

Sorting Signals in the MHC Class II Invariant Chain Cytoplasmic Tail and Transmembrane Region Determine Trafficking to an Endocytic Processing Compartment

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Abstract. Targeting of MHC class II molecules to the endocytic compartment where they encounter processed antigen is determined by the invariant chain (Ii). By analysis of Ii-transferrin receptor (TR) chimera trafficking, we have identified sorting signals in the Ii cytoplasmic tail and transmembrane region that mediate this process. Two non-tyrosine-based sorting signals in the Ii cytoplasmic tail were identified that mediate localization to plasma membrane clathrin-coated pits and promote rapid endocytosis. Leu⁷ and Ile⁸ were required for the activity of the signal most distal to the cell membrane whereas Pro¹⁵ Met¹⁶ Leu¹⁷ were important for the membrane-proximal signal.

The same or overlapping non-tyrosine-based sorting signals are essential for delivery of Ii-TR chimeras, either by an intracellular route or via the plasma membrane, to an endocytic compartment where they are rapidly degraded. The Ii transmembrane region is also required for efficient delivery to this endocytic processing compartment and contains a signal distinct from the Ii cytoplasmic tail. More than 80% of the Ii-TR chimera containing the Ii cytoplasmic tail and transmembrane region is delivered directly to the endocytic pathway by an intracellular route, implying that the Ii sorting signals are efficiently recognized by sorting machinery located in the *trans*-Golgi.

MAJOR histocompatibility complex (MHC)¹ class II molecules are polymorphic cell surface glycoproteins expressed primarily on specialized antigen-presenting cells such as macrophages, dendritic cells, and B lymphocytes. They bind peptides derived from exogenous proteins and present them to CD4⁺ helper T cells as part of the mechanism for recognizing foreign antigens and stimulating an immune response. MHC class II molecules displayed on the cell surface are $\alpha\beta$ heterodimers, but during their intracellular transport to the cell surface, they are transiently associated with a third nonpolymorphic polypeptide, the invariant chain (Ii). Ii is a type II membrane protein with different isoforms that arise from alternative splicing of one exon and the use of two translation initiation sites (Strubin et al., 1986; O'Sullivan et al., 1987; Koch et al., 1987).

Studies of mutant mice lacking Ii have established that Ii plays a critical role in the surface expression of class II molecules and in the ability of class II molecules to present native protein antigens (Viville et al., 1993; Bikoff et al., 1993). Ii determines the membrane trafficking of MHC class II $\alpha\beta$ dimers (Bakke and Dobberstein, 1990; Lotteau et al., 1990) and also blocks their peptide-binding site during transit through the biosynthetic pathway (Roche and Cresswell, 1990; Teyton et al., 1990; Roche et al., 1992). Ii is assembled with class II α and β polypeptides in the endoplasmic reticulum, and, after its transport through the Golgi region, the $\alpha\beta$ Ii complex is sorted out of the constitutive biosynthetic pathway to an acidic compartment within the endocytic system (Cresswell, 1985; Lamb et al., 1991; Pieters et al., 1991). Within this prelysosomal (Peters et al., 1991), early lysosomal (Harding and Geuze, 1993), or endosomal compartment (Guagliardi et al., 1990), Ii dissociates from class II $\alpha\beta$ dimers, permitting binding of peptides from exogenous foreign antigens generated in the endocytic pathway. Class II $\alpha\beta$ dimers loaded with peptide are then transported to the cell surface, while the free Ii is rapidly degraded (Blum and Cresswell, 1988; Pieters et al., 1991). Although $\alpha\beta$ Ii complexes are thought to be delivered to an endocytic compartment predominantly by an intracellular route, small amounts

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1. *Abbreviations used in this paper:* CEF, chicken embryo fibroblasts; CT, cytoplasmic tail; Ii, human invariant chain; Ii_{CT}, the cytoplasmic tail of Ii; Ii_{TM}, Ii transmembrane region; MHC, major histocompatibility complex; M6PR, mannose-6-phosphate receptor; RT, room temperature; Tf, human transferrin; TR, human transferrin receptor; TM, transmembrane region.

of Ii and $\alpha\beta$ Ii complexes have been detected on the cell surface, and rapid internalization of $\alpha\beta$ Ii complexes has been reported (Wraight et al., 1990; Lotteau et al., 1990; Roche et al., 1993). These data suggest that at least some newly synthesized Ii molecules are transported to an endocytic compartment via the plasma membrane, a route analogous to that taken by some integral lysosomal membrane proteins, e.g., lysosomal acid phosphatase (Braun et al., 1989; Peters et al., 1990).

Ii contains one or more sorting signals within its amino-terminal cytoplasmic domain required for localization to the endocytic system (Bakke and Dobberstein, 1990; Lotteau et al., 1990; Simonsen et al., 1993). If Ii with a truncated cytoplasmic domain is coexpressed with class II $\alpha\beta$ dimers, Ii fails to dissociate, and $\alpha\beta$ Ii complexes are expressed on the cell surface (Roche et al., 1992). However, the sorting signals within the cytoplasmic domain of Ii have not been well characterized, and neither the trafficking pathway taken by MHC class II $\alpha\beta$ Ii complexes nor the endocytic membrane compartment where $\alpha\beta$ dimers encounter antigen have been clearly defined.

In this study, we have constructed Ii-human transferrin receptor (TR) chimeras with the aim of characterizing Ii sorting signals. We show that the cytoplasmic domain of Ii contains two independent non-tyrosine-based sorting signals, both of which mediate internalization via clathrin-coated pits of the plasma membrane. Both signals contain two adjacent large hydrophobic nonaromatic residues suggesting that this may be a common feature of non-tyrosine-based sorting signals. Trafficking of Ii-TR chimeras to an endocytic compartment where they are degraded is dependent upon these signals but occurs predominantly by a direct intracellular route, implying that the non-tyrosine-based sorting signals are efficiently recognized by sorting machinery located in the *trans*-Golgi. This endocytic compartment is distinct from the sorting and recycling endosomal compartments traversed by the wild-type TR, and, therefore, is located within the prelysosomal/lysosomal branch of the endocytic pathway. In addition, we show that efficient delivery of Ii-TR chimeras to this endocytic compartment requires an independent signal in the Ii transmembrane region.

Materials and Methods

Human Ii-TR Constructs

A Cla I fragment containing the entire coding region of the wild-type human TR was cloned into the phagemid pBluescript SK (Stratagene, La Jolla, CA) (Jing et al., 1990). Oligonucleotide site-directed mutagenesis was used to generate the cytoplasmic tail and transmembrane domain of Ii (Ii_{CT} and Ii_{TM}) chimeric constructs from a pBluescript SK phagemid template of the wild-type TR by the method of Kunkel (1985) using the Muta-gene mutagenesis kit (Bio-Rad Labs., Richmond, CA). The same method was used to generate the Ii_{CT+TM} chimeric construct and mutant Ii_{CT} chimeric constructs from pBluescript SK phagemid templates of the cytoplasmic tail of Ii (Ii_{CT}). Oligonucleotides were prepared on a model 391 PCR-MATE DNA synthesizer using phosphoramidite chemistry and purified by polyacrylamide gel electrophoresis (Appl. Biosystems, Inc., Foster City, CA). Mutants were selected by restriction mapping or differential hybridization, and Cla I fragments encoding the mutant receptors were excised and cloned into the retroviral expression vector BH-RCAS (Hughes et al., 1990). The mutations were verified by dideoxynucleotide sequencing (Sanger et al., 1977; Tabor and Richardson, 1987) of the BH-RCAS constructs using the Sequenase kit (US Biochemical Corp., Cleveland, OH) according to the manufacturer's directions.

Expression of Wild-type TR and Ii-TR Chimeras in CEF

Chicken embryo fibroblasts (CEF) were prepared from fertilized eggs (SPAFAS, Norwich, CT) and grown in DME supplemented with 1% (vol/vol) chicken serum, 1% (vol/vol) defined bovine calf serum (Hyclone, Logan, UT), and 2% (vol/vol) tryptose phosphate broth (Difco, Detroit, MI). CEF were transfected with 30 μ g retroviral construct DNA per 10-cm tissue culture plate of 40% confluent cells using the polybrene-dimethyl sulfoxide method (Kawai and Nishizawa, 1984). One to two weeks after transfection with the BH-RCAS constructs, the CEF stably expressed the wild-type TR and chimeric Ii-TR constructs as a result of infection by recombinant virus. Surface expression levels of the wild-type TR and chimeric Ii-TR constructs was determined by measuring the binding of ¹²⁵I-labeled human transferrin (Tf) at 4°C. Diferric human Tf (Miles Scientific, Naperville, IL) was labeled with ¹²⁵I to a specific activity of 2–4 μ Ci/ μ g using Enzymobeads (Bio-Rad Labs.) according to the manufacturer's directions. CEF were plated in triplicate at a density of 7.5×10^4 cells/cm² in 24-well tissue culture plates 24 h before the binding assay (Costar Corp., Cambridge, MA). Cells were incubated in serum-free DME for 1 h at 37°C, and then washed once with ice-cold 0.15 M NaCl, 0.01 M sodium phosphate buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA-PBS). ¹²⁵I-labeled Tf (4 μ g/ml) in 0.15 ml of BSA-PBS was added to triplicate wells and incubated at 4°C for 60 min. Cells were then washed three times with 0.5 ml of ice-cold BSA-PBS, removed from the wells with 0.5 ml of 1 M NaOH, and the radioactivity was counted in a gamma counter.

Determination of Steady-state Distribution of Ii-TR Chimeras

The apparent internalization efficiencies of the wild-type TR and chimeric Ii-TR constructs were estimated from measurements of the steady-state distribution of receptors at 37°C (Tanner and Leinhard, 1987). CEF were plated in triplicate wells as described for the binding studies. The cells were first incubated in serum-free DME, and then incubated with 4 μ g/ml ¹²⁵I-labeled Tf in BSA-PBS for 1 h at 37°C. The labeling medium was removed, and the cells were washed three times with 1 ml of ice-cold BSA-PBS, and then incubated twice for 3 min with 0.5 ml 0.2 M acetic acid-0.5 M NaCl (pH 2.4) to remove surface-bound ¹²⁵I-labeled Tf (Hopkins and Trowbridge, 1983), and removed from the wells with 1 M NaOH. Radioactivity in the acid wash and in the cell lysate was determined. More prolonged incubation with the acid wash did not change the amount of ¹²⁵I released. At steady state, the rate of internalization, k_{int} , of cell surface Tf-TR complexes, $[TR]_{sur}$, equals the rate of externalization, k_{ext} , of the internal pool of apoTf-TR complexes, $[TR]_{int}$; i.e., $k_{int} \cdot [TR]_{sur} = k_{ext} \cdot [TR]_{int}$, assuming an insignificant rate of degradation of internalized receptors during the time required to achieve steady-state. The values of $[TR]_{sur}$ and $[TR]_{int}$ can be obtained from steady-state binding of TR under saturating conditions at 37°C. As k_{ext} of apoTf-TR complexes is independent of signals in the TR cytoplasmic domain (Jing et al., 1990), k_{ext} values of mutant and wild-type receptors are identical so that their k_{int} values are proportional to their steady state distribution, $[TR]_{int}/[TR]_{sur}$; i.e., mutant internalization efficiency percent = $[TR]_{int}(\text{mutant}) \cdot [TR]_{sur}(\text{wild-type}) \times 100/[TR]_{sur}(\text{mutant}) \cdot [TR]_{int}(\text{wild-type})$. Kinetic studies demonstrated that the steady-state distributions of wild-type TR as well as the Ii-TR chimeras were achieved within 20 min and did not change if cells were incubated up to 1 h.

Iron Uptake Assay

The internalization efficiencies of wild-type TR and Ii-TR chimeras were also determined by measuring their ability to mediate iron uptake. Human apo-Tf was labeled with ⁵⁹Fe (FeCl₃; Amersham Corp., Arlington Heights, IL) to a specific activity of 5–10 μ Ci/ μ g using nitritriacetate (Bates and Schlachet, 1973). Cells were plated in triplicate wells as described for the binding studies. The following day, cells were washed twice in prewarmed (37°C) serum-free DME, and then incubated in DME containing 0.1% BSA and 4 μ g/ml ⁵⁹Fe-Tf at 37°C for 0, 1, 2, 3, and 4 h. At the indicated times, the medium was removed, and cells were washed three times with ice-cold BSA-PBS. Cells from triplicate wells for each time point were removed in 0.5 ml 1 M NaOH, and the radioactivity was counted in a gamma counter. The relative levels of wild-type TR and chimeric Ii-TR constructs expressed on the various CEF populations were determined in each experiment by measuring ¹²⁵I-labeled Tf binding at 4°C for each well. After preincubation for 1 h at 37°C in serum-free DME, triplicate wells of cells were incubated with 4 μ g/ml ¹²⁵I-labeled Tf on ice for 1 h, and then washed three

times with 1 ml of ice-cold BSA-PBS, and the radioactivity bound to the cells was counted in a gamma counter.

Measurement of Tf Proteolysis after Internalization

CEF were plated in triplicate wells as described for the binding studies. Cells were preincubated in serum-free DME for 30 min at 37°C, and then incubated with ¹²⁵I-labeled Tf (4 µg/ml) in BSA-PBS for 1 h at 37°C. The medium was removed and the cells were washed three times with ice-cold BSA-PBS and incubated at 37°C with prewarmed (37°C) DME containing 0.1% BSA and 50 µg/ml unlabeled Tf for 0, 5, 10, 15, 20, 40, or 60 min. After incubation, the medium was collected, protein was precipitated in 10% TCA and removed by centrifugation, and then the acid-soluble and acid-insoluble radioactivity was counted in a gamma counter. The surface-bound and internalized Tf in CEF was determined by the acid wash procedure described for the steady-state distribution assay.

¹²⁵I Labeling of Surface TR and Measurement of Degradation Rate

CEF expressing chimeric Ii-TR constructs were ¹²⁵I surface-labeled using lactoperoxidase for 10 min essentially as described (Omary and Trowbridge, 1981), except that iodination was performed in 6-cm tissue culture dishes in a total volume of 0.5 ml. CEF were then washed four times with ice-cold PBS and incubated at 37°C in complete medium for 0, 2, 3, 4, or 6 h. At each time point, cells were solubilized on ice with 1% NP-40-PBS, and the chimeric Ii-TR constructs were immunoprecipitated using B3/25 monoclonal antibody specific for the extracellular domain of the human TR (Trowbridge and Omary, 1981). Immunoprecipitates were analyzed on 7.5% SDS-polyacrylamide gels, which were then dried and exposed to preflashed XAR film (Eastman Kodak, Rochester, NY).

Metabolic Labeling and Immunoprecipitation

CEF were washed twice with methionine-free DME, and then pulse-labeled for 30 min in 1 ml of DME containing 0.12 mCi/ml *trans*-³⁵S-label (ICN Biomedicals, Irvine, CA) and 2% defined calf serum. Pulse-labeled cells were chased for 0, 2, 4, 8, or 24 h in complete medium. In one experiment, cells were preincubated for 4 h in medium containing 100 µg/ml leupeptin, and then pulse-labeled and chased for 0, 1, 2, 3, or 4 h in leupeptin-containing medium. At each time point, cells were solubilized on ice with 1% NP-40-PBS. The wild-type TR and chimeric Ii-TR constructs were immunoprecipitated from postnuclear supernatants using B3/25 monoclonal antibody and analyzed on 7.5% SDS-polyacrylamide gels (Trowbridge and Omary, 1981). Dried gels were exposed to preflashed XAR film (Eastman Kodak, Rochester, NY). Quantitation of radioactivity was performed on a model 425 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Fraction of Ii-TR Chimeras Transiently Expressed On Cell Surface

The surface-accessible pools of wild-type TR and Ii-TR chimeras were calculated from the ¹²⁵I-labeled Tf bound to intracellular and surface receptors at steady-state. The surface-accessible pool of chimera relative to wild-type TR, $[(Ii-TR)_{SA}]$, equals the cell surface chimera-Tf complexes, $[(Ii-TR)_{sur}]$, plus internalized chimera-Tf complexes $[(Ii-TR)_{int}]$, multiplied by 100, divided by cell surface TR-Tf complexes $[TR]_{sur}$, plus internalized TR-Tf complexes, $[TR]_{int}$; i.e., $[(Ii-TR)_{SA}] = \frac{[(Ii-TR)_{sur}] + [(Ii-TR)_{int}]}{[TR]_{sur} + [TR]_{int}} \times 100$ assuming insignificant degradation of intracellular receptors. This condition is met as steady-state conditions are achieved in ~20 min at 37°C (Jing et al., 1990; see also above). In 20 min, <10% of the total ¹²⁵I-labeled Tf bound to the most rapidly degraded chimera, IiCT+TM, is released into the medium as TCA soluble material (see Fig. 7). The fraction of Ii-TR chimeras trafficking via the cell surface is the surface accessible pool, $[(Ii-TR)_{SA}]$, divided by the rate of biosynthesis of the Ii-TR chimera multiplied by the rate of degradation of $[(Ii-TR)_{SA}]$; i.e., $(Ii-TR)_{SA} \times$ degradation rate of synthesis.

As an example of the calculation, the surface expression of IiCT+TM is 12% relative to wild-type TR, and at steady-state 76% of the IiCT+TM chimera and 62% of the wild-type TR are internalized (see Results and Table I). Thus, $[(IiCT+TM)_{SA}] = \frac{(12 + [12 \times 76/24]) \times 100}{100 + [100 \times 62/38]} = 19\%$. Quantitation of the data presented in Fig. 2 showed that eightfold more IiCT+TM chimera was synthesized than wild-type TR, whereas quantitation of the data presented in Fig. 8 showed that the $[(IiCT+TM)_{SA}]$ is degraded eightfold faster than wild-type TR. Therefore, the

fraction of IiCT+TM chimeras trafficking via the cell surface relative to wild-type TR is $19 \times 8/8\%$ or 19%.

Indirect Immunofluorescence

CEF and CEF expressing wild-type or Ii-TR chimeras were plated onto glass coverslips and cultured overnight. The cells were fixed with 2% formaldehyde treatment for 15 min at room temperature (RT), quenched in 0.27% NH₄Cl, 0.37% glycine, pH 7.2, and incubated in P/H/S (PBS/10% horse serum/0.1% saponin) (for permeabilized samples) or P/H (for nonpermeabilized samples) for 30 min at RT. The samples were next incubated with monoclonal antibody B3/25 (undiluted hybridoma supernatants) followed by a second incubation with rat anti-mouse IgG₁-FITC (1:20 dilution; Zymed Labs.). Each incubation step was followed by three washes in P/H/S or P/H for 10 min each. In all cases, antibodies were diluted in P/H/S or P/H, and primary and secondary antibodies were incubated for 1 h and 30 min, respectively. Coverslips were mounted in 1 mg/ml p-phenylene-diamine in a 1:10 mixture of PBS:glycerol and sealed with nail polish. Slides were analyzed on a Nikon Optiphot microscope equipped with epifluorescence and a DAGE MTI series SIT video camera (Dage-MTI, Michigan City, IN) coupled to an Image 1-AT (Universal Imaging, Media, PA) image analysis system. Micrographs were prepared with a Sony Color Video Printer (UP-5000).

For double-label indirect immunofluorescence, cells were fixed, permeabilized, and incubated with the following combinations of antibodies: rabbit anti-human TR antiserum (1:500 dilution), goat anti-rabbit Ig-FITC (1:20 dilution; American Qualex), mouse monoclonal antibody anti-LEP100 (undiluted tissue culture supernatant, Developmental Studies Hybridoma Bank, University of Iowa), goat anti-mouse IgG-Texas red (1:100; Zymed Laboratories). Slides were analyzed on a Leitz fluorescence microscope equipped with a Vario Orthomat II camera system and a dual wavelength (FITC/Texas red) epifluorescence filter module. Micrographs were prepared with Ektachrome P1600 Professional film processed at ASA 800.

Manufacture of Protein A-Colloidal Gold Conjugates and Immunohistochemistry

Colloidal gold (6 and 10 nm diameter) sols were made as described by Slot and Geuze (1985). Protein A gold and rabbit anti-mouse Ig gold antibody conjugates (1978) were made by standard methods (de Mey, 1986) and cryosections made and immunolabeled essentially as described by Tokuyasu (1978). The sections were incubated 30 min with a rabbit antiserum against human TR (Trowbridge, I.S., unpublished results) followed by 6-nm gold protein A for 20 min, and then rinsed for 20 min with five changes of PBS and incubated with anti-LEP 100 mouse monoclonal antibody for 30 min followed by 10-nm gold rabbit anti-mouse Ig antibody for 20 min before an additional five rinses in PBS. Omitting either first or second specific antibodies indicated nonspecific labeling was less than 5%. Staining and embedding were carried out as described previously (Hopkins, 1983) and the sections were examined at 80 keV in a Philips 301 electron microscope. Quantitation of the IiCT+TM chimera in clathrin-coated pits was determined on conventional plastic sections from cells incubated with B3/25-gold complexes at 4°C as described earlier (Miller et al., 1991).

Results

Ii-TR Chimeras Are Expressed on the Cell Surface and Rapidly Internalized

To investigate the signal-dependent trafficking of Ii, we constructed Ii-TR chimeras consisting of the Ii cytoplasmic tail, the TR extracellular domain, and the transmembrane region from either TR or from Ii (Fig. 1). We reasoned that Ii-TR chimeras would be expressed in sufficient amounts on the cell surface to identify Ii sorting signals using quantitative assays which measure Tf internalization and iron uptake (Jing et al., 1990). As there is increasing evidence that the same or closely related signals are recognized by the clathrin-based sorting machinery at the plasma membrane and *trans*-Golgi (Letourneur and Klausner, 1992; for review see Trowbridge et al., 1993), we hoped that this approach

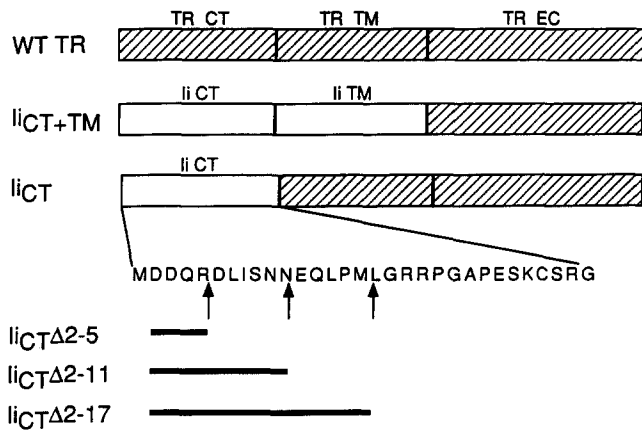


Figure 1. Wild-type TR and chimeric Ii-TR constructs. Schematic representation of the wild-type TR and of chimeric Ii-TR constructs containing the cytoplasmic tail of the p31 isoform of human Ii, the extracellular domain of human TR, and the transmembrane domain from either Ii or TR. Arrows indicate the carboxy-terminal end of deletion mutants of the Ii_{CT} chimera, and bars show the amino acids deleted.

would also allow identification of Ii sorting signals operative along the intracellular trafficking pathway.

Wild-type human TR and Ii-TR chimeras containing either the Ii_{CT} or Ii_{CT+TM} were stably expressed in CEF using BHR-CAS, a replication-competent retroviral vector derived from Rous sarcoma virus (Jing et al., 1990; Hughes et al., 1990). Binding studies at 4°C using ¹²⁵I-labeled human Tf indicated that both Ii-TR chimeras were expressed on the cell surface of infected CEF, but at lower levels than wild-type TR (51 ± 13% Ii_{CT} and 12 ± 1% Ii_{CT+TM} relative to wild-type TR).

Internalization of the Ii-TR chimeras was assayed by measuring the steady-state distribution of internalized receptors and their ability to mediate iron uptake (Jing et al., 1990; Collawn et al., 1990). The Ii_{CT} chimera was efficiently internalized, as judged by either iron uptake or steady-state assays (Table I), indicating that the Ii cytoplasmic domain contains internalization signal(s) that can promote rapid endocytosis of the human TR. Electron microscopy (see Fig. 6, inset) clearly demonstrates that Ii-TR chimeras internalize via clathrin-coated pits, 15% of the label on the plasma membrane being located within these domains. The apparent

internalization efficiency of the Ii_{CT+TM} chimera was significantly higher than either wild-type TR or the Ii_{CT} chimera, implying that the transmembrane region of Ii also influences trafficking.

Ii Cytoplasmic Tail Contains Two Non-Tyrosine-based Internalization Signals

To locate the Ii cytoplasmic tail internalization signal(s), deletion mutants of the Ii_{CT} chimera were constructed (Fig. 1). Deletion of residues 2-5 or residues 2-11 reduced the apparent internalization efficiency of the Ii_{CT} chimera to ~50%, whereas deletion of residues 2-17 completely abolished rapid endocytosis (Table II). These results suggest that the Ii cytoplasmic tail contains two internalization signals, one located between residues 2-11, the other between residues 12-17.

Since the Ii cytoplasmic tail does not contain aromatic residues, the internalization signals identified by analysis of the Ii_{CT} deletion mutants cannot be tyrosine-based. However, the Ii cytoplasmic tail contains three leucine residues (residues 7, 14, and 17) within the first 17 amino-terminal residues (Fig. 1), suggesting that internalization of the Ii-TR chimeras may be mediated by signals related to the di-leucine-based sorting signals previously identified in the cytoplasmic tails of the T cell receptor CD3γ chain and the cation-independent and cation-dependent mannose-6-phosphate receptors (Letourneur and Klausner, 1992; Johnson and Kornfeld, 1992a,b). Although first identified as a lysosomal targeting signal, the CD3γ chain di-leucine signal has been shown to function as an internalization signal (Letourneur and Klausner, 1992; White, S., J. F. Collawn, and I. S. Trowbridge, unpublished results). Di-leucine-based sorting signals have not been extensively characterized; however, isoleucine can substitute for the second leucine, but not the first, in the CD3γ di-leucine signal without loss of lysosomal targeting activity, whereas alteration of either leucine to alanine substantially reduces activity (Letourneur and Klausner, 1992). Because of the stricter requirement for leucine in the first position of the CD3γ chain signal, we initially thought that the two internalization signals identified by deletion analysis may involve Leu⁷Ile⁸ and Leu¹⁴Pro¹⁵, respectively. To investigate this possibility, the two residues of either one or both of these di-peptides were altered to alanine. Strikingly, independently altering each di-peptide to two alanine residues reduced the apparent internalization efficiency

Table I. Comparisons of Steady-state Distribution and ⁵⁹Fe Uptake of Ii-TR Chimeric Receptors Expressed in CEF

TR constructs	Steady-state analysis		Iron uptake	
	% Internalized Tf	Internalization efficiency (%) [*]	Fe atoms internalized/Hr/surface receptor	Relative Fe uptake (%)
Wild-type TR	62 ± 0.2 [‡] (3) [§]	100	22 ± 2.6 (3)	100
Ii _{CT}	62 ± 1.2 (3)	100	22 ± 4.4 (3)	100
Ii _{CT+TM}	76 ± 1.0 (3)	194	64 ± 3.1 (3)	290
TRΔ3-59	19 ± 1.3 (4)	14	6 ± 1.2 (5)	16

^{*} The internalization efficiencies of Ii-TR chimeras were calculated as $[TR]_{int}(\text{mutant}) \cdot [TR]_{sur}(\text{wild-type}) \times 100 / [TR]_{sur}(\text{mutant}) \cdot [TR]_{int}(\text{wild-type})$ as described in Materials and Methods.

[‡] Mean ± standard error.

[§] Number of independent experiments.

^{||} Data for the "tailless" TR mutant are taken from Collawn et al. (1990) and shown for comparison. Internalization efficiencies for the tailless mutant are calculated based on the steady-state and iron uptake values for wild-type TR in the same series of experiments.

Table II. Comparisons of Steady-state Distribution and ⁵⁹Fe Uptake of Mutant Ii_{CT} Chimeric Receptors Expressed in CEF

Ii _{CT} constructs	Steady-state analysis		Iron uptake	
	% Internalized Tf	Internalization efficiency (%) [*]	Fe atoms internalized/Hr/surface receptor	Relative Fe uptake (%)
Ii _{CT}	62 ± 1.2 [‡] (3) [§]	100	22 ± 4.4 (3)	100
Ii _{CT} Δ2-5	48 ± 0.9 (3)	57	ND	ND
Ii _{CT} Δ2-11	39 ± 0.8 (3)	39	ND	ND
Ii _{CT} Δ2-17	16 ± 0.3 (3)	12	3 ± 0.7 (3)	14
Ii _{CT} LI→AA	45 ± 1.2 (3)	50	11 ± 1.8 (3)	50
Ii _{CT} LP→AA	48 ± 0.7 (3)	57	11 ± 2.4 (3)	50
Ii _{CT} LI,LP→AA,AA	23 ± 0.7 (3)	18	4 ± 0.5 (3)	18
TRΔ3-59 [†]	19 ± 1.3 (4)	14	6 ± 1.2 (5)	16

^{*} The internalization efficiencies of the mutant Ii_{CT} chimeras were calculated relative to the Ii_{CT} chimera, i.e., mutant internalization efficiency = [TR]_{int} (mutant Ii_{CT}) / [TR]_{int} (Ii_{CT}) × 100 / [TR]_{sur} (mutant Ii_{CT}) · [TR]_{int} (Ii_{CT}).

[‡] Mean ± standard error.

[§] Number of independent experiments.

^{||} Not determined.

[†] Data for the tailless TR mutant are taken from Collawn et al. (1990) and shown for comparison. Internalization efficiencies for the tailless mutant are calculated based on the steady-state and iron uptake values for wild-type TR in the same series of experiments.

of the Ii_{CT} chimera by ~50%, whereas alteration of both dipeptides completely abrogated high efficiency endocytosis (Table II). These results supported the idea that the Ii cytoplasmic tail contains two non-tyrosine-based signals with similar internalization activity that are related to the CD3γ chain di-leucine signal.

To further characterize the two sorting signals, each of the two residues that might be important for their activity were independently changed to alanine in mutant receptors in which the second signal had been inactivated by alanine substitutions. The results of this analysis clearly showed that both Leu⁷ and Ile⁸ were required for the signal most distal from the transmembrane region (Table III). However, whereas alteration of Pro¹⁵ to alanine abolished the internalization activity of the membrane-proximal signal, alteration of Leu¹⁴ to alanine increased its efficiency. This result implied that the membrane-proximal signal was novel and led us to examine whether the two hydrophobic residues, Met¹⁶ and Leu¹⁷, on the carboxy-terminal side of Pro¹⁵ were required. As shown in Table III, alteration of either Met¹⁶ or Leu¹⁷ to alanine completely abrogated the activity of the

membrane-proximal sorting signal in the Ii cytoplasmic tail. We conclude, therefore, that one internalization signal in the Ii cytoplasmic tail involves Leu⁷ and Ile⁸, whereas Pro¹⁵, Met¹⁶, or Leu¹⁷ are all important for the second signal.

Consistent with the distal signal being closely related to the CD3γ chain di-leucine signal, Ile⁸ could be modified to leucine without loss of activity. Interestingly, the non-conservative mutation of Pro¹⁵ to leucine did not reduce the internalization activity of the membrane-proximal signal (Table III).

Ii-TR Chimeras Are Rapidly Degraded in a Post-Golgi Endocytic Compartment

Ii is thought to be transported as an αβIi complex via the *trans*-Golgi directly to an acidic endocytic compartment where it dissociates and is rapidly degraded (Cresswell, 1985; Lamb et al., 1991; Pieters et al., 1991). To determine whether the cytoplasmic tail of Ii was sufficient to target TR to this endocytic compartment and whether the non-tyrosine-based sorting signals were involved, metabolic pulse-

Table III. Characterization of Ii Cytoplasmic Tail Sorting Signals by Single Amino Acid Substitutions

Mutant Ii _{CT} constructs [*]	% Internalized Tf	Internalization efficiency (%) [‡]
Ii _{CT}	72.8 ± 3.1 [§] (5)	100
L ⁷ →A	33.7 ± 2.3 (3)	19
I ⁸ →A	22.2 ± 2.4 (5)	11
L ¹⁴ →A	70.6 ± 2.5 (4)	91
P ¹⁵ →A	21.2 ± 1.3 (6)	10
M ¹⁶ →A	9.3 ± 1.0 (5)	4
L ¹⁷ →A	10.7 ± 0.6 (5)	4
I ⁸ →L	56.1 ± 2.3 (5)	48
P ¹⁵ →L	54.1 ± 1.9 (5)	44

^{*} The L⁷→A, I⁸→A, and I⁸→L mutations were introduced into the mutant Ii_{CT} LP→AA construct (internalization efficiency of 57% relative to the Ii_{CT} chimera; see Table II), and the L¹⁴→A, P¹⁵→A, P¹⁵→L, M¹⁶→A, and L¹⁷→A mutations were introduced into the mutant Ii_{CT} LI→AA construct (internalization efficiency of 50% relative to the Ii_{CT} chimera; see Table II).

[‡] Internalization efficiencies of mutant Ii_{CT} chimeras are expressed relative to the Ii_{CT} chimeras as described in Table II.

[§] Mean ± standard error.

^{||} Number of independent experiments.

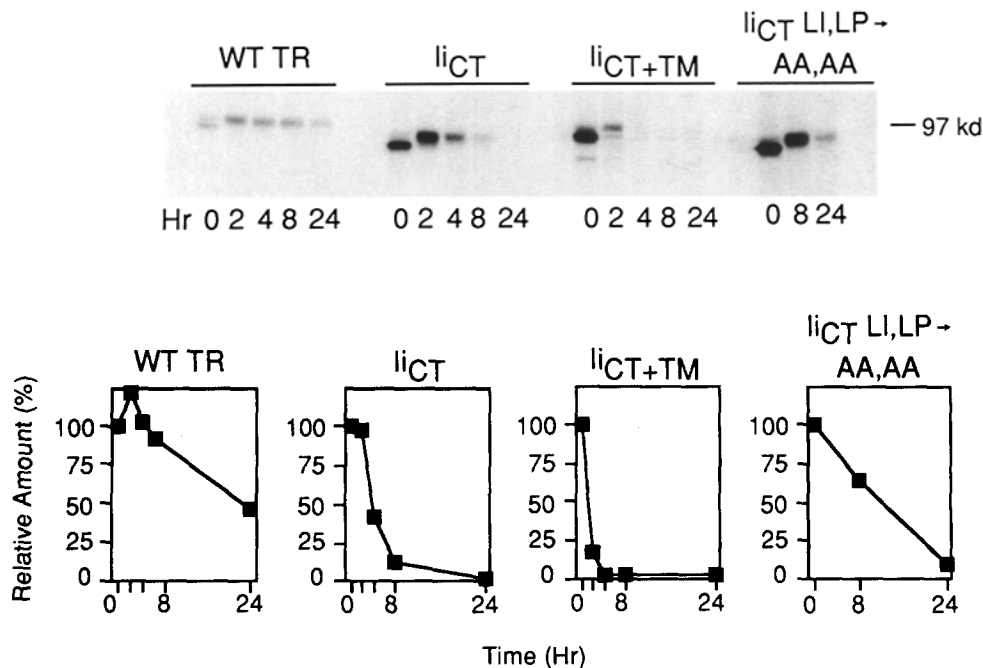


Figure 2. Rapid degradation of Ii-TR chimeras in a post-Golgi endocytic compartment. Equivalent cell numbers of CEF expressing either wild-type TR or the Ii-TR chimeras, Ii_{CT}, Ii_{CT+TM}, or Ii_{CT} LI,LP→AA,AA were pulse-labeled for 30 min with *trans*-³⁵S-label and chased for various periods of time. TRs were then immunoprecipitated from post-nuclear supernatants and analyzed on SDS-polyacrylamide gels as described in Materials and Methods. Dried gels were exposed to preflashed XAR film for two days (Eastman Kodak, Rochester, NY). Immunoprecipitates were quantitated on a model 425 PhosphorImager (Molecular Dynamics). The data shown are from one of two similar experiments.

chase experiments were performed. CEF expressing either TR, Ii_{CT}, Ii_{CT+TM} or the Ii_{CT} LI,LP→AA,AA mutant were pulse-labeled with *trans*-³⁵S-label for 30 min and chased for various lengths of time; TR and Ii-TR chimeras were then isolated by immunoprecipitation and analyzed by SDS-PAGE (Fig. 2). Notably, both the Ii_{CT+TM} and Ii_{CT} chimeras were rapidly degraded with half-lives of <2 h and ~4 h, respectively, similar to the rate of turnover of native Ii in antigen-presenting cells. In contrast, the wild-type TR had a half-life of ~24 h, consistent with previous estimates of wild-type human TR turnover rates in CEF (Jing and Trowbridge, 1990). The Ii_{CT+TM} chimera was consistently degraded >2-fold more rapidly than the Ii_{CT} chimera, implying that the Ii transmembrane region influences intracellular trafficking. After 2 h (Fig. 2), the *M_r* of TR and Ii-TR chimeras increased to that of the mature glycoprotein (Omary and Trowbridge, 1981) and the molecules became partially resistant to endoglycosidase H digestion (data not shown). This indicates that the Ii-TR chimeras traverse the Golgi where glycosylation is completed and are degraded in a post-Golgi compartment. The Ii-TR chimera lacking the non-tyrosine-based signals was degraded more slowly, indicating that these signals are required for delivery to this compartment. Further, the rate of synthesis of the Ii_{CT} and Ii_{CT+TM} chimeras was 4- and 8-fold higher, respectively, than that of wild-type TR even though the chimeric receptors were expressed in lower amounts on the cell surface suggesting most of the chimeric molecules were trafficking by a direct intracellular route to the endocytic compartment where they were degraded.

Degradation of Ii is partially inhibited by lysosomotropic agents such as chloroquine, monensin, and leupeptin (Blum and Cresswell, 1988; Nguyen and Humphreys 1989; Pieters et al., 1991; Zachgo et al., 1992). Similarly, leupeptin significantly inhibited the degradation of the Ii_{CT+TM} chimera, as indicated by the slower rate of disappearance of the

intact molecule and the appearance of partial proteolytic products derived from the TR external domain (Fig. 3), which is consistent with degradation occurring in an endocytic compartment. Comparable results were obtained with chloroquine treatment (data not shown).

Ii-TR chimeras trafficking through the Golgi complex were identified using electron microscopy immunocytochemistry. Gold complexes bearing antibody specific for the external domain of TR were applied to cryosections and showed Ii-TR distributed throughout the flattened cisternae of the Golgi stack and within coated vesicles in the *trans*-Golgi area (see Fig. 6).

The intracellular and cell surface distribution of the Ii-TR chimeras was determined by immunofluorescence microscopy. The Ii_{CT+TM} chimera was found predominantly in intracellular vesicles, as indicated by the virtual lack of staining of non-permeabilized cells (Fig. 4 A), compared to the strong punctate staining of permeabilized cells (Fig. 4 B).

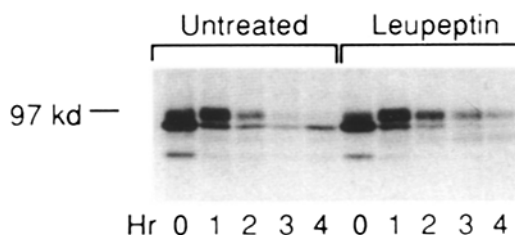


Figure 3. Leupeptin inhibits degradation of the Ii_{CT+TM} chimera. Equivalent cell numbers of CEF expressing Ii_{CT+TM} were preincubated for 4 h at 37°C in culture medium with (+) or without (-) leupeptin (100 μg/ml) and then pulse-labeled with *trans*-³⁵S-label and chased as described in legend to Fig. 2 ± leupeptin. TRs were then immunoprecipitated and analyzed on SDS-polyacrylamide gels as described in Fig. 2. The data shown are from one of two similar experiments.

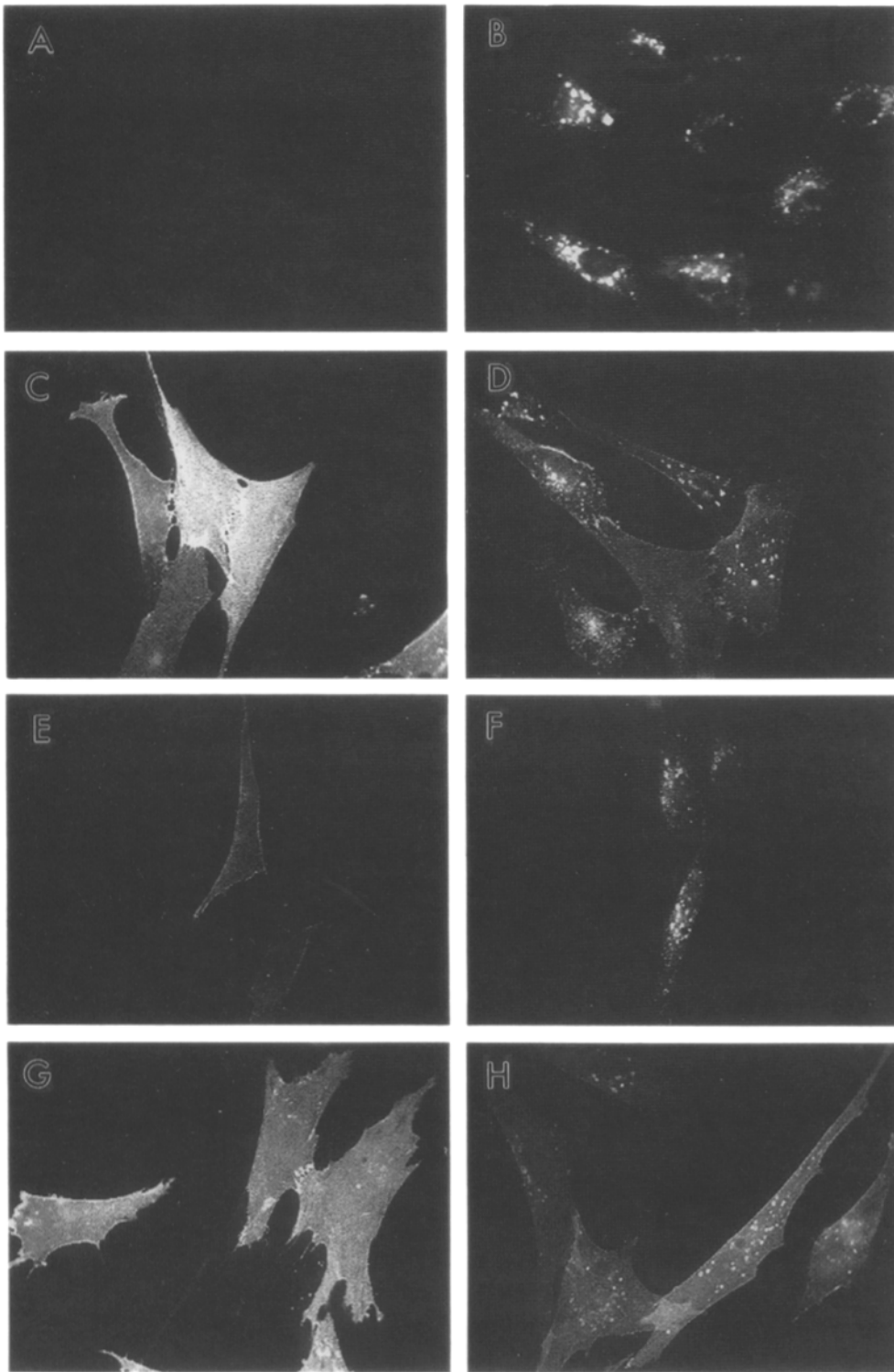


Figure 4. Cellular distribution of Ii-TR chimeras by immunofluorescence analysis. CEF expressing Ii_{CT}+TM (A and B), wild-type TR (C and D), Ii_{CT} (E and F), or Ii_{CT} LI,LP→AA,AA (G and H) chimeras were fixed and stained with an anti-TR (B3/25) and a FITC-conjugated second antibody for the surface-stained cells (A, C, E, and G), or were fixed, permeabilized, and stained with the same antibody combination for the intracellular staining (B, D, F, and H).

CEF expressing the Ii_{CT} chimera exhibited more surface staining (Fig. 4 E), but this chimera was also found predominantly in intracellular vesicles (Fig. 4 F). The Ii_{CT} LI,LP→AA,AA chimera was expressed mainly on the cell surface (Fig. 4 G), but some staining of intracellular vesicles was still observed (Fig. 4 H). Two-color immunofluorescence studies comparing the intracellular distribution of the Ii_{CT}+TM

chimera with LEP100, an endogenous chicken lysosomal integral membrane protein (Lippincott-Schwartz and Fambrough, 1986, 1987; Mathews et al., 1992), showed significant colocalization, indicating that the hybrid molecules traffic along the prelysosomal segment of the endocytic pathway (Fig. 5 C). Conversely, wild-type TR showed virtually no colocalization with LEP100 (Fig. 5 A). The Ii_{CT} chimera

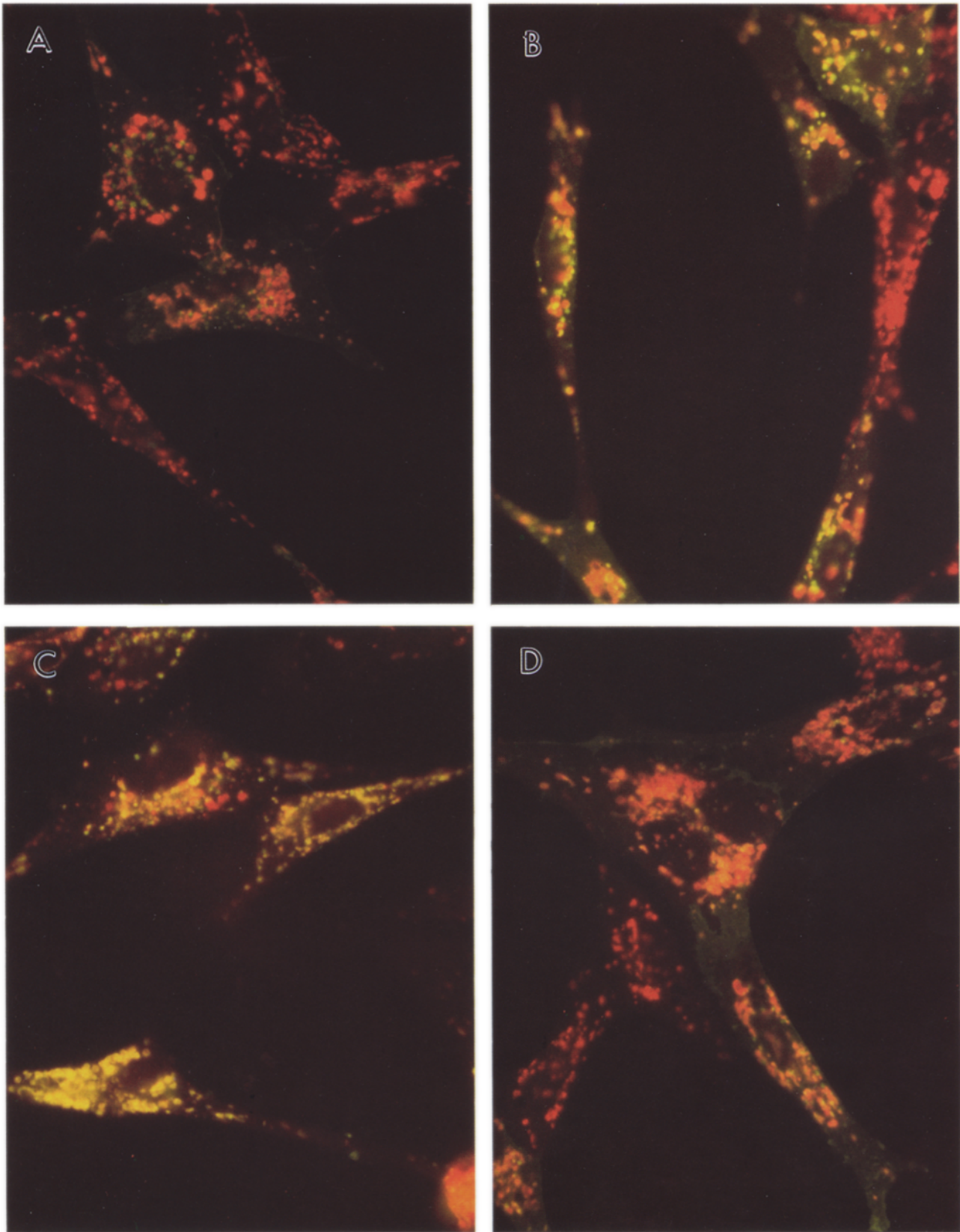


Figure 5. Co-localization of Ii-TR chimeras with LEP100. CEF expressing wild-type TR (A), Ii_{CT} (B), Ii_{CT+TM} (C), or Ii_{CT} LI,LP→AA,AA (D) chimeras were fixed, permeabilized, and stained with rabbit anti-TR antisera followed by fluorescein-labeled goat anti-rabbit Ig or mouse anti-LEP100 followed by Texas red-labeled goat anti-mouse IgG. Colocalization of the two proteins is indicated by yellow fluorescence.

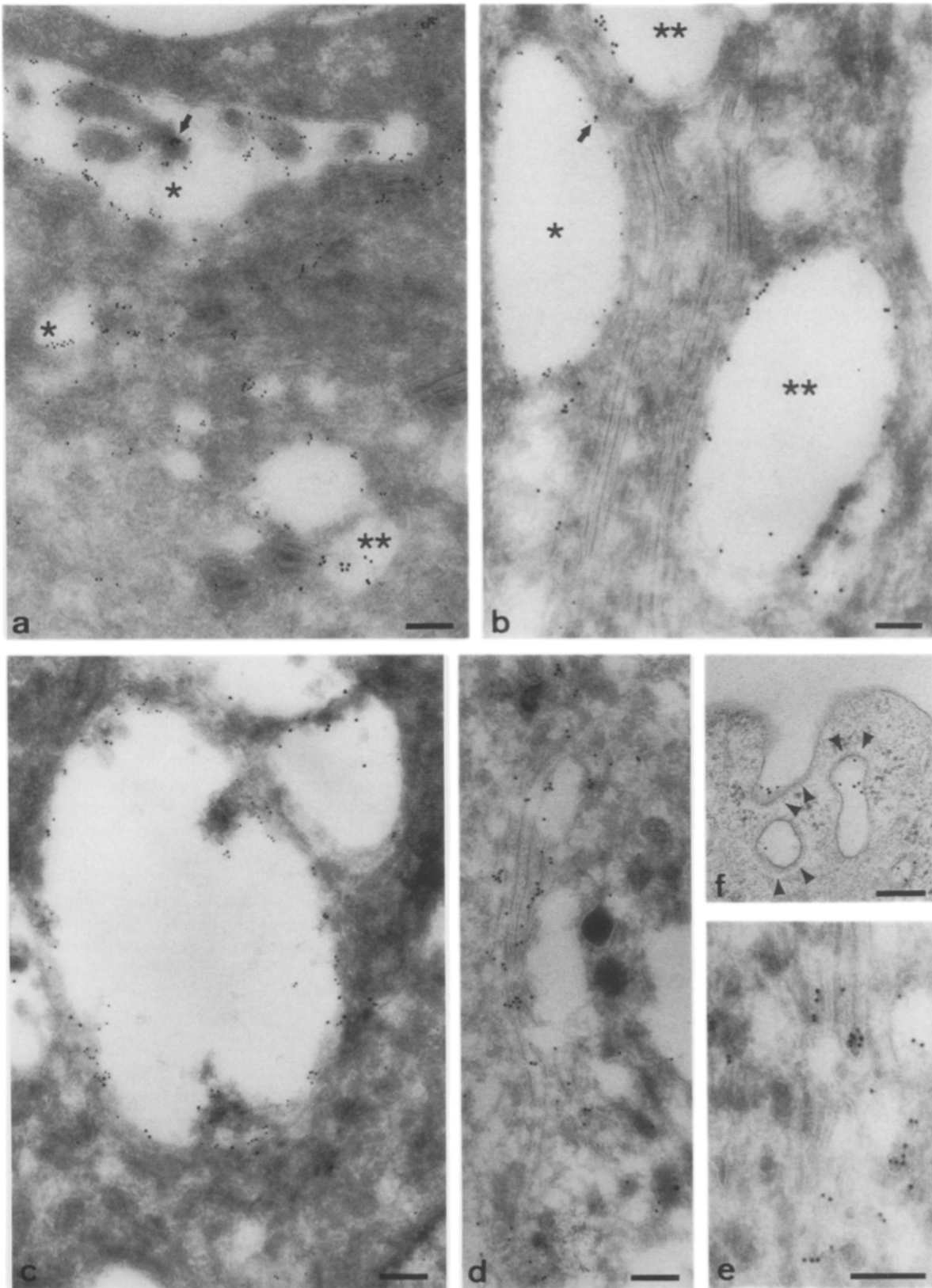


Figure 6. Immunoelectronmicroscopy analysis of Ii_{CT+TM} chimera, TR and LEP100. (a and b) Cells transfected with wild-type TR. Small (6 nm) gold particles show distribution of TR, larger (10 nm) particles show distribution of LEP100. * Indicates endocytic compartments containing almost exclusively TR label (arrows indicate the few LEP100 specific particles). ** Indicates compartments containing only LEP100. (c-f) Cells transfected with Ii_{CT+TM} chimera. (c) Cryosection showing distribution of Ii_{CT+TM} chimera (6-nm gold particles) and LEP100 (10-nm gold particles) within the same compartment. (d and e) Cryosections showing distribution of Ii_{CT+TM} in Golgi stack and trans-Golgi network. (f) Conventional plastic section from cells incubated with B3/25-gold complexes at 4°C. Arrowheads indicate distribution of clathrin-coated domains or pits and vesicles associated with the plasma membrane. Bar, 0.2 μm.

also partially colocalized with LEP100 (Fig. 5 B), whereas the I_{iCT} LI,LP \rightarrow AA,AA (Fig. 5 D) gave a staining pattern similar to that of wild-type TR.

To quantitatively determine the altered trafficking of I_{iCT+TM} chimera, the steady-state distribution of TR and I_{iCT} TR chimeras was determined by gold label immunocytochemistry using antibody specific for the external domain of human TR. The extent to which the TR and I_{iCT+TM} chimera were colocalized with LEP100, an antigen located predominantly in the prelysosomal branch of the endocytic pathway (Lippincott-Schwartz and Fambrough, 1986, 1987), was quantified by double labeling. As shown in Fig. 6, the distribution of TR, I_{iCT+TM} , and LEP100 is clearly restricted to the perimeter membranes of endocytic elements. Quantitative analysis shows that while relatively low amounts (11.2%) of TR can be found in LEP100-containing compartments, 64% of the labeled compartments in cells transfected with I_{iCT+TM} contain both I_{iCT+TM} and LEP100.

Ii-TR Chimeras Traffic from the Plasma Membrane to the Endocytic Compartment Where They Are Degraded

To determine whether I_{iCT} TR chimeras expressed on the cell surface traffic to the endocytic compartment where degradation occurs, cells were incubated with ^{125}I -labeled Tf at 37°C for 1 h to load the endocytic pathway with receptor-ligand complexes. The cells were then rapidly washed, and the reappearance of intact and degraded Tf in the medium

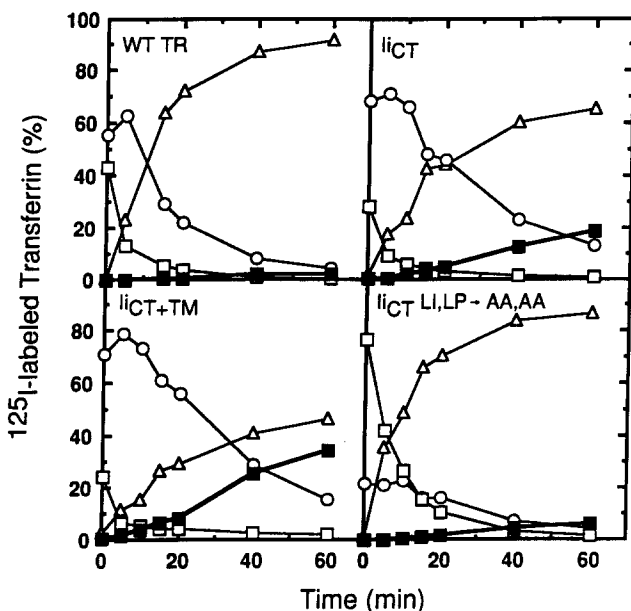


Figure 7. Degradation of Tf bound to I_{iCT} TR chimeras. Equivalent cell numbers of CEF expressing either wild-type TR, I_{iCT} , I_{iCT+TM} , or I_{iCT} LI,LP \rightarrow AA,AA were preincubated in serum-free DME for 30 min at 37°C, and then incubated with ^{125}I -labeled Tf for 1 h at 37°C. The cells were then washed and reincubated at 37°C in DME containing 50 μ g/ml unlabeled Tf for various times. Acid-soluble radioactivity (\blacksquare) or acid-insoluble ^{125}I -labeled Tf (\triangle) released into the medium, as well as surface-bound ^{125}I -labeled Tf (\square) and internalized ^{125}I -labeled Tf (\circ) were determined as described in Materials and Methods and are expressed as a percent of total radioactivity recovered. Each point is the average of triplicate determinations from a representative experiment.

was monitored by measuring TCA insoluble and soluble radioactivity. As expected, the apo-Tf released into the medium from cells expressing the wild-type TR was undergraded (Fig. 7), as TR-apo-Tf complexes are efficiently recycled back to the cell surface through the sorting and recycling endosomal compartments (Jing et al., 1990; Hopkins and Trowbridge, 1983; Dunn et al., 1989; Weissman et al., 1986; Trowbridge et al., 1993). In striking contrast, $\sim 35\%$ of the ^{125}I -labeled Tf released from cells expressing the I_{iCT+TM} chimera was degraded, implying that this fraction of the chimeric receptors traffic directly from the cell surface to an endocytic compartment where they are degraded. A significantly smaller fraction ($\sim 20\%$) of Tf bound to the I_{iCT} chimera was degraded (Fig. 7), indicating that the I_{iCT} transmembrane region influences trafficking from the cell surface to this compartment. The I_{iCT} TR chimera lacking non-tyrosine-based sorting signals was degraded only to a slightly higher extent than wild-type TR.

To confirm the inference from these studies that the I_{iCT} TR chimeras traffic from the plasma membrane to an endocytic compartment where they are degraded, CEF expressing I_{iCT} , I_{iCT+TM} , or I_{iCT} LI,LP \rightarrow AA,AA were surface-iodinated and the rate of degradation of receptors transiently located at the plasma membrane directly determined. As shown in Fig. 8, surface-iodinated I_{iCT} chimera (half-life ~ 6 h) was degraded more rapidly than the chimera lacking non-tyrosine-based signals. Surface-iodinated I_{iCT+TM} was degraded at an even faster rate (half-life $\sim 2-3$ h).

The Ii Transmembrane Region Determines Rapid Degradation of TR

The I_{iCT+TM} chimera was degraded more rapidly than the I_{iCT} chimera, whether the chimeras were delivered directly from the *trans*-Golgi to the endocytic pathway or via the cell surface, implying that the I_{iCT} transmembrane region was influencing trafficking along both pathways. To determine whether the I_{iCT} transmembrane region was sufficient to promote delivery of TR to the degradative endocytic compartment, a chimera consisting of the TR external domain and cytoplasmic tail and the I_{iCT} transmembrane region (I_{iCT+TM}) was constructed. The I_{iCT+TM} chimera was expressed on the cell surface at a level of $23 \pm 6\%$ relative to the wild-type TR and was internalized with a relative efficiency of $76.0 \pm$

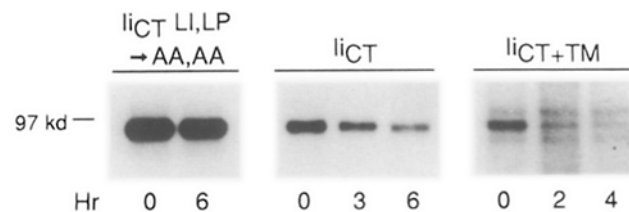


Figure 8. Degradation of ^{125}I surface-labeled I_{iCT} TR chimeras. Equivalent cell numbers of CEF expressing either I_{iCT} , I_{iCT+TM} or I_{iCT} LI,LP \rightarrow AA,AA chimeras were surface-iodinated, and then incubated for the times indicated. The I_{iCT} TR chimeras were immunoprecipitated from post-nuclear supernatants and analyzed on SDS-polyacrylamide gels as described in Materials and Methods. Dried gels were exposed to preflashed XAR film (Eastman Kodak, Rochester, NY) for one day (I_{iCT} LI,LP \rightarrow AA,AA and I_{iCT}) or 2 d (I_{iCT+TM}).

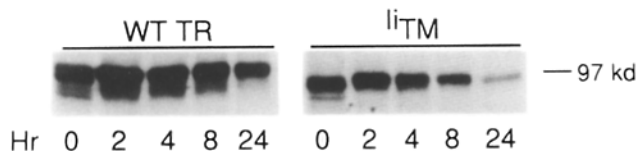


Figure 9. Ii transmembrane region targets the TR to a post-Golgi degradative compartment. Equivalent cell numbers of CEF expressing either wild-type TR or the IiTM chimeric receptor were pulse-labeled with trans-³⁵S-label and chased as described in the legend for Fig. 2. TRs were then immunoprecipitated from post-nuclear supernatants and analyzed on SDS-polyacrylamide gels as described in Materials and Methods. Dried gels were exposed to preflashed XAR film for two days (Eastman Kodak, Rochester, NY), and immunoprecipitates were quantitated on a model 425 PhosphorImager (Molecular Dynamics).

0.5%. Importantly, pulse-chase analysis showed that the fully glycosylated IiTM chimera was rapidly degraded (Fig. 9) with a half-life of ~6 h, similar to that of the Ii_{CT} chimera.

Discussion

By constructing Ii-TR chimeras comprised of the cytoplasmic domain of Ii, the external domain of the human TR, and the transmembrane region of either Ii or TR, we have been able to take advantage of the quantitative assays available for measuring the rate of internalization of TRs from the cell surface. This experimental strategy has allowed us to demonstrate that the Ii cytoplasmic tail can promote rapid internalization of TR and to identify residues within the cytoplasmic tail of Ii that are required for this activity.

Our data indicate that the Ii cytoplasmic tail contains two independent non-tyrosine-based sorting signals that promote rapid internalization. The same or overlapping signals are recognized in the *trans*-Golgi and mediate direct sorting along an intracellular route to the endocytic pathway. One signal involves Leu⁷ and Ile⁸ and appears to be analogous to the di-leucine lysosomal targeting signal identified in the cytoplasmic domain of the CD3 γ chain (Letourneur and Klausner, 1992). Deletion of residues 2-5 from the I_{CT} chimera cytoplasmic tail reduces the internalization activity to ~50%, implying that, as for the di-leucine signal of the CD3 γ chain (Letourneur and Klausner, 1992), residues to the amino-terminal side of Leu⁷Ile⁸ are required for activity. The second sorting signal requires Pro¹⁵, Met¹⁶, and Leu¹⁷, and, therefore, differs significantly from the di-leucine and leucine-isoleucine motifs identified previously (Letourneur and Klausner, 1992; Johnson and Kornfeld, 1992a,b; Ogata and Fukuda, 1994). Nevertheless, all these signals, including both Ii signals, contain two adjacent large nonaromatic hydrophobic residues and may, therefore, represent a family of related structural motifs specified by short linear arrays of amino acids that differ in specific sequence, analogous to tyrosine-based signals (Trowbridge et al., 1993). Additional mutagenesis is required, however, to define these sorting signals more precisely, to identify any common features they may share, and to delineate any differences between signals recognized in the *trans*-Golgi or at the plasma membrane. At present, we believe it most appropriate to refer to them as non-tyrosine-based signals, a neutral

term that serves to distinguish them from tyrosine-based signals.

Our results are consistent with those of Bakke and Dobberstein (1990), who identified amino acids 12-15 in the Ii cytoplasmic tail as important for targeting Ii to the endocytic pathway. Lotteau et al. (1990) also concluded that a sorting signal was located within residues 10-16 of the Ii cytoplasmic tail. The distal signal involving Leu⁷Ile⁸ was not identified in either of these studies, however (see also Romagnoli et al., 1993), illustrating the difficulty of detecting multiple signals without quantitative assays. Recently, in agreement with our data, Pieters et al. (1993) have independently concluded that the Ii cytoplasmic tail contains two sorting signals, one involving Leu⁷Ile⁸, the other localized to residues 12-29.

The extent to which newly synthesized Ii molecules, alone or complexed to MHC class II $\alpha\beta$ dimers, traffic to the endocytic pathway by an indirect route via the cell surface has been a matter of debate (Peters et al., 1991; Pieters et al., 1991; Roche et al., 1993). Ii-TR chimeras trafficking to the plasma membrane can be quantitated because receptors appearing transiently at the cell surface can be labeled with exogenous Tf. Thus, the surface-accessible pools of the Ii-TR chimeras can be determined by measuring ¹²⁵I-labeled Tf bound to surface and internalized chimeric receptors under steady-state conditions. The surface-accessible pool of the Ii_{CT+TM} chimera was only 19% of the wild-type TR pool despite the fact that the ~8-fold increase in the degradation rate of the Ii_{CT+TM} surface-accessible pool is offset by a similar increased rate of biosynthesis relative to wild-type TR (see Materials and Methods for details of the calculation). Assuming that essentially all wild-type TRs bind Tf under steady-state labeling conditions, it can be calculated that only 19% of the Ii_{CT+TM} chimera traffics via the cell surface, and, therefore, that >80% of the chimera must be delivered by a direct intracellular route to the endocytic compartment where it is degraded. Similarly, ~50% of the Ii_{CT} chimera is degraded without ever being displayed on the cell surface. The lysosomal membrane glycoprotein, Lgp-A (LEP100, LAMP-1), also traffics along the endocytic pathway to the lysosome by a direct intracellular route or via the cell surface, although which route is the major pathway is less clear and may depend upon expression level (Williams and Fukuda, 1990; Mathews et al., 1992; Harter and Mellman, 1992).

The fate of Ii-TR chimeras which traffic via the plasma membrane can also be determined by loading them with exogenous Tf and monitoring the reappearance of intact and degraded Tf in the medium. The results of such studies indicated that ~35% of Ii_{CT+TM} chimeras transiently displayed on the plasma membrane were degraded after internalization, whereas the remaining ~65% were recycled back to the cell surface. An even higher fraction (~80%) of the Ii_{CT} chimera recycled back to the cell surface. These results indicate that the Ii-TR chimeras displayed on the plasma membrane recycle several times before being sorted to the endocytic compartment where they are degraded. In this respect, Ii-TR chimeras expressed on the cell surface behave similarly to lysosomal acid phosphatase which is transported to the cell surface and undergoes multiple rounds of internalization and recycling before being transferred to the lysosome (Braun et al., 1989).

Studies on the trafficking of the cation-independent mannose-6-phosphate receptor (M6PR) have clearly established

that a direct route from the *trans*-Golgi to the endocytic pathway exists (Kornfield and Mellman 1989). It has been shown that M6PR are concentrated within clathrin-coated buds in the *trans*-Golgi (Rijnboutt et al., 1992; Klumperman et al., 1993) and there is evidence suggesting that they are then delivered to an early part of the endocytic pathway, most probably the TR-containing endosome (Ludwig et al., 1991; Méresse and Hoflack, 1993). Previous work on Ii (Guagliardi et al., 1990; Pieters et al., 1991; Zachgo et al., 1992; Romagnoli et al., 1993) also suggest that entry into the endocytic pathway is via the TR-containing endosome.

Our data show that the cytoplasmic domain of Ii contains two signals recognized in the *trans*-Golgi that are the same or overlap with the internalization signals recognized by clathrin-coated domains at the plasma membrane. This relationship implies that the signals active in the *trans*-Golgi are recognized by clathrin-based sorting machinery. It is also apparent, however, that for direct transfer of Ii from the *trans*-Golgi to the endocytic pathway to operate with full efficiency, additional signal information within the transmembrane region is required. The Ii transmembrane region also increases the efficiency of trafficking from the plasma membrane to the degradative endocytic compartment. Golgi retention signals have previously been localized to the transmembrane region (Machamer and Rose, 1987; Swift and Machamer, 1991; Munro, 1991; Nilsson et al., 1991; Wong et al., 1992). However, there is no precedent for the transmembrane region of an integral membrane protein influencing sorting in the *trans*-Golgi or trafficking along the endocytic pathway.

Our evidence shows that Ii, like the M6PR (Kornfield and Mellman, 1989), enters the TR-containing endosome from both the *trans*-Golgi and the plasma membrane. However, the data also indicate that the Ii-TR chimeras which enter directly from the *trans*-Golgi are delivered to a degradative compartment more efficiently than those which enter from the plasma membrane. Our recent observations on the TR-containing endosome in CEF (Hopkins et al., 1994) show that it is a morphologically complex compartment and suggest that it consists of at least two interconnected sub-compartments in which internalized TRs are processed at different rates. Receptors entering the TR-containing endosome can either be recycled rapidly ($t_{1/2}$ in 10 min) or remain for more prolonged periods ($t_{1/2}$ in 30 min) before returning to the surface. The data on Ii-TR chimeras can thus be explained by proposing that most of the chimeras which enter from the cell surface travel via the rapidly recycling pathway and that on each cycle only ~30% of them are sorted towards the lysosome. Direct transfer from the *trans*-Golgi, though more efficient, probably occurs via the sub-compartment of the endosome which lies within its immediate vicinity in the pericentriolar area and through which TR are processed more slowly.

Identification of a sorting signal in the Ii transmembrane region that complements the non-tyrosine-based sorting signals in the Ii cytoplasmic tail provides a plausible explanation for two earlier observations. Pieters et al. (1993) showed that the Ii cytoplasmic tail was sufficient to target a heterologous protein, influenza neuraminidase, to the endocytic pathway. However, they noted significant differences between the localization of the Ii-neuraminidase chimera and Ii within the endocytic pathway and concluded that other

sorting information may be contained within the Ii transmembrane region and/or extracellular domain. In a second study, Nilsson et al. (1991) reported that an Ii chimera containing the cytoplasmic tail of β -1,4 galactosyltransferase, an enzyme normally localized to the *trans*-Golgi by a retention signal within its transmembrane region, paradoxically had a subcellular distribution similar to wild-type Ii (p31). The galactosyltransferase cytoplasmic tail contains a dileucine sequence which may function as a sorting signal and when complemented by the Ii transmembrane region signal promote similar trafficking to wild-type Ii.

Finally, Dintzis and Pfeffer (1990) observed that the M6PR cytoplasmic tail was not sufficient to target the epidermal growth factor receptor to prelysosomes and suggested that additional sorting information in the M6PR transmembrane region and/or extracellular domain may be required.

The identification of the Ii sorting signals required for targeting MHC class II molecules to the compartment where they encounter antigen may have practical implications since, in principle, it should be possible to target endogenously synthesized recombinant proteins containing Ii sorting signals to this compartment for degradation and selective presentation by MHC class II to T helper cells.

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