 μg of Tf with 40 μg of chloramine T (Sigma) and 0.5–1.0 ml of Na2125I (Amersham) in a total volume of 150 μl of phosphate-buffered saline (PBS). The reaction was stopped by adding 80 μg of sodium metabisulfite (Fisher Scientific, Fair Lawn, NJ) in 10 μl of PBS. 125I-Labeled Tf was separated from free 125I on a Sephadex G-25 column equilibrated in PBS. To determine the steady-state cell-surface distribution of human Ii-TR chimeras, filter-grown cells were incubated in DMEM for 1 h at 37 °C, then shifted to 4 °C and washed with PBS (PBS with 1 mM CaCl2 and 1 mM MgCl2) containing 0.5% bovine serum albumin (BSA) (BSA-PBS). Cells were then incubated for 1 h at 4 °C with 4 μg/ml 125I-labeled Tf in BSA-PBS added to either side of the monolayer (150 μl basolaterally or 350 μl apically). Under these conditions, less than 0.1% of the 125I-labeled Tf crossed the monolayers. Cells were then washed 3 times at 4 °C with BSA-PBS (2 ml/monolayer surface), and the amount of radioactivity specifically bound to the cells was determined by excising the filters and counting them in a γ-counter.

Internalization Efficiencies of Human Ii-TR Chimeras—MDCK cells expressing human Ii-TR chimeras were plated at a density of 7.5 × 104 cells/cm2 in a 24-well Costar tissue culture plate and cultured at 37 °C for 1 day. The cells were then incubated for 1 h at 37 °C in 1 ml of serum-free DMEM, then incubated with 150 μl of 4 μg/ml 125I-labeled Tf in DMEM containing 0.5% BSA (BSA-DMEM) for 1 h at 37 °C. The media was removed, and the cells were washed three times at 4 °C with 1 ml of BSA-PBS, then incubated at 4 °C twice for 3 min with 0.5 ml of 0.2 mM acetic acid, 0.5 mM NaCl (pH 2.4) to remove surface-bound 125I-labeled Tf (32). Cells were removed from the wells with 2 washes in 0.5 ml of 1 M NaOH, and radioactivity in the acid wash and the cell lysate was determined. Prolonged incubation with the acid wash did not affect the radioactivity released (33). The internalization efficiencies of mutant human Ii-TR chimeras relative to the wild-type human TR were determined from the steady-state intracellular distribution of 125I-labeled Tf and calculated as described previously (25, 33).

Polarized Cell-surface Delivery of Newly Synthesized Human Ii-TR Chimeras—Filter-grown MDCK cells expressing human Ii-TR chimeras were incubated for 30 min at 37 °C in methionine- and cysteine-free DMEM (monolayer surface), then incubated for 1 h with 125I-labeled methionine-cysteine (ICN Biomedicals, Irvine, CA) and 1% dialyzed fetal bovine calf serum. Monolayers were then washed 2 times with DMEM and chased for 20 or 40 min at 37 °C in DMEM containing a 10-fold excess of unlabeled methionine and cysteine. The cells were then chilled with 2 washes in ice-cold DMEM at 4 °C and incubated for 30 min in DMEM containing 100 μg/ml trypsin added to either the apical or the basolateral surface (Worthington Biochemical Corp., Freehold, NJ). DMEM containing 100 μg/ml trypsin inhibitor (Sigma) was added to the opposite surface. Tryptic cleavage of surface TR generates a soluble ~70-kDa extracellular domain fragment which was immunoprecipitated from the apical and basolateral media using B3/25 mAb and analyzed on 10% SDS-polyacrylamide gels (34). Dried gels were exposed to flushed XAR film (Eastman Kodak, Rochester, NY), and quantitation of radioactivity was performed on a model 425 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). No TR tryptic fragments were detected in immunoprecipitates of media containing trypsin inhibitor.

Polarized Recycling of Internalized Human Ii-TR Chimeras—Filter-grown MDCK cells expressing human Ii-TR chimeras were incubated for 1 h at 37 °C in DMEM, then for 1 h at 37 °C with 4 μg/ml 125I-labeled Tf in BSA-DMEM (150 μl basolaterally and 350 μl apically). Cells were then washed 3 times at 4 °C with BSA-DMEM (2 ml/mmonolayer surface), and surface-bound Tf was removed with >95% efficiency using deroxoramine mesylate as described previously (31, 33). Cells were then washed 3 times with BSA-DMEM at 4 °C and incubated at 37 °C for 90 min in BSA-DMEM containing 100 μg/ml unlabeled Tf (1 ml/mmonolayer surface).
Fig. 1. Schematic diagram of the II_CT chimera from Odorizzi et al. (25) consisting of the 31-residue II cytoplasmic tail (open box) fused to the TR transmembrane region and extracellular domain (shaded boxes). Shown below is the amino acid sequence of the II cytoplasmic tail. Residues that have been deleted from this domain are shown by brackets, and amino acids that have been substituted by alanine are indicated by closed circles.

Fig. 2. Sorting information in the II cytoplasmic tail targets II-TR chimeras to the basolateral surface of MDCK cells. Filter-grown MDCK cells expressing II_CT chimeras were incubated either apically or basolaterally for 1 h at 4 °C with 125I-labeled Tf (4 µg/ml). After washing away unbound Tf, the amount of 125I-labeled Tf specifically bound on each surface was determined (mean ± S.E. of three independent experiments). Values for the distributions of wild-type and the II CT chimeras in filter-grown MDCK monolayers (30) had been expressed (31). The steady-state cell-surface distributions of wild-type and the tailless (Δ3–59) TR, shown for comparison, are from Odorizzi et al. (31).

RESULTS

Sorting Information in the II Cytoplasmic Tail Targets II-TR Chimeras to the Basolateral Surface of MDCK Cells—To investigate the trafficking of II in polarized epithelial cells, we expressed in MDCK cells wild-type and mutant II-TR chimeras (Fig. 1) that had previously been expressed in CEF (25) using a retroviral vector derived from RSV(A) (29, 35). Recombinant retrovirus produced by CEF was used to infect MDCK cells in which the 157-amino acid isoform of the receptor for RSV(A) (30) had been expressed (31). The steady-state cell-surface distributions of II-TR chimeras in filter-grown MDCK monolayers were subsequently determined by measuring the binding of 125I-labeled human Tf at the apical and basolateral surfaces at 4 °C. As shown in Fig. 2, a chimera (II_CT) consisting of the 30-residue amino-terminal cytoplasmic tail of II (p33 isoform) and the transmembrane and extracellular domains of TR was localized predominantly at the basolateral surface, similar to the polarized cell-surface distribution of the wild-type TR (31), demonstrating that the cytoplasmic tail of II contains basolateral sorting information. Deletion of residues 2–11 of the II cytoplasmic tail resulted in a modest decrease in the polarized steady-state basolateral cell-surface expression of chimeric receptors (Fig. 2). However, deletion of residues 2–17 led to almost complete loss of basolateral polarity (Fig. 2), although a small but significantly greater fraction of this mutant chimera was found at the basolateral border when compared with the distribution of the tailless TR (Δ3–59) in MDCK cells (31).

Located within residues 2–17 of the II cytoplasmic tail are two dihydrophobic sorting signals (Leu7-Ile8 and Pro15-Met16-Leu17) which independently promote rapid internalization in non-polarized cells (23–25). However, Leu7-Ile8 are not required for basolateral localization, as alteration of both residues to alanine did not affect the polarized steady-state expression of II_CT chimeras at the basolateral surface (Fig. 2). We next investigated whether Pro15-Met16-Leu17 is involved in basolateral targeting by examining the cell-surface distributions of previously constructed II_CT chimeras in which each of these residues had been individually altered to alanine in the context of an II cytoplasmic tail in which Leu7-Ile8 had also been changed to alanine (25). Fig. 2 shows that alteration of Pro15 to alanine resulted in only a small decrease in the selective steady-state basolateral distribution of II_CT chimeras, suggesting that this residue has, at most, a minor role in basolateral sorting. However, a random cell-surface distribution resulted from the substitution of alanine for Met16. A similar result was obtained if Met16 had been altered to alanine in the context of the wild-type II cytoplasmic tail in which Leu7-Ile8 were intact (Fig. 2), confirming that Met16, but not Leu7-Ile8, is required for selective basolateral expression of II_CT chimeras. Fig. 2 shows that Leu17 is required for basolateral polarity as well, although alteration of this residue more significantly impaired basolateral localization. The steady-state level of the L17A mutant chimera on the basolateral surface is lower than predicted from the independent measurements of the biosynthetic delivery and recycling of the mutant (see Figs. 4 and 5) and, therefore, may reflect differences in either the rates of internalization or degradation of the mutant chimera from the basolateral and apical surfaces. Previous studies of II-TR chimeras in non-polarized cells indicated that Gly18 has no role in high efficiency endocytosis (25). Similarly, substitution of alanine for Gly18 had no effect on the basolateral expression of chimeric receptors in MDCK cells (Fig. 2). These results, therefore, indicate that Met16 and Leu17 are important for the polarized basolateral localization of II_CT chimeras in MDCK cells, whereas Pro15 is not required.

The Structural Requirements for Internalization and Basolateral Sorting of II-TR Chimeras in MDCK Cells Are Distinct—In non-polarized cells, both Leu7-Ile8 and Pro15-Met16-Leu17 independently mediate high efficiency endocytosis (23–25). Therefore, since only Met16-Leu17, but not Leu7-Ile8, were found to be important for basolateral localization, we next investigated whether rapid endocytosis in MDCK cells was mediated by the same internalization signals previously char-
act in concert to mediate rapid endocytosis in MDCK cells. Met16-Leu17 and the Membrane-Proximal Region of the Ii Cytoplasmic Tail Mediate Basolateral Sorting of Newly Synthesized Ii-TR Chimeras in MDCK Cells—The polarized basolateral steady-state cell-surface distribution of TR in MDCK cells has been demonstrated to result from the basolateral sorting of both newly synthesized and recycling receptors (31). Therefore, we first investigated the biosynthetic cell-surface delivery of Ii-TR chimeras by pulse-labeling newly synthesized chimeric receptors with 35S-labeled methionine-cysteine and chasing them to the surface of filter-grown monolayers for 20 or 40 min at 37 °C. Subsequently, the apical or basolateral surface was incubated in trypsin at 4 °C for 30 min with trypsin (100 μg/ml). Trypsin inhibitor (100 μg/ml) was included in the opposite media. B3/25 mAb immunoprecipitates of the TR extracellular fragments in the media collected from each surface were analyzed on SDS-polyacrylamide gels and quantitated by PhosphorImager analysis (mean ± S.E. of three independent experiments). No material could be detected in immunoprecipitates from the media containing trypsin inhibitor. Shown are the data from the 20-min chase which gave identical values for basolateral delivery after a 40-min chase.

FIG. 4. Met16-Leu17 and the membrane-proximal region of the Ii cytoplasmic tail mediate basolateral sorting of newly synthesized Ii-TR chimeras in MDCK cells. Newly synthesized Ii-TR chimeras expressed in filter-grown MDCK cells were labeled with 35S-labeled methionine/cysteine for 30 min and chased to the cell surface for 20 or 40 min at 37 °C. Receptors at the apical or basolateral surface were then cleaved at 4 °C for 30 min with trypsin (100 μg/ml). Trypsin inhibitor (100 μg/ml) was included in the opposite media. B3/25 mAb immunoprecipitates of the TR extracellular fragments in the media collected from each surface were analyzed on SDS-polyacrylamide gels and quantitated by PhosphorImager analysis (mean ± S.E. of three independent experiments). No material could be detected in immunoprecipitates from the media containing trypsin inhibitor. Shown are the data from the 20-min chase which gave identical values for basolateral delivery after a 40-min chase.

FIG. 5. Met16-Leu17, but not the membrane-proximal region, are required for basolateral sorting of Ii-TR chimeras in the endocytic pathway of MDCK cells. Filter-grown MDCK cells expressing Ii-TR chimeras were incubated basolaterally at 37 °C for 1 h with 125I-labeled Tf. Monolayers were then washed at 4 °C, and surface-bound 125I-labeled Tf was removed with deferoxamine mesylate. Cells were then incubated at 37 °C for 90 min, and the appearance of 125I-labeled Tf in the apical and basolateral media was determined (mean ± S.E. of three independent experiments). More than 90% of the internalized 125I-labeled Tf recycled after 90 min at 37 °C.

FIG. 5. Met16-Leu17, but not the membrane-proximal region, are required for basolateral sorting of Ii-TR chimeras in the endocytic pathway of MDCK cells. Filter-grown MDCK cells expressing Ii-TR chimeras were incubated basolaterally at 37 °C for 1 h with 125I-labeled Tf. Monolayers were then washed at 4 °C, and surface-bound 125I-labeled Tf was removed with deferoxamine mesylate. Cells were then incubated at 37 °C for 90 min, and the appearance of 125I-labeled Tf in the apical and basolateral media was determined (mean ± S.E. of three independent experiments). More than 90% of the internalized 125I-labeled Tf recycled after 90 min at 37 °C.
deletion of residues 2–17 completely impaired polarized basolateral recycling (Fig. 5), indicating that the basolateral sorting information in the membrane-proximal region of the Ii cytoplasmic tail which is recognized in the biosynthetic pathway is not recognized by the basolateral sorting machinery in the endocytic pathway. As expected, Leu7–Ile8 were not required for basolateral recycling, nor was Pro15. In contrast, alteration of either Met16 or Leu17 abolished polarized recycling of Ii-TR chimeras to the basolateral surface (Fig. 5). Therefore, Met16, Leu17, but not the membrane-proximal region of the Ii cytoplasmic tail, mediate polarized basolateral sorting in the endocytic pathway.

**DISCUSSION**

By analysis of human Ii-TR chimeras expressed in MDCK cells, we have provided evidence that Ii contains a dihydrophobic basolateral sorting signal, Met16–Leu17, that overlaps but is distinct from an internalization signal previously identified in the Ii cytoplasmic tail. The Met16-Leu17 signal promotes selective delivery of newly synthesized Ii-TR chimeras to the basolateral surface and is required for the polarized basolateral recycling of internalized chimeric receptors. The Met16-Leu17 basolateral sorting signal in Ii is similar to the dileucine signal identified in FcR (15, 16). Both signals promote biosynthetic transport to the basolateral surface and overlap internalization signals previously identified within these proteins. However, based upon an extensive mutagenic analysis, the structural requirements for basolateral sorting and internalization mediated by the dileucine signal in FcR were indistinguishable (15, 16). In contrast, recognition of the Met16-Leu17 signal in Ii as a basolateral sorting signal could be distinguished from the overlapping internalization signal by the absolute requirement for Pro15 for rapid endocytosis but not for basolateral sorting. As Gly is also not required for basolateral sorting activity, Met16-Leu17 appear sufficient to create a basolateral sorting signal, although whether or not other more distant residues may be important for its recognition is unknown. Evidence has been reported that acidic residues amino-terminal to the dihydrophobic internalization signals in Ii are important for their activity in some structural contexts (36).

The preferential basolateral delivery (~75%) of newly synthesized Ii-TR chimeras in which residues 2–17 had been deleted suggests that the membrane-proximal region of the Ii cytoplasmic tail contains a basolateral sorting signal that is recognized within the biosynthetic pathway. Nonetheless, the basolateral sorting signal comprised of Met16 and Leu17 also appears to be involved in biosynthetic sorting to the basolateral surface, as alteration of either residue to alanine decreased the polarity of cell-surface delivery of newly synthesized chimeras. However, whereas alteration of Met16 resulted in an intermediate decrease in the polarity of biosynthetic transport, biosynthetic cell-surface delivery was random upon substitution of alanine for Leu17. One possible explanation for this result is that this mutation of Leu17 significantly altered the conformation of the Ii cytoplasmic tail, thereby inactivating the membrane-proximal basolateral sorting signal. A comparison of the amino acid sequence of the membrane-proximal region of the Ii cytoplasmic tail, which contains several charged residues but no large hydrophobic amino acids (Fig. 6), reveals no sequence similarities with other basolateral sorting signals. One possibility that cannot be excluded is that the amino-terminal methionine residue of the Ii cytoplasmic tail contributes to the targeting of newly synthesized IiΔ2–17 chimera to the basolateral surface by virtue of its juxtaposition to the membrane-proximal region. Thus, defining the structural basis of the membrane-proximal basolateral sorting signal detected in the IiΔΔ2–17 chimera and determining whether the activity is present in the wild-type cytoplasmic tail will likely require substantial additional mutagenic analysis. Regardless of the structural basis of the membrane-proximal basolateral sorting signal, the fact that this region only mediates basolateral sorting in the biosynthetic pathway implies that the structural requirements for basolateral sorting in the biosynthetic and endocytic pathways differ.

Interestingly, the internalization signal consisting of Leu7–Ile8 in the cytoplasmic tail of Ii, which was previously identified in non-polarized cells (23–25) and is required for rapid endocytosis in MDCK cells, is not involved in basolateral sorting. Thus, as in the case of tyrosine-based internalization signals, dihydrophobic internalization signals do not necessarily function simultaneously as basolateral sorting signals. NMR analyses of a synthetic peptide corresponding to residues 1–27 in the Ii cytoplasmic tail suggest that Leu7 and Ile8 are within a nascent helix, whereas the Pro15-Met16-Leu17 sequence is part of a type I β turn (37), which may be significant since a β turn has been proposed to be a common structural feature of basolateral sorting signals (13). However, substitution of alanine for Pro15, which destabilizes the turn (37) and abrogates rapid internalization (25), only modestly impairs basolateral sorting, suggesting that a turn structure is not critical for recognition of the Met16-Leu17 signal by the basolateral sorting machinery. The known structural requirements for the dihydrophobic internalization and basolateral sorting signals located in the Ii cytoplasmic domain are summarized in Fig. 6.

Although both Leu7–Ile8 and Pro15-Met16-Leu17 are required for rapid endocytosis in MDCK cells and cannot act separately, previous studies indicated that each signal is independently active as an internalization signal in non-polarized cells (24, 25). The reason for this functional difference is not clear but may reflect species-specific or cell type-specific differences in the ability of plasma membrane clathrin-coated pits to recognize each motif. Another example of how the recognition of Ii dihydrophobic internalization signals can be cell-dependent is that in HeLa cells, in contrast to CEF, although each signal was found to be independently active, their activity in the wild-type Ii cytoplasmic tail was not additive, implying that the signals cannot be recognized simultaneously in HeLa cells (36). Such cell-dependent differences have also been observed for internalization of lysosomal acid phosphatase in MDCK cells and BHK cells (11).

The tyrosine-based internalization signal is the most thoroughly characterized class of membrane protein sorting signal (reviewed in Ref. 38), and structural predictions as well as two-dimensional NMR analyses of synthetic peptides suggest that these 4- or 6-residue motifs adopt a tight turn conformation, displaying the critical aromatic or large hydrophobic...
amino acids in the first and last positions on the same side of the turn. In addition to tyrosine-based internalization signals, several dihydrophobic sorting signals have been found to mediate high efficiency endocytosis (15, 16, 25, 38–43). One common feature shared between each type of signal is the location of two hydrophobic residues within close proximity to each other, and this structural similarity may underlie the functional equivalence of these signals within plasma membrane clathrin-coated pits. As in the case of the dileucine signal in FcγR (15, 16) and the Met<sup>16</sup>-Leu<sup>17</sup> signal in I<sub>ii</sub>, several tyrosine-dependent basolateral sorting signals have been identified that are closely related to endocytic motifs (6, 44–46). Thus, structural similarity may also underlie the basolateral sorting activity of both dihydrophobic and tyrosine-dependent signals, suggesting that each type of signal is recognized by a common basolateral sorting mechanism.

Whereas alteration of Leu<sup>2</sup>-Ile<sup>3</sup> had no effect on the polarized basolateral recycling of I<sub>ii</sub>-TR chimeras, deletion of residues 2–11 resulted in a small but significant decrease in the basolateral sorting of internalized chimeric receptors. In contrast, this deletion did not affect the basolateral sorting of newly synthesized chimeras. This observation suggests that recognition of the Met<sup>16</sup>-Leu<sup>17</sup> basolateral sorting signal in the endocytic pathway can be influenced by mutations not involving residues comprising the signal itself, confirming recent studies of the activity of the TR basolateral sorting signal in the biosynthetic and endocytic pathways of MDCK cells.<sup>2</sup> One possible explanation for the diminished activity of these two signals upon alterations outside the region important for basolateral sorting is that an indirect conformational change significantly alters the structure of the cytoplasmic tail. Thus, unlike tyrosine-based internalization signals, which are self-determined structural similarity may also underlie the basolateral sorting activity of both dihydrophobic and tyrosine-dependent signals, suggesting that each type of signal is recognized by a common basolateral sorting mechanism.

Coat proteins are likely to be involved in the recognition and sorting of basolateral membrane proteins. Although both clathrin and COPI coats have been proposed to mediate basolateral sorting of newly synthesized membrane proteins (8), evidence for their role in biosynthetic basolateral transport has yet to be provided. In non-polarized cells, biochemical studies suggest that COPI subunits associate with endosomal membranes (47, 48), while morphological studies have identified clathrin-coated buds on the endosome compartment (49). We have recently identified clathrin and γ-adaptin on the sorting endosome complexes of MDCK cells, and functional data indicate that this coat protein complex is required for basolateral sorting of internalized TR.<sup>3, 4</sup> Whether dihydrophobic basolateral sorting signals are recognized by the same clathrin-based sorting machinery remains to be determined.

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