The invariant chain (Ii) targets newly synthesized major histocompatibility complex class II complexes to a lysosome-like compartment. Previously, we demonstrated that both the cytoplasmic tail (CT) and transmembrane (TM) domains of Ii were sufficient for this targeting and that the CT contains two di-leucine signals, DQRDLI8 and EQLPML17 (Odorizzi, C. G., Trowbridge, I. S., Xue, L., Hopkins, C. R., Davis, C. D., and Collawn, J. F. (1994) J. Cell Biol. 126, 317–330). In the present study, we examined the relationship between signals required for endocytosis and those required for lysosomal targeting by analyzing Ii-transferrin receptor chimeras in quantitative transport assays. Analysis of the Ii CT signals indicates that although DQRDLI8 is necessary and sufficient for endocytosis, either di-leucine signal is sufficient for lysosomal targeting. Deletions between the two signals reduced endocytosis without affecting lysosomal targeting. Transplantation of the DQRDLI sequence in place of the EQLPML signal produced a chimera that trafficked normally, suggesting that this di-leucine sequence coded for an independent structural motif. Structure-function analysis of the Ii TM region showed that when Ii TM residues 11–19 and 20–29 were individually substituted for the corresponding regions in the wild-type transferrin receptor, lysosomal targeting was dramatically enhanced, whereas endocytosis remained unchanged. Our results therefore demonstrate that the structural requirements for Ii endocytosis and lysosomal targeting are different.

Major histocompatibility complex class II molecules are cell surface molecules that function to bind peptide antigens and present them to CD4+ helper T cells. Newly synthesized major histocompatibility complex class II αβ complexes are targeted to lysosome-like compartments (1–3) by the invariant chain (Ii) (reviewed in Ref. 4). Upon arrival, Ii is degraded, and αβ chains acquire peptide antigens (5–11). αβIi complexes are delivered to the lysosomal compartment from the TGN directly (11) or indirectly from the cell surface, where they are rapidly endocytosed (6, 12, 13). Both direct and indirect pathways are utilized for delivery of class II to a processing compartment (6, 11, 13, 14).

Support for the idea that Ii is sorted to the latter stages of the endocytic pathway comes from studies in which the Ii cytoplasmic tail was replaced with the TR cytoplasmic tail (15). Newly synthesized class II molecules containing this TR-Ii chimera are delivered to the cell surface and efficiently internalized, but Ii proteolysis is blocked, and class II antigen presentation is inhibited (15). This suggests that the TR cytoplasmic tail, which contains a tyrosine-based internalization signal (16, 17), is not sufficient for delivery of the class II complexes to a lysosome-like processing compartment and that additional sorting information is required.

Two Ii cytoplasmic tail targeting signals have been identified using Ii-neuraminidase chimeras (18) and Ii-TR chimeras (14), Leu7-Ile8 and Met16-Leu17. These di-leucine signals were first identified in the CD3 γ-chain and the cation-dependent mannose 6-phosphate receptor and shown to be important for lysosomal and late endosomal targeting, respectively (19, 20). They consist of two hydrophobic residues, usually leucine-leucine or leucine-isoleucine, that are often preceded by four hydrophilic residues (19). For Ii, residues amino-terminal to the di-leucine-like motifs are important for sorting (14, 21, 22), suggesting that Ii di-leucine signals like those found in the CD3 γ- and δ-chains consist of six residues, DQRDLI8 and EQLPML17.

NMR structural data on the Ii cytoplasmic tail suggests that the membrane-distal signal lies within a nascent helix, whereas the membrane-proximal motif is a part of a turn (22). Tyrosine-based signals have been proposed to be turn structures as well (16, 23–25), and have been shown to form independent structural motifs because one tyrosine-based signal can often substitute for another (26–28). Whether di-leucine-based signals form independent structural motifs is unknown. Furthermore, how tyrosine- and di-leucine-based signals are related is unclear, especially because they appear to be recognized by distinct cytosolic factors (29).

Although native Ii is a trimer, studies of mixed trimers containing wild-type and tailless Ii demonstrate that trimers containing at least two wild-type Ii molecules are targeted properly in fibroblasts (30), illustrating that molecules containing two or three Ii cytoplasmic tails traffic the same. Furthermore, the quaternary structural requirements for Ii sorting appear to be flexible because tetramers containing the Ii cytoplasmic tail spliced to the transmembrane and extracellular domains of neuraminidase traffic the same as wild-type Ii trimers (18).

The purpose of this study was to define how internalization signals differ from those required for delivery to the latter stages of the endocytic pathway. We examined two regions of the Ii that had previously been shown to be important for targeting to the lysosomal compartment, the cytoplasmic tail (CT) and the transmembrane region (TM) (14). We took advantage of the fact that we could quantitatively monitor 1) internalization using radiolabeled transferrin (Tf), and 2) lysosomal
targeting by monitoring the half-lives of the Ii-TR chimeras (14). Mutational and functional analysis of II CT suggests that some mutations affected endocytosis, some affected lysosomal targeting, and some affected both. Analysis of the II TM region indicated that two distinct regions within the TM were sufficient to confer lysosomal targeting on a reporter molecule without affecting internalization rates. The results demonstrate that the structural requirements for II endocytosis and lysosomal targeting are not the same.

**EXPERIMENTAL PROCEDURES**

**Human II-TR Constructs—**Mutants were prepared as described previously (14) by the method of Kunkel (31). Polymerase chain reaction fragments encoding the various cytoplasmic tail deletion mutants were subcloned into a tailless TR construct that contained BP/II and NheI sites 5 bp from the start site and at the beginning of the transmembrane region (amino acid position 64–65 in the TR sequence), respectively (CCL-1). Introduction of the NheI site created a substitution mutation (Ala for Gly at position 64, GGA-AGT → GCT-AGC). This substitution had no effect on internalization or expression levels. Mutants were screened and selected by restriction mapping or sequencing and cloned into the expression vector BH-RCAS (32). All mutations were verified by dideoxynucleotide sequencing of the entire cytoplasmic or transmembrane domain in BH-RCAS constructs (33, 34).

**Expression of II Chimeras and Wild-type TR—**II chimeras and wild-type TR were expressed in chicken embryo fibroblasts (CEF’s) as described previously (35). Surface expression of II chimeras and mutants was determined by measuring the binding of 125I-labeled Tf at 4°C (35).

**Internalization Assay—**The rate of Tf internalization was determined using the IN/SUR method (36). Diferric human Tf (Miles Scientific, Naperville, IL) was labeled with 125I to a specific activity of 2–4 Ci/mg using Enzymobeads (Bio-Rad) according to the manufacturer’s directions. CEF’s were plated in triplicate at a density of 7.5 × 10^4 cells/cm² in 24-well tissue culture plates 24 h before the assay (Costar Corp., Cambridge, MA). Cells were incubated in serum-free Dulbecco’s modified Eagle’s medium for 1 h at 37°C. Cells were then lysed with 1 M NaOH. Radioactivity in the acid wash (SUR) and in the cell lysate (IN) was determined. The ratio of internalized (IN) to surface (SUR) counts indicates the relative protein expression of each of the constructs compared with the wild-type TR, TR or II-TR chimeras were then immunoprecipitated from postnuclear supernatants and quantitated on SDS-polyacrylamide gels by Phosphorimager analysis. To determine the relative degradation rate, we measured the relative half-lives of each of the constructs using a metabolic pulse-chase procedure. The relative biosynthetic and degradation rates were then compared with the wild-type TR control.

For example, if the surface-labeled counts of the chimera are 25% that of the TR, the relative biosynthetic rate is 4 times that of the TR, and the relative degradation rate is 8 times (t½, chimera = 3 h; t½, TR = 24 h), then the percentage of the chimeras reaching the cell surface (surface-accessible pool) is 50% (4/4 × 7/4 × 5% × 100%). In this case, 50% of the chimeras traffic directly to the lysosome, and 50% are delivered to the cell surface before delivery to the lysosome. This calculation shows that all of the TR that is synthesized reaches the cell surface and that little degradation of the chimeras occurs during the 1 h of labeling (125I-labeled TR) from the surface (surface-labeled counts).

**RESULTS**

**II-TR Chimeras Are Expressed on the Cell Surface and Rapidly Internalized—**To determine whether the structural features of II that are important for endocytosis are the same as those required for targeting to the latter stages of the endocytic pathway, we constructed II-TR chimeras consisting of either the wild-type or mutant II cytoplasmic tails spliced to the transmembrane and extracellular domains of TR (Fig. 1). Wild-type human TR and II-TR chimeras were stably expressed in CEF’s using BH-RCAS, a replication-competent retroviral vector derived from the Rous sarcoma virus (38). Binding studies at 4°C using 125I-labeled human Tf indicated that all of the II-TR chimeras were expressed on the cell surface, although at lower levels than the wild-type TR (data not shown).

Internalization rates of the II-TR chimeras were monitored using the IN/SUR method of Wiley and Cunningham (36). Analysis of the chimera with the wild-type II chain cytoplasmic tail (IICT) indicated that it was internalized as rapidly (kᵢ = 0.117 min⁻¹) as the wild-type TR (kᵢ = 0.107 min⁻¹; Table I). This internalization rate compares favorably with human TR internalization rates determined in other cell types (25). A tailless TR (Δ22–59), for comparison, was internalized poorly (kᵢ = 0.006 min⁻¹; Table I).

**Residues 20–29 of the II Cytoplasmic Tail Are Not Required for Endocytosis or Lysosomal Targeting—**Two regions of the II chain cytoplasmic tail, residues 3–8 and 12–17, have been reported to be important for sorting in the endocytic pathway (14, 18, 21, 22, 39). Additional signals may be contained in the membrane-proximal region of the II cytoplasmic tail (15, 40).

To determine whether the first 19 residues of the II chain cytoplasmic tail were sufficient for both endocytosis and lysosomal targeting, we prepared three deletion mutants, IICT Δ20–29, IICT Δ20–24, and IICT Δ25–29 (Fig. 1B). Each deletion mutant was tested in internalization assays and found to have nearly wild-type internalization activity (74–120%; Table I), indicating that residues 20–29 were not required.

Because II is transported to an acidic endocytic compartment, where it is rapidly degraded (5–8), we next determined the effect of these deletions on the half-lives of the chimeras in metabolic pulse-chase experiments. CEF’s expressing either TR, IICT, IICT Δ20–29, IICT Δ20–24, or the IICT Δ25–29 mutant were pulse-labeled with 35S-trans label for 30 min and chased in complete medium for the indicated periods of time; TR and II-TR chimeras were then isolated by immunoprecipitation and analyzed by SDS-PAGE (Fig. 2). The IICT chimera was rapidly degraded with an average half-life of 3.1 ± 0.4 h (Table II, average ± S.E.), similar to the half-life of native II in antigen presenting cells (41), whereas the IICT Δ20–29 chimera was degraded with a half-life of 6.7 ± 0.2 h (Table II, average ± S.E.). Interestingly, the II-TR chimeras with smaller deletions were degraded more rapidly (IICT Δ32–20, IICT Δ32–24, t½ = 3.0 ± 0.1 h; IICT Δ32–25–29, t½ = 3.7 ± 0.9 h), suggesting that none of these residues from 20 to 29 per se were important for targeting to the processing compartment. In contrast, the half-life of the wild-type TR was greater than 20 h (t½ = 21.2 ± 1.6 h, Table II).

After 2 h (Fig. 2), the Mᵢ of TR and II-TR chimeras increased to that of the mature glycoprotein (42), indicating that the II-TR chimeras traverse the Golgi where glycosylation is complete and are degraded in a post-Golgi compartment. Degradation of...
The Ii CT chimera occurred within the endocytic pathway because treatment of the cells with 50 mM NH₄Cl during the labeling and chase period increased the half-life dramatically (data not shown).

The Membrane-distal Signal, DQRDLI, Is Necessary for Endocytosis and Sufficient for Lysosomal Targeting—Two dileucine-like signals, Leu⁷-Ile⁸ and Met¹⁶-Leu¹⁷, are reported to promote Ii internalization (14, 18). These studies, however, relied on radiolabeled bivalent antibody uptake (18) or steady-state distributions (14) to monitor endocytosis. To monitor internalization rates more precisely, we measured ¹²⁵I-labeled Tf uptake for the Ii CT Ala⁷-Ala⁸ mutant and the Ii CT Ala¹⁶-Ala¹⁷ mutant (Fig. 1B). Comparison of the internalization rates indicated that IiCT Ala⁷-Ala⁸ mutant was internalized poorly (\(k_e = 0.013\)), whereas the IiCT Ala¹⁶-Ala¹⁷ mutant had significant activity (\(k_e = 0.066\)) (Fig. 3, A versus B). The Ii CT Ala¹⁶-Ala¹⁷ mutant had 65% activity (IiCT \(k_e = 0.101\)) (Fig. 3B), whereas the IiCT Ala⁷-Ala⁸ mutant had 13% activity, similar to the background activity of a tailless TR (\(k_e = 0.008\)) (Fig. 3C). The Ii CT mutant was internalized at essentially the same rate as the wild-type TR (Fig. 3B, \(k_e = 0.101\) versus 0.114, respectively). The results indicate that the membrane-distal signal, Leu⁷-Ile⁸, is required for efficient endocytosis, whereas the membrane-proximal signal, Met¹⁶-Leu¹⁷, is not.

We next determined the effect of the Ala⁷-Ala⁸ and Ala¹⁶-Ala¹⁷ mutations on lysosomal targeting by analyzing these chimeras in metabolic pulse-chase experiments. The results indicate that both chimeras were rapidly degraded (\(t_{1/2}; 5.2\) h (Fig. 4A) and \(t_{1/2} \sim 7.0\) h (Fig. 4B), respectively), although neither was degraded as rapidly as the wild-type Ii chimera.

![Diagram](image-url)
Experiment (of three) is shown. To XAR film overnight (Kodak). Immunoprecipitates were quantitated on a Model 425 PhosphorImager (Molecular Dynamics). A representative from postnuclear supernatants and analyzed on SDS-polyacrylamide gels as described under “Experimental Procedures.” Dried gels were exposed to XAR film overnight (Kodak). Immunoprecipitates were quantitated on a Model 425 PhosphorImager (Molecular Dynamics). A representative experiment (of three) is shown.

FIG. 2. Residues 20–29 of the invariant chain cytoplasmic tail are not required for efficient lysosomal targeting. Equivalent cell numbers of CEFs expressing wild-type TR, IICT, IICTΔ20–29, IICTΔ20–24, or IICTΔ25–25 chimeras were pulse-labeled for 30 min with 35S-trans label and chased with complete medium for various periods of time as indicated (in hours). Wild-type TR or IICT chimeras were then immunoprecipitated from postnuclear supernatants and analyzed on SDS-polyacrylamide gels as described under “Experimental Procedures.” Dried gels were exposed to XAR film overnight (Kodak). Immunoprecipitates were quantitated on a Model 425 PhosphorImager (Molecular Dynamics). A representative experiment (of three) is shown.

Table II

| TR constructs | Half-life | Wild-type |
|---------------|----------|-----------|
| Wild-type TR  | 21.2 ± 1.6 (16) | 100 |
| IICT         | 3.1 ± 0.4 (5)  | 15 |
| IICTΔ20–29   | 6.7 ± 0.2 (3)  | 32 |
| IICTΔ20–24   | 3.0 ± 0.1 (3)  | 14 |
| IICTΔ22–29   | 3.7 ± 0.9 (3)  | 17 |
| IICTΔAla7–Ala8 | 5.8 ± 0.7 (3)  | 27 |
| IICTΔAla15–Ala17 | 6.9 ± 0.1 (3)  | 33 |
| IICTΔ39     | 3.0 ± 0.4 (3)  | 14 |
| IICTΔ9–11   | 3.5 ± 0.5 (3)  | 17 |
| IICTΔ25–29  | 3.4 ± 0.4 (3)  | 16 |
| IICTΔ32DQRDL17 | 15.3 ± 2.8 (4) | 72 |
| IICTΔEQPML6,12DQRDL17 | 5.6 ± 1.7 (4) | 26 |
| IICTΔ      | 15.7 ± 3.0 (3) | 74 |
| IICTΔ1–10   | 2.8 ± 0.4 (4)  | 12 |
| IICTΔ11–19  | 2.5 ± 1.4 (3)  | 12 |
| IICTΔ20–24  | 2.5 ± 0.6 (3)  | 12 |
| IICTΔ25–29  | 2.5 ± 0.6 (3)  | 12 |

*Mean value ± S.E.; number in parentheses is the number of independent experiments.

(average = 3.1 h, Table II). This demonstrated that either region was sufficient for lysosomal targeting.

To determine whether the cell surface pool of IICTΔAla7–Ala8 chimeras was delivered to the lysosomal compartment despite its poor internalization activity, we incubated cells expressing IICTΔAla7–Ala8 IICT, or wild-type TR with 125I-labeled Tf for 1 h at 37 °C. This procedure loaded the endocytic pathway with receptor-ligand complexes (14). The cells were then rapidly washed, and the reappearance of intact and degraded Tf in the medium was monitored by measuring TCA insoluble and soluble radioactivity, respectively. As expected, apo-Tf released into the medium from cells expressing the wild-type TR was undegraded (Fig. 5), because TR-apo-Tf complexes are efficiently recycled back to the cell surface through sorting and recycling compartments (35, 37, 43, 44). Only about 2% of the counts were in the TCA soluble fraction after 2 h (TCA soluble counts). In contrast, ~16% of the 125I-labeled Tf released from cells expressing the IICTΔAla7–Ala8 chimera was degraded, implying that this percentage of chimeras traffics directly from the cell surface to the lysosomal compartment where they are degraded. Although this is less efficient degradation than the IICT chimera (~32%), these results demonstrate that the surface-expressed IICTΔAla7–Ala8 chimera is also efficiently delivered to the lysosomal compartment, albeit with reduced kinetics. This suggests three things. First, endocytosis does not appear to be the rate-limiting step in lysosomal transport of Tf. Second, only one of the cytoplasmic tail signals, either LI or ML, is necessary for efficient lysosomal delivery. Third, the two signals appear to be additive with regard to lysosomal targeting efficiency.

The Membrane-distal Signal, DQRDL1, Is Necessary for Sorting at the TGN—Because the majority of major histocompatibility complex class II complexes are delivered from the TGN to the lysosomal compartment without appearing on the cell surface, we tested to see whether the mutations that affected endocytosis also affected sorting at the TGN. Cells expressing wild-type TR, IICT, IICTΔAla7–Ala8, IICTΔAla15–Ala17, IICTΔ20–29, IICTΔ20–24, and IICTΔ25–29 were incubated with 125I-labeled Tf for 1 h to label the endocytic pathway. The cells were then washed with BSA-PBS at 4 °C to remove the unbound Tf, lysed in 1% Nonidet P-40, and counted in a gamma counter. Comparison between the individual cell lines gave an estimate of the total amount of chimeras on the surface and in endosomal compartments (Table III, surface-labeled counts). In companion dishes, cells were pulse-labeled with 35S-trans label for 30 min. TR or II-TR chimeras were then immunoprecipitated from postnuclear supernatants and quantitated on SDS-polyacrylamide gels by PhosphorImager analysis to determine the relative biosynthetic rates of each of the constructs. In a third set of dishes, we measured the relative half-lives of each of the constructs using a metabolic-pulse-chase procedure. The relative biosynthetic rates and degradation rates were then compared with the wild-type TR control. Based on the assumption that all the wild-type TR reaches the cell surface and is labeled with Tf, we estimate that only ~34% of the IICTΔ is delivered to the cell surface (surface-accessible pool; see under “Experimental Procedures” for details of the calculation) (Table III). The only mutation which increases the surface-accessible
pool, is the Ala\(^7\)-Ala\(^8\) mutation, the same one that disrupted endocytosis, suggesting that the signals for TGN sorting and endocytosis are closely related.

The Membrane-distal Sequence, DQRDLI, Can Substitute for the Membrane-proximal Signal without Affecting Endocytosis or Lysosomal Targeting—The di-leucine signals in Ii appear to be related to the identified six-residue signal in the CD3 \(\gamma\)-chain, DKQTLL (19). To test whether the two Ii di-leucine related sequences, DQRDLI and EQLPML, coded for independent structural motifs, such as those that have been identified for tyrosine-based signals (25–28, 45), we replaced one sequence, EQLPML, with the other, DQRDLI (IiCT\(^{12}\)DQRDLI\(^{17}\)).

We also prepared a mutant in which the two sequences were substituted for each other (IiCT\(^3\)EQLPML\(^8\), 12DQRDLI\(^{17}\), Fig. 1B).

Analysis of these two mutants in internalization assays indicated that Ii CT\(^{12}\)DQRDLI\(^{17}\) and Ii CT\(^3\)EQLPML\(^8\), 12DQRDLI\(^{17}\) were both endocytosed (Table I, \(k_e = 0.077\) and 0.050, respectively), although neither was equivalent to the wild-type Ii (Table I, \(k_e = 0.117\)). Analysis of the Ii CT\(^{12}\)DQRDLI\(^{17}\) mutant in a metabolic pulse-chase experiment indicated that it was rapidly degraded (\(t_{1/2}; 3\) h, Fig. 6A). Whereas the Ii CT\(^3\)EQLPML\(^8\), 12DQRDLI\(^{17}\) mutant had an extended half-life (\(t_{1/2}; 12\) h, Fig. 6A). These results demonstrated that the DQRDLI sequence can substitute for the EQLPML signal without a significant loss of either sorting event, whereas swapping the two sequences results in a partial loss of internalization activity and an almost total loss of lysosomal targeting. This suggests that the sequence EQLPML could not substitute for the DQRDLI signal.

Deletion of Ser\(^9\) (D\(^9\)), Ser\(^9\)-Asn\(^10\) (D\(^9\)–10), or Ser\(^9\)-Asn\(^10\)-Asn\(^11\) (D\(^9\)–11) Inhibits Endocytosis without affecting Lysosomal Targeting—To determine whether residues between the two di-leucine signals were required for either targeting event, we prepared mutants in which one, two, or three residues were deleted (IiCT\(^D\(^9\), IiCT\(^{D\(^9\)}\)–10, and IiCT\(^D\(^9\)–11, respectively; Fig. 1B). Analysis of these mutants indicates that these deletions result in a 32, 52, and 36% loss of internalization activity, respectively (Table I, \(k_e = 0.079\), 0.061, and 0.075, respectively). Analysis in metabolic pulse-chase experiments indicated that the half-lives remain unchanged (Fig. 6B; IiCT\(^D\(^9\), \(t_{1/2} = 3.0\) h, IiCT\(^D\(^9\)–10, \(t_{1/2} = 2.8\) h, and IiCT\(^D\(^9\)–11, \(t_{1/2} = 3.5\) h, suggesting that these modifications affected endocytosis without corresponding effects on lysosomal targeting).

Two 10-Amino Acid Regions from the Ii Transmembrane Are Sufficient to Promote Lysosomal Targeting of the TR—To localize the lysosomal targeting signal in the Ii TM domain (14), we prepared Ii-TR chimeras that contained \(\sim 10\) amino acid segments from the Ii TM region transplanted into the corresponding regions of the wild-type TR (Fig. 7). Each of these chimeras was expressed in CEFs using BH-RCAS. Binding studies at
The results indicate that the half-life of the IiTM 20—29 chimera was extended from 1.5 h to approximately 8 h (Fig. 9), indicating that the degradation was occurring within the endocytic pathway. Ammonium chloride treatment extended the half-life of the IiTM 11–19 chimera as well (data not shown). A summary of the half-lives of the Ii-TR chimeras is shown in Table II. Analysis of the TM chimeras in internalization assays demonstrated that none of the mutations dramatically affected internalization (Table IV, 86–129% activities), indicating that the increased degradation of the chimeras was not caused by a corresponding increase in internalization.

**DISCUSSION**

By analyzing Ii-TR chimeras in quantitative assays, we report that the two cytoplasmic tail di-leucine signals appear to be recognized at distinct cellular sites. We provide evidence that the membrane-distal signal, which includes Leu7-Ile8, is important for endocytosis and lysosomal targeting, whereas the membrane-proximal signal, which includes Met16-Leu17, appears to be important for lysosomal targeting (Fig. 10). The distinction between the two signals is best illustrated by the fact that modification of the membrane-distal signal, Leu7-Ile8 to Ala7-Ala8, resulted in a 9-fold reduction in internalization activity, whereas modification of the membrane-proximal signal, Met16-Leu17 to Ala16-Ala17, had less than a 2-fold effect. Interestingly, Leu7-Ile8 signal also appeared to be required for sorting at the TGN because its disruption resulted in a 3-fold increase in the amount of chimera that reached the cell surface. In contrast to sorting at the TGN and plasma membrane, both di-leucine signals appear to promote lysosomal targeting, although neither alone was as efficient as both in combination.

Met16-Leu17 has been reported to be important for internalization (14, 18, 21, 40, 46), but these studies were based on bivalent antibody binding or measurement of steady-state distributions. Bivalent antibodies have been shown to promote lysosomal targeting of receptors that normally recycle (47–51), including the TR (37, 43). In the present study, we directly measured transferrin uptake, a process that does not affect the endocytosis or trafficking of the TR (52). This allowed us to differentiate internalization signals from lysosomal signals because we followed the fate of the chimeras with a monovalent ligand (53), transferrin. Furthermore, because steady-state distribution measurements for estimation of internalization rates are based on the assumption that all of the cell surface protein efficiently recycles (14) (a requirement that is not met with lysosomally directed proteins, as is the case here), we measured the internalization rates directly.

One surprising finding regarding lysosomal targeting was the fact that although the IiCT Ala7-Ala8 chimera was poorly internalized, it was still rapidly degraded. The half-life of this chimera was the same as the IiCT Ala16-Ala17 chimera, which was internalized rapidly. This suggests that the rate-limiting step for lysosomal targeting is not endocytosis. Further support for this idea comes from studies on mutant hemagglutinins by Zwart et al. (54), who demonstrated that targeting to the latter stages of endocytic pathway does not simply correlate with the concentration of mutant hemagglutinins in the early endosome (54). With their hemagglutinin mutants, they demonstrated that there was no correlation between internalization rate and degradation rate and, furthermore, that the signal for these two processes were distinct. Although the IiCT Ala7-Ala8 chimeras most dramatically illustrated the demarcation between these two processes, a number of the other Ii mutants (∆8, ∆9–10, ∆9–11) had compromised internalization activity without any lysosomal targeting loss. Studies on P-selectin and the epidermal growth factor receptor also support the idea that distinct regions of these two molecules are required for endo-

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**FIG. 5. Degradation of Tf bound to Ii-TR chimeras.** Equivalent cell numbers of CEFs expressing either wild-type TR, Ii-CT, or Ii-CT Ala7-Ala8 chimeras were preincubated in serum-free Dulbecco’s modified Eagle’s medium for 30 min at 37 °C and then incubated with 125I-labeled Tf for 1 h at 37 °C. The cells were then washed and reincubated at 37 °C in Dulbecco’s modified Eagle’s medium containing 50 μg/ml unlabeled Tf for various times. Acid-soluble radioactivity (□) or acid-insoluble 125I-labeled Tf (○) released into the medium, as well as surface-bound 125I-labeled Tf (△) or acid-insoluble 125I-labeled Tf (○), were determined as described under “Experimental Procedures” and are expressed as a percentage of total radioactivity recovered. Each data point is the average of triplicate determinations from a representative experiment (of two).

4 °C using 125I-labeled human Tf indicated that all of the chimeras were expressed on the cell surface of transfected CEFs, but at lower levels than the wild-type TR (data not shown).

Analysis of the chimeras in metabolic pulse-chase experiments indicated that two regions within the Ii TM region were sufficient to target the TR to a processing compartment, residues 11 though 19 and residues 20 though 29 (Fig. 8; IiTM 11–19, t1/2 = 3.0 h; IiTM 20–29, t1/2 = 3.5 h). For comparison, the half-lives of the wild-type TR and IiTM 1–10 chimera were 24 and 17 h, respectively. The chimera that contained Ii TM residues 1–10 and 20–29 had an average half-life of 2.5 h (Table II), suggesting that the TM 20–29 mutation was sufficient in either context for lysosomal targeting. As before, the Ii-TR chimeras were maturely glycosylated, indicating that they had traversed the Golgi complex and were not simply misfolded proteins. To confirm that proteolysis of the transmembrane chimeras was occurring in a lysosomal compartment, we determined their half-lives in the presence of ammonium chloride.
cytosis and lysosomal targeting (55, 56). These studies, as well as our own on Ii, suggest, therefore, that monitoring endocytosis of cell surface molecules is not a reliable method for monitoring down-regulation or lysosomal targeting.

By monitoring the relative amounts of Ii-TR chimeras that reached the cell surface, we show that the same signal important for internalization, Leu7-Ile8, is also important for recognition at the TGN. A number of studies have suggested that di-leucine signals can be recognized at the cell surface (19, 57) and the TGN (20, 58). How recognition at the two cellular sites differs remains unclear. One attractive model is that the position of the signal within the tail specifies at which site it is recognized (59). Dietrich et al. (59) propose that receptors containing membrane-distal di-leucine signals are sorted directly from the TGN to the endosome/lysosome, whereas receptors containing membrane-proximal di-leucine signals require phosphorylation for internalization and lysosomal targeting. Consistent with this model, the Ii cytoplasmic tail is phosphorylated on a serine residue, although the site is not known (60). Although our study does not support this model because the membrane-distal signal appears to be required for sorting at both sites, it does support the idea that the position of the signal influences how and where it is recognized.

One of the goals of this study was to determine whether the two di-leucine signals in Ii were equivalent. Although position clearly influenced how each was being recognized, we also wondered whether the particular sequences themselves were specific. To address this point, we substituted the sequence from the membrane-distal signal, DQRDLI, for the membrane-proximal signal, EQLPML. Our results demonstrated that this mutation did not disrupt either targeting event, suggesting that there was nothing unique about the second signal. These results also suggested that di-leucine signals, like tyrosine-based signals, can often substitute for each other. Interestingly, however, swapping the signals resulted in a loss of both targeting events. The most likely explanation for this comes from the NMR data on a 27-residue peptide corresponding to the Ii cytoplasmic tail, which suggests that the first 14 residues lie within a nascent helix, whereas the membrane-proximal signal which includes Pro15-Met16-Leu17 is a part of a turn (22). Thus, placing a proline residue at position 6 of the Ii cytoplasmic tail probably disrupted the secondary structure, thereby inhibiting recognition for all sorting events.

Deletion of residues 20–29 of the Ii cytoplasmic tail indicated that none of these residues were specifically required for endocytosis or lysosomal targeting. The mutant lacking residues 20–29, however, was degraded more slowly than either of the two deletions encompassing this region, suggesting two things. First, none of the residues per se were required for lysosomal targeting; second, the position of the two di-leucine regions relative to the transmembrane region influenced lysosomal targeting. This is consistent with studies on LAMP-1 that suggest a strict positional requirement of a signal for lysosomal targeting events (61). Interestingly, the membrane-proximal region of Ii has also been reported to be recognized by the basolateral sorting machinery in the biosynthetic pathway in MDCK cells (40). Our studies suggest that this region is not necessary for TGN recognition in fibroblasts because the relative amounts of this chimera that reached the cell surface were the same as the wild-type Ii (Table III).

Our analysis of the Ii transmembrane domain indicates that two 10-amino acid regions within this domain, residues 11–19 and residues 20–29, are sufficient to target the TR to lysosomal compartment without affecting the relative internalization rate. The structural features within these regions that mediate this effect are not known. We demonstrated that the chimeras were properly glycosylated, which suggests that none of them were misfolded proteins. We also showed that the degradation of the chimeras occurred within the endocytic pathway because it could be inhibited with weak bases. The only other known signals to be identified in transmembrane regions are Golgi retention signals (reviewed in Ref. 62). Interestingly, the important feature of Golgi localization signals appears to be the presence of uncharged polar residues (Asn, Thr, and Gln), which when mutated result in the loss of Golgi retention (63, 64). The Ii has an unusual number of polar residues, although they are not lined along one face of the predicted helix as they

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**TABLE III**

**Comparisons of the surface-accessible pools of Ii-TR chimeras, one of two independent experiments.**

| Construct              | Surface-labeled counts | Relative biosynthetic ratea | Relative degradation rateb | Surface-accessible pool |
|------------------------|------------------------|----------------------------|---------------------------|-------------------------|
| Wild-type TR           | 61,028                 | 1.00                       | 1.00                      | 100                     |
| IiCT                  | 15,734                 | 0.39                       | 3.53                      | 34                      |
| IiCTAla15-Ala17       | 68,018                 | 0.19                       | 4.24                      | 89                      |
| IiCTAla7-Ala8         | 54,202                 | 0.11                       | 3.16                      | 31                      |
| IiCTA20–29            | 23,553                 | 0.18                       | 7.07                      | 50                      |
| IiCTA20–24            | 20,419                 | 0.17                       | 5.73                      | 32                      |
| **Relative degradation rate**
| **Surface-accessible pool** |

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a Biosynthetic rate per biosynthetic rate of chimera.

b Half-life of chimera.

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**Fig. 6. Structural requirements of the invariant chain cytoplasmic tail necessary for lysosomal targeting.** Wild-type TR, IiCT 12–DQRDLI17, or IiCT 12–EQLPML16 12–DQRDLI17 (A) or wild-type TR, IiCT Δ9, IiCT Δ9–10, or IiCT Δ9–11 (B) chimeras were pulse-chased, immunoprecipitated from postnuclear supernatants, and analyzed on SDS-polyacrylamide gels as described in Fig. 2. A representative experiment (of three) is shown.
are in the case of the cis-Golgi protein M (64). The implication from Golgi localization studies is that polar residues mediate protein-protein interactions resulting in oligomerization of protein complexes within the lipid bilayer (62). Oligomerization of the TM chimeras could result in a similar retention mechanism within the endocytic pathway, resulting in a loss of recycling efficiency.

Because the three-dimensional structure of the nonameric complex of the major histocompatibility complex class II molecule is not known, the significance of a signal within the transmembrane domain is not clear. However, because the interaction site between the α-β chains and the Ii is in the extracellular domain and proteolysis of the Ii extracellular

FIG. 7. Schematic diagram of the invariant chain-transferrin receptor transmembrane chimeras. Schematic representation of the transferrin receptor with various regions replaced by the corresponding sequence from the invariant chain. Unshaded areas represent regions of Ii-TR derived from TR; shaded areas represent regions derived from Ii. The sequence shown is the invariant chain transmembrane sequence. Residues 1–10, 11–19, and 20–29 of the transferrin receptor were replaced with the corresponding sequences from the invariant chain. Constructs are referred to in text by the corresponding names shown at the left.

FIG. 8. Residues 11–19 and 20–29 of the invariant chain transmembrane region are sufficient to mediate targeting of the TR to a post-Golgi processing compartment. Equivalent cell numbers of CEFs expressing the WT TR, Ii TM 1–10, Ii TM 11–19, or Ii TM 20–29 chimeras were pulse-chased, immunoprecipitated from postnuclear supernatants, and analyzed on SDS-polyacrylamide gels as described in Fig. 2. A representative experiment (of three) is shown.

FIG. 9. Ammonium chloride inhibits degradation of the Ii TM 20–29 chimera. CEFs expressing the Ii TM 20–29 chimera were preincubated with 50 mM NH₄Cl (±50 mM NH₄Cl) and then pulse-labeled with ³⁵S-trans label and chased in the presence (±50 mM NH₄Cl) or absence (control) of NH₄Cl. The chimeras were then immunoprecipitated and analyzed on SDS-polyacrylamide gels as described in Fig. 2. A representative experiment (of two) is shown.

TABLE IV
Comparisons of the internalization rates of the transferrin receptor-invariant chain transmembrane chimeras

| TR-invariant chain constructs | Internalization rate constant | Internalization efficiency % |
|-----------------------------|------------------------------|----------------------------|
| Wild-type TR                | 0.110 ± 0.016 (6)*           | 100                        |
| Ii TM                       | 0.157 ± 0.020 (6)            | 125                        |
| Ii TM 1–10                  | 0.115 ± 0.018 (6)            | 105                        |
| Ii TM 11–19                 | 0.142 ± 0.026 (6)            | 129                        |
| Ii TM 20–29                 | 0.095 ± 0.009 (6)            | 86                         |
| Ii TM 1–10, 20–29           | 0.105 ± 0.015 (6)            | 95                         |

* Mean value ± S.E.; number in parentheses is the number of independent experiments.

FIG. 10. Summary of the structural features of the internalization and lysosomal targeting signals within the Ii cytoplasmic tail and transmembrane region. The cytoplasmic tail of Ii contains one region that is necessary for efficient endocytosis (overlined) and two regions that are required for efficient lysosomal targeting (underlined). Mutation of either region decreases lysosomal targeting efficiency by approximately 50%. Deletions between the two regions inhibit endocytosis (up to 50%) without affecting lysosomal targeting. The sequence DQRDLI can replace EQLPML without a significant loss of internalization or lysosomal targeting activity. The position of the two regions relative to the TM region influences both internalization and lysosomal targeting. Two regions within the Ii transmembrane domain are important for lysosomal targeting (underlined). Each TM region is individually sufficient to promote lysosomal of the TR. Neither TM region has any effect on endocytosis.
domain occurs first, one possibility is that the transmembrane signal operates once the extracellular portions of Ii are gone and the α-β chains have been released. This would ensure that the residual portions of Ii would be degraded in the lysosome. Clearly, degradation of the Ii-TR chimera proceeds more rapidly when the Ii transmembrane region is included (14). In conclusion, the results reported here suggest that the relative location of the di-leucine signal within the Ii cytoplasmic tail influences how it is recognized by the sorting machinery of the cell. Furthermore, our study suggests that TR chimeras containing Ii cytoplasmic tail or transmembrane signals are efficiently delivered to the lysosomal compartment. Because the cellular machinery required for sorting in the endosomal compartment has yet to be identified, it is unclear how signals from the two different domain structures would be recognized.

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