Analysis of the Structural Requirements for Lysosomal Membrane Targeting Using Transferrin Receptor Chimeras*

Suhaila White‡, Sean R. Hatton§, Masood A. Siddiqui§, Cynthia D. Parker§, Ian S. Trowbridge‡, and James F. Collawn§

From the ‡Department of Cancer Biology, The Salk Institute, San Diego, California 92186-5800 and §Department of Cell Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005

The sorting of membrane proteins to the lysosome requires tyrosine- or dileucine-based targeting signals. Recycling receptors have similar signals, yet these proteins seldom enter the latter stages of the endocytic pathway. To determine how lysosomal and internalization signals differ, we prepared chimeric molecules consisting of the cytoplasmic tails of CD3 γ-chain, lysosomal acid phosphatase, and lysosomal-associated membrane glycoprotein-1, each fused to the transmembrane and extracellular domains of the transferrin receptor (TR).

Each chimera was expressed on the cell surface and rapidly internalized. Metabolic pulse-chase experiments showed that the CD3 γ-chain and lysosomal acid phosphatase chimeras, unlike the lysosomal-associated membrane glycoprotein chimera, were rapidly degraded in a post-Golgi compartment following normal glycosylation. Transplantation of signals from CD3 γ-chain and lysosomal acid phosphatase into the TR cytoplasmic tail in place of the native signal, Y20TRF23, indicated that each signal was sufficient to promote endocytosis but not lysosomal targeting of the resulting mutant. Transplantation of two CD3 signals at specific sites in the TR cytoplasmic tail or a single tyrosine-based signal in a truncated TR tail, however, was sufficient to promote lysosomal targeting. Our results therefore suggest that the relative position of the signal within the cytoplasmic tail is a critical feature that distinguishes lysosomal targeting signals from internalization signals.

The trafficking of integral membrane proteins to lysosomes occurs independently of the mannose 6-phosphate receptor (1, 2). Newly synthesized lysosomal membrane proteins are delivered to lysosomes either by a direct intracellular route or by an indirect route via the plasma membrane (3–8). Membrane proteins that traffic to lysosomes by the direct intracellular route are thought to be sorted from the constitutive biosynthetic pathway in the trans-Golgi via clathrin-coated vesicles (9). Membrane proteins that are delivered to the lysosome from the cell surface cluster in clathrin-coated pits and internalize in a manner indistinguishable from recycling receptors. In the sorting endosome, however, lysosomally directed membrane proteins segregate from recycling receptors (10, 11).

Studies on the transferrin receptor recycling pathway demonstrate that recycling receptors and lipids recycle back to the cell surface with the same kinetics, suggesting that recycling of membrane proteins occurs by a bulk flow process (12). Furthermore, deletion of the TR Y1 cytoplasmic tail has dramatic effects on TR internalization, but is without effect on TR recycling (13, 14). Functional analysis of LAMP-1 mutants clearly demonstrates that modifications in the cytoplasmic tail convert a lysosomally targeted protein into a protein trapped in the recycling pathway (11), suggesting that additional targeting information is required for entry to the later stages of the endocytic pathway and recycling receptors lack this information.

Analysis of the trafficking of mutant lysosomal membrane proteins with altered cytoplasmic tails has identified two classes of sorting signals involved in lysosomal targeting. One class contains a critical tyrosine residue that is structurally related to tyrosine-based internalization signals, while the other contains two adjacent leucine or related large hydrophobic residues (for reviews, see Refs. 15 and 16). Tyrosine-based lysosomal-targeting signals have been identified in LAMP-1 and LAMP-1 (4, 5, 17, 18). Non-tyrosine-based or dileucine sorting signals have been identified in the cation-independent and -dependent mannose 6-phosphate receptors (19, 20), the T cell receptor CD3 γ-chain (21), LIMP II (22), FcRII-B2 receptor (23, 24), GLUT-4 (25), the insulin receptor (26), and major histocompatibility complex class II invariant chain (27–29). Dileucine-based signals, like tyrosine-based signals, can function as efficient internalization signals (23–26, 28, 29), although the two signal types appear to be recognized by distinct cytosolic factors (30).

Despite the extensive series of studies described above, the structural requirements for lysosomal targeting have not been clearly defined. For example, whereas the published literature underscores the close relationship between tyrosine-based internalization signals and lysosomal targeting signals, the structural features which distinguish the two classes of signal have not been revealed by alanine-scanning mutagenesis. The most striking example of this is the recent analysis of the structural requirements for lysosomal targeting, endocytosis and basolateral sorting of rat Lgp120 (LAMP-1) (5). Complete alanine scanning of the 11-residue cytoplasmic tail of rat Lgp120 indicated that alteration of only two residues, Tyr-8 and Ile-11, to alanine decreased the efficiency of lysosomal targeting, endocytosis, and basolateral sorting, with each sorting step being equally affected. Mutation of Gly-7 to alanine

* This work was supported in part by National Institutes of Health Grants R29-DK47339 (to J.F.C.) and R01-AI40389 (to I. S. T.), grants from the Sankyo Ltd. and the Arthritis Foundation (to J. F. C.), and a National Science Foundation Predoctoral Fellowship (to S. R. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Cell Biology, University of Alabama at Birmingham, BHSB 392, UAB Station, Birmingham, AL 35294-0005. Tel.: 205-934-1002; Fax: 205-934-0950; E-mail: jcollawn@uab.edu.

‡ The abbreviations used are: TR, transferrin receptor; Tf, transferrin; CEF, chicken embryo fibroblasts; LAMP, lysosomal associated membrane glycoprotein; LAP, lysosomal acid phosphatase; DMEM, Dulbecco’s modified Eagle’s medium.
resulted in a loss of direct targeting, but not indirect targeting, to the lysosome (5).

In this study we have investigated the structural requirements for lysosomal targeting by analyzing chimeras consisting of the cytoplasmic tail of LAP, LAMP-1, or the CD3 γ-chain each fused to the transmembrane region and external domain of the TR. We have also transfected putative internalization and lysosomal-targeting signals from each of these proteins into the TR in place of the native TR internalization signal, YTRF. Tyrosine-based signals from LAMP-1 and LAP were also transfected into a truncated TR cytoplasmic domain, Δ3–18, 29–59. Our studies demonstrate that the position of the signal within the cytoplasmic tail is a critical feature that distinguishes lysosomal targeting signals from internalization signals.

EXPERIMENTAL PROCEDURES

Construction of TR Chimeras and Mutant TRs Containing Transplanted Signals—Mutant human TR constructs were prepared as described previously (13) by the method of Kunkel (31). Mutants were selected either by differential hybridization or by restriction mapping and cloned into the expression vector, BH-RCAS (32, 33). The mutants were verified by dideoxynucleotide sequencing (34, 35) of the BH-RCAS constructs using the Sequenase kit (U.S. Biochemical Corp., Cleveland, OH) according to the manufacturer’s directions.

LAMP-TR and LAP-TR chimeras (Fig. 1A) were prepared by oligonucleotide-directed mutagenesis using the tailless (Δ3–59) TR mutant phagemid as a template (13). The CD3 γ-chain-TR chimera (Fig. 1A) was constructed using the polymerase chain reaction as described previously (36). A polymerase chain reaction was performed on the CD3 γ-chain cDNA (kindly provided by Dr. Bob Hyman, Department of Cancer Biology, Salk Institute). Our polymerase chain reaction-generated construct did not contain the carboxyl-terminal three residues (Arg-Lys-Lys) of the CD3 γ-chain since these residues had previously been shown to be unnecessary for lysosomal targeting, and in the context of a type I membrane protein, acted as an endoplasmic reticulum retention signal (21). Unique NotI and AluII sites were introduced in the 5′ (5′-CAT-GTC-AGC-CAG-GTA-GTA-CTG-CCG-3′) and 3′ (3′-CAT-CTT-AGC-CAG-CTG-TTC-CTT-3′) primers, respectively. This polymerase chain reaction-generated fragment was then subcloned into Bluescript SK+ with a human TR insert containing these two sites (encoding a GAG-ATG-GTC-AGC-CTT-AAG-GCGG) encoding a 49-residue cytoplasmic tail with the sequence Met-Met-Ala-Ser-Leu-Lys-Arg (13); kindly provided by Greg Odorizi and Albert Lai (Department of Cancer Biology, The Salk Institute). The addition of the two restriction sites adds three residues, Ala, Ser, Leu to the tailless TR (Δ3–59; (13)) and the CD3 γ-chain tail sequence was inserted between the Ser and Leu residues (Fig. 1B–D).

The TR mutants containing transplanted signals were prepared by oligonucleotide-directed mutagenesis (Fig. 1, B and C). The γYQTF3, the γYRHV2, the D18KQTLL23, and the Y20QTI23 mutagenic primers were prepared using the wild-type TR phagemid as template. The γYQTF3 and γYRHV2 mutants (Fig. 1D) were prepared by oligo-directed mutagenesis of the Δ3–18, 29–59 mutant TR phagemid as template (38). The D18KQTLL23, γYQTLK24 mutant was prepared using the D18KQTLL23 mutant phagemid as template (Fig. 1C). Preparations of the γYTRF32 mutant, the γYTRF34 mutant, and the γYTRF20 mutant had been previously described (36).

Expression of Wild-type TR, TR Chimeras, and TR Mutants in Chicken Embryo Fibroblasts (CEF)—TR chimeras and mutants were expressed in CEF as described previously (13). Surface expression levels of wild-type TR and TR chimeras and mutants were confirmed by immunoprecipitation using the B3/25 monoclonal antibody and analyzed on 7.5% SDS-polyacrylamide gels (40). Dried gels were exposed on XAR film (Eastman Kodak Co.). Quantification of immunoprecipitates was performed on a model 425 Polaroid Dyer (Polaroid, Cambridge, MA).

Indirect Immunofluorescence—CEF and CEF expressing the CD3 γ-chain-TR, LAP-TR, and LAMP-TR chimeras and the wild-type TR were plated onto glass coverslips and cultured overnight. Analysis by indirect immunofluorescence was performed as described previously (28).

RESULTS

TR Chimeras Containing LAMP-1, LAP, and the CD3 γ-Chain Cytoplasmic Tails Are Expressed on the Cell Surface and Rapidly Internalized—To determine if the cytoplasmic tails of lysosomal membrane proteins were sufficient to promote TR internalization, we constructed three chimeric proteins consisting of the cytoplasmic domains of LAMP-1, LAP, or the CD3 γ-chain, fused to the transmembrane and extracellular domains of the human TR (Fig. 1A). Each of these chimeras was expressed in CEF using BH-RCAS, a replication-competent retroviral vector derived from the Rous sarcoma virus (13, 33). Cell surface expression of each chimera was confirmed using 125I-labeled Tf binding at 4 °C (data not shown).

Internalization efficiencies of the chimeras were determined by measuring the steady-state distribution of internalized Tf and their ability to mediate iron uptake (13). As shown in Table I, all of the chimeras were rapidly internalized with relative activities ranging from 50 to 131% (compared with the wild-type TR). These data are consistent with previous studies showing that the cytoplasmic tails of LAMP-1, LAP, and the CD3 γ-chain contain internalization signals (4, 7, 18, 21) and indicate that each of these cytoplasmic tails is sufficient to promote rapid TR endocytosis. They also suggest that the cytoplasmic tails from type I membrane proteins are sufficient to promote internalization of type II membrane proteins, as has been previously shown for the β-amyloid precursor cytoplasmic tail (37).

CD3 γ-Chain-TR and LAP-TR Chimeras Are Degraded in a Post-Golgi Endocytic Compartment—To determine whether the cytoplasmic tails of CD3 γ-chain, LAP, and LAMP-1 were sufficient to target the chimeras to the prelysosomal/lysosomal compartment, metabolic pulse-chase experiments were performed. CEF expressing the TR chimeras and the wild-type TR were pulse-labeled with Tran35S-label for 30 min and chased for various time periods; wild-type TR and TR chimeras were then isolated by immunoprecipitation using an antibody to the TR extracellular domain and analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2 the CD3 γ-chain-TR and LAP-TR chimeras were more rapidly degraded (t1⁄2 < 10 h, respectively) than the wild-type TR (t1⁄2 ~24 h) and
the LAMP-TR chimera (t½ = 22 h), suggesting that only the CD3 γ-chain and LAP tails were sufficient to target the TR to the lysosomal compartment. After 2 h (Fig. 2), the Mₜ of the wild-type TR and each of the chimeras increased to that of the mature glycoprotein (40), indicating that all of the chimeras traverse the Golgi compartment where oligosaccharide processing is completed before degradation occurs.

To confirm that degradation was occurring in a lysosomal compartment, we monitored the rate of degradation in the presence of a lysosomotropic agent, ammonium chloride. As shown in Fig. 3 the half-lives of the CD3 γ-chain-TR and LAP-TR chimeras were extended from 1.5 h to 21 h and 5.5 h to 21 h, respectively, in the presence of 50 mM NH₄Cl. Addition of 100 μg/ml protease inhibitor, leupeptin, also increased the half-lives of the CD3 γ-chain-TR and LAP-TR chimeras (data not shown).

Next, we compared the intracellular distribution of the chimeras to LEP100, an endogenous chicken lysosomal integral membrane protein (6, 7, 41). The CD3 γ-chain-TR and the LAP-TR chimeras both showed significant co-localization with LEP100 (Fig. 4, A and B, respectively), whereas the LAMP-TR chimera and wild-type TR did not (Fig. 4, C and D, respectively), suggesting that the CD3 γ-chain-TR and LAP-TR traffic along the prelysosomal segment of the endocytic pathway. The low levels of co-localization of the wild-type TR and LAMP-TR with LEP100 are consistent with previous results using gold label immunocytochemistry, which demonstrated that −11% of
Lysosomal Targeting Signals

**Fig. 2.** Rapid degradation of the CD3 γ-chain-TR and LAP-TR chimeras occurs in a post-Golgi endocytic compartment. Equivalent cell numbers of CEF expressing wild-type (WT) TR, CD3 γ-chain-TR chimera, LAP-TR chimera, or LAMP-TR chimera were pulse-labeled for 30 min with Tran°S-label and chased for various periods of time (h) as indicated. TR or TR chimeras were then immunoprecipitated from post-nuclear 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 five) is shown.

**Fig. 3.** Ammonium chloride (NH4Cl) inhibits degradation of CD3 γ-chain-TR and LAP-TR chimeras. Equivalent cell numbers of CEF expressing CD3 γ-chain-TR or LAP-TR chimeras were preincubated in 50 mM NH4Cl in medium (+NH4Cl) or medium alone (−NH4Cl) for 1 h at 37 °C and then pulse-labeled with Tran°S-label and chased in the presence of NH4Cl in medium (+NH4Cl) or in medium alone (−NH4Cl). The chimeras were then immunoprecipitated from post-nuclear supernatants and analyzed on SDS-polyacrylamide gels as described in Fig. 2. A representative experiment (of three) is shown.

The wild-type TR is found in the LEP100-containing compartment (28).

**Transplanted Signals from LAMP-1, LAP, and the CD3 γ-Chain Promote TR Internalization but Not Lysosomal Targeting**—To determine the minimum sorting information required for lysosomal targeting, we transplanted the tyrosine-based signals from LAMP-1 (the amino acid residues YQTI) (5, 42), LAP (residues YRHV) (18), and CD3 γ-chain (residues YQPLK) (21), and the dileucine-based signal (residues DKQTLL) from the CD3 γ-chain (21) into the TR cytoplasmic tail as described in Fig. 7. As shown in Table II, each of the transplanted signals, DKQTLL (CD3 γ-chain), YQPLK (CD3 γ-chain), YQTI (LAMP-1), and YRHV (LAP) were effective at promoting TR internalization (relative activities of 153, 109, 46, and 80%, respectively, compared with the wild-type TR), demonstrating that the dileucine signal was the most effective internalization signal.

To determine if the transplanted signals were sufficient to promote lysosomal targeting of TR, metabolic pulse-chase experiments were performed. The results demonstrate that CD3 γ-chain dileucine signal, DKQTLL, in contrast to the CD3 γ-chain-TR chimera, is degraded slowly (t1/2 = 16 h; Fig. 5). Similarly, the rate of degradation of the mutant TRs containing the LAP signal, YRHV, or the CD3 γ-chain signal, YQPLK, were slow (t1/2 = 15.5 h and >24 h, respectively) like the wild-type TR (t1/2 > 24 h). This indicated that none of the individual signals were sufficient to deliver the TR to the prelysosomal/lysosomal compartment.

Two Signals from the CD3 γ-Chain are Sufficient for Lysosomal Targeting—Since the CD3 γ-chain tail was sufficient for delivery of the TR to the lysosomal compartment, but the individual signals were not, we next asked if insertion of two CD3 γ-chain signals into the TR tail would reconstitute lysosomal targeting. To perform this experiment, we took advantage of the fact that introduction of a YTRF internalization signal at position 31–34 in the TR cytoplasmic tail restores endocytosis of an internalization-defective mutant receptor to wild-type levels, suggesting that a signal in this position is efficiently recognized at the cell surface (36). Similar results were reported by Pytowski et al. (43), who demonstrated that introduction of a tyrosine residue for the first or last residue at position 31–34 created an effective internalization signal.

Based on these two studies, we introduced the CD3 γ-chain signal, YQPLK, at position 31–35 of the D18KQTLL23 mutant, and analyzed the resulting mutant, D18KQTLL23,Y31QPLK35 (Fig. 1C), in a metabolic pulse-chase experiment. As shown in Fig. 6, the D18KQTLL23,Y31QPLK35 mutant was similar to the CD3 γ-chain-TR chimera in that it was rapidly degraded (t1/2 = 2 h) and its degradation could be inhibited in the presence of NH4Cl (t1/2 = 17 h). Unexpectedly, the internalization rate of this mutant was twice that of the CD3 γ-chain-TR chimera (Table II).

Transplantation of an Additional YTRF Signal at Position 31–34 Promotes Lysosomal Targeting of the TR—Since the D18KQTLL23,Y31QPLK35 mutant promoted lysosomal targeting, we next asked if two tyrosine-based signals could provide the same sorting information. To test this, we examined the half-lives of three previously described mutants that contain an additional copy of the YTRF sequence inserted at different locations within the TR cytoplasmic domain (36) (Fig. 1C). As shown in Fig. 7, the Y31TRF34 mutant had a half-life of 6 h, whereas the Y31TRF15 and Y31TRF50 mutants had half-lives of 17 h and >24 h, respectively (Fig. 7). Treatment with NH4Cl increased the half-life of the Y31TRF44 mutant to 14 h (data not shown). These results suggest that having a second signal at
position 31–34 or 31–35 in the TR cytoplasmic tail restores lysosomal targeting and that placement of a second signal at other cytoplasmic tail positions does not.

**TR Chimeras with Tyrosine-based Signals Proximal to the Transmembrane Region Are Rapidly Degraded**—Because a number of lysosomal membrane proteins have short cytoplasmic tails with tyrosine-based signals in close proximity to the transmembrane region, we next asked if transplantation of signals into a truncated TR cytoplasmic tail, D3–18, 29–59, promoted lysosomal targeting (Fig. 1D). D3–18, 29–59 TR has previously been shown to promote efficient internalization (13). As shown in Table II, each of the truncation mutants, Y4QTI7 and Y4RHV7, promoted TR internalization. Furthermore, metabolic pulse-chase experiments revealed that each was rapidly degraded (t1/2 = 3.5, 3, and 3 h, for the Y4QTI7, Y4RHV7, and D3–18, 29–59 mutants, respectively) (Fig. 8). These results suggest that even the wild-type TR signal, YTRF, is sufficient for lysosomal targeting if presented in the proper context.

**CD3γ-Chain-TR and LAP-TR Chimeras, and the D18KQTLL23, Y31QLPK35, and Y31TRF34 Mutants That Reach the Cell Surface Are Targeted to Lysosomes**—Although the metabolic pulse-chase experiments indicated that several of the TR chimeras were rapidly degraded in a post-Golgi compartment, we wanted to determine if the chimeras that reached the cell surface were also delivered to the lysosomal compartment. To address this, we performed the following experiment. Cells expressing the chimeras were incubated with 125I-labeled Tf at 37°C for 1 h to load the endocytic pathway with receptor-ligand complexes (28). The cells were then rapidly washed and the reappearance of intact and labeled Tf in the medium was determined by measuring trichloroacetic acid-insoluble and soluble radioactive counts. For the CD3γ-chain-TR and LAP chimeras, 31 and 20% of Tf bound, respectively, were degraded before being released into the medium (trichloroacetic acid-soluble counts; Fig. 9A), whereas only 2.6% of Tf bound to the

### Table II

| Human TR constructs | Internalized human TR at steady state | Internalization efficiencya | Iron uptake | Internalization efficiencyb |
|---------------------|---------------------------------------|----------------------------|-------------|-----------------------------|
| Wild-type (Y20TRF23) | 64 ± 0.8 (15) | 100 | 18 ± 0.9 (9) | 100 |
| Y20QTI23           | 45 ± 1.0 (3) | 46 | 14 ± 3.8 (6) | 78 |
| Y20RHV23           | 59 ± 1.3 (8) | 80 | 7.3 ± 0.7 (3) | 41 |
| Y20QPLK24          | 66 ± 2.4 (3) | 109 | ND          | ND          |
| D18KQTLL23, Y31QLPK35 | 73 ± 1.2 (12) | 153 | 27 ± 1.8 (3) | 150 |
| ∆3–18, 29–59 (YTRF23) | 83 ± 2.1 (3) | 276 | ND          | ND          |
| ∆3–18, 29–59 (Y4QTI7) | 64 ± 1.4 (3) | 100 | ND          | ND          |
| ∆3–18, 29–59 (Y4RHV7) | 51 ± 0.9 (3) | 58 | 19 ± 4.1 (6) | 106 |
| D3–18, 29–59 (Y4TRF34) | 54 ± 3.5 (7) | 66 | 7.3 ± 1.0 (3) | 41 |

a Calculated from steady-state assays as described under “Experimental Procedures.”

b Calculated from iron uptake assays.

Mean value ± S.E.

d Number of independent experiments.

e ND, not determined.
wild-type TR was. Thus, a substantial fraction of CD3 γ-chain-TR and LAP-TR chimeras are directly targeted to lysosomes after internalization. Analysis of the D18KQTLL23 mutant, the D18KQTLL23,Y31QPLK35 mutant, and Δ3–18, 29–59 TR indicated that 5, 12, and 16% of Tf bound was degraded before being released into the medium (Fig. 9B). This result suggested that the D18KQTLL23,Y31QPLK35 mutant and Δ3–18, 29–59 TR were delivered to the lysosomal compartment from the cell surface, albeit less efficiently than the CD3 γ-chain chimera.

DISCUSSION

The studies reported here were undertaken to define more precisely the properties of lysosomal targeting signals. Our data indicate that the addition of the CD3 γ-chain and LAP cytoplasmic tails onto a type II membrane protein, the TR, is sufficient for efficient targeting of the chimeras to the lysosomal compartment. Interestingly, the LAMP-1 cytoplasmic domain did not confer this same targeting specificity. Williams and Fukuda (17) first demonstrated that the addition of the 11-residue cytoplasmic tail of LAMP-1 to a reporter molecule, the soluble human gonadotropin α-chain spliced to the VSV-G protein transmembrane domain, was sufficient to deliver the chimera to the lysosomes. They also demonstrated that a tyrosine was a critical part of the signal and that the position of the tyrosine residue within the cytoplasmic tail was critical for lysosomal targeting.

Studies by Guarnieri et al. (42) on the murine LAMP-1 extended these findings to show that the lysosomal targeting signal consisted of the last 4 residues in the 11-residue tail that formed the pattern Y-X-X-hydrophobic. Furthermore, there ap-
peared to be a strict requirement for the placement of this motif relative to the transmembrane region since a 7-residue spacer was necessary for proper recognition (42). Rohrer et al. (11) demonstrated that the spacer requirement was important for sorting in the sorting endosome and less so at the cell surface. In our studies, placement of this 4-residue signal, YQTI, in 61-residue TR tail as well as placement of the entire 11-residue LAMP-1 tail in a 4-residue "tailless" TR tail all produced chimeras that were efficiently internalized but not lysosomally targeted. Interestingly, the only case in which this motif was recognized as a lysosomal targeting signal was in a truncated TR cytoplasmic tail, placing the YQTI motif 7 residues from the transmembrane region. This suggested that the spacer requirement found in type I membrane proteins (11, 42) is also true for type II membrane proteins such as the TR. The same spacing requirement, however, was not important for endocytosis, since chimeras containing the YQTI motif were internalized with the same efficiency when the motif was placed at 2, 7, or 38 amino acids from the transmembrane region.

The evidence that both the CD3 δ-chain and LAP chimeras are efficiently delivered to the lysosome comes from both biochemical and morphological experiments. First, metabolic pulse-chase experiments demonstrated that the chimeras were rapidly degraded in a post-Golgi compartment. Clearly, formation of the maturely glycosylated chimeras confirmed that they were not simply misfolded proteins that failed to exit the endoplasmic reticulum. Second, degradation of the chimeras occurred in a lysosomal compartment since the degradation could be inhibited with the weak base, ammonium chloride. Third, ~31% of the CD3 δ-chain TR chimeras that were displayed on the cell surface were degraded after internalization. Fourth, the intracellular distribution of the CD3 δ-chain-TR and LAP-TR chimeras overlapped significantly with a marker of the prelysosomal/lysosomal compartment, LEP100, whereas the wild-type TR and LAMP-TR chimera did not.

Our results on the CD3 γ-chain-TR chimeras are consistent with those of Letourneur and Klausner (21) who reported that the CD3 γ- and δ-chains of the T cell antigen receptor cytoplasmic tails contained two lysosomal-sorting signals. Using chimeras consisting of the extracellular and transmembrane regions of the Tαc antigen (IL-2 receptor α-chain) spliced to the cytoplasmic tails of these subunits, they identified a tyrosine-based motif, YQLPK, and a dileucine motif, DKQTLT, that functioned to deliver the chimera to the lysosome without going to the cell surface. Mutants that contained only one of these signals were delivered to the cell surface, rapidly internalized, and subsequently delivered to the lysosomes. The dileucine motif alone, therefore, served as an effective internalization signal as well as a lysosomal targeting signal in the context of the CD3 γ-chain. Our studies show that the DKQTLT sequence is also sufficient to mediate rapid internalization of the TR, indicating that tyrosine-based signals and dileucine signals appear to be functionally equivalent. Our results, however, differ in that the dileucine motif alone was not sufficient to target the TR to the lysosome. The half-life of this chimera was 18 ± 2.0 h (mean ± S.E. for five independent experiments), suggesting that additional targeting information is required.

The other mutant that was delivered to the lysosome, the LAP-TR chimera, was not as efficiently delivered to the lysosomal compartment as the CD3 γ-chain chimera (t½ = 8.2 ± 0.6 h (mean ± S.E. of six independent experiments)). Insertion of the LAP cytoplasmic tail into a "tailless" TR tail (Fig. 1) placed the YRHV signal 9 residues from the transmembrane. Interestingly, placement of this signal in the truncated TR tail, positioned this signal 7 residue from the transmembrane and resulted in a more rapid degradation (t½ = 5.3 ± 1.8 h, mean ± S.E. of three independent experiments). In the context of the wild-type LAP protein, this signal is 8 residues from the transmembrane region. That this sequence, YRHV, was not specifically required for lysosomal targeting in the context of the truncated TR cytoplasmic tail was demonstrated by the fact that the LAMP-1 targeting signal, YQTI, as well as the native TR internalization signal, YTRF, were also sufficient for this sorting process.

Several studies have shown that the direct intracellular route used by newly synthesized cation-independent mannose 6-phosphate receptors is from the trans-Golgi to the early endosomal compartment (4, 44). This may represent the pathway taken by lysosome membrane proteins, implying that the sorting signals for lysosomal targeting would need to be recognized at the sorting endosome, as was suggested in the studies by Rohrer et al. (11) on LAMP-1. Our data support this idea since all of the mutants successfully reached the endosome, yet many failed to be delivered further into the endocytic pathway. For efficient lysosomal delivery, our chimeras contained one of the following structural features: 1) a dileucine-based signal and a tyrosine-based signal separated by 5–7 residues, 2) two tyrosine-based signals separated by 7 residues, or 3) a single tyrosine-based signal 7–9 residues from the transmembrane region. Interestingly, however, not all two-signal chimeras conferred lysosomal targeting specificity, suggesting that there was a strict positional requirement of these signals for proper targeting.

In conclusion, our data support the following model. Constitutively recycling receptors contain internalization signals that allow them to enter the endocytic pathway. Once they reach the sorting endosome, another sorting decision is made. Since the TR lacking a cytoplasmic domain recycles to the cell surface as effectively as a wild-type TR (Jing et al. (13)), then recycling would be expected to be the default pathway. The results presented here demonstrate that the same signal types that function as efficient internalization signals can function as lysosomal targeting signals, but the cytoplasmic tail positional requirements for lysosomal signals appear to be much more restricted. This suggests that similar motifs have been adapted for use in endocytosis and lysosomal targeting (and perhaps trans-Golgi network sorting), and that positional changes of the motif within the cytoplasmic tail affect recognition at each site differently.

REFERENCES
1. Kornfeld, S. (1989) Annu. Rev. Cell Biol. 5, 483–525
2. Fukuda, M. (1991) J. Biol. Chem. 266, 21327–21330
3. Green, S. A., Zimmer, K., Griffiths, G., and Mellman, I. (1987) J. Cell Biol. 105, 1227–1240
4. Harter, C., and Mellman, I. (1992) J. Cell Biol. 117, 311–325
5. Honing, S., and Hunziker, W. (1995) J. Cell Biol. 128, 321–332
6. Lippincott-Schwartz, J., and Fambrough, D. M. (1987) Cell 49, 669–677
7. Mathews, P. M., Martinie, J. B., and Fambrough, D. M. (1992) J. Cell Biol. 118, 1027–1040
8. Braun, M., Waeber, A., and von Figura, K. (1989) EMBO J. 8, 3633–3640
9. Honing, S., Griffiths, J., Geuze, H. J., and Hunziker, W. (1996) EMBO J. 15, 5230–5239
10. Futter, C. E., Felder, S., Schlessinger, J., Ullrich, A., and Hopkins, C. K. (1993) J. Cell Biol. 120, 77–83
11. Rohrer, J., Schweizer, A., Russell, D., and Kornfeld, S. (1996) J. Cell Biol. 132, 565–576
12. Mayer, S., Presley, J. F., and Maxfield, F. R. (1993) J. Cell Biol. 121, 1257–1269
13. Jing, S., Spencer, T., Miller, K., Hopkins, C., and Trowbridge, I. S. (1990) J. Cell Biol. 110, 283–294
14. Johnson, L. S., Dunn, K. W., Pytowski, B., and McGraw, T. E. (1993) Mol. Biol. Cell 4, 1251–1266
15. Trowbridge, I. S., Collawn, J. F., and Hopkins, C. K. (1993) Annu. Rev. Cell Biol. 9, 129–161
16. Sandoval, I. V., and Bakke, O. (1994) Trends Cell Biol. 4, 292–297
17. Williams, M. A., and Fukuda, M. (1990) J. Cell Biol. 111, 955–966
18. Lehmann, L. E., Eberle, W., Krull, S., Prill, V., Schmidt, B., Sander, C., von Figura, K., and Peters, C. (1992) EMBO J. 11, 4381–4389
19. Johnson, K. F., and Kornfeld, S. (1992) J. Cell Biol. 119, 249–257
20. Johnson, K. F., and Kornfeld, S. (1992) J. Biol. Chem. 267, 17110–17115
21. Letourneur, F., and Klausner, R. D. (1992) Cell 69, 1143–1157

Lysosomal Targeting Signals
22. Ogata, S., and Fukuda, M. (1994) J. Biol. Chem. 269, 5210–5217
23. Matter, K., Yamamoto, E. M., and Mellman, I. (1994) J. Cell Biol. 126, 991–1004
24. Huniczek, W., and Fumeys, C. (1994) EMBO J. 13, 2963–2969
25. Corvera, S., Chawla, A., Chakrabarti, R., Joly, M., Buxton, J., and Czech, M. P. (1994) J. Cell Biol. 126, 979–989
26. Haft, C. R., Klausner, R. D., and Taylor, S. I. (1994) J. Biol. Chem. 269, 26286–26294
27. Pieters, J., Bakke, O., and Dobberstein, B. (1994) J. Cell Sci. 106, 831–846
28. Lai, A., Sisodia, S. S., and Trowbridge, I. S. (1995) J. Biol. Chem. 268, 21686–21692
29. Marks, M. S., Woodruff, L., Ohno, H., and Bonifacino, J. S. (1996) J. Cell Biol. 135, 341–354
30. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
31. Hughes, S. H., and al., e. (1990) J. Cell Biol. 111, 1811–1823
32. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci U. S. A. 74, 5463–5467
33. Tabor, S., and Richardson, C. C. (1987) Biochemistry 26, 4767–4771
34. Collawn, J. F., Lai, A., Domingo, D., Fitch, M., Hatton, S., and Trowbridge, I. S. (1995) J. Biol. Chem. 268, 21686–21692
35. Lai, A., Sisodia, S. S., and Trowbridge, I. S. (1995) J. Biol. Chem. 270, 3565–3573
36. Collawn, J. F., Stangel, M., Kuhn, L. A., Esekogwu, V., Jing, S., Trowbridge, I. S., and Tainer, J. A. (1995) J. Cell Biol. 63, 1061–1072
37. Tanner, L. I., and Lienhard, G. E. (1987) J. Biol. Chem. 262, 8975–8980
38. Lippincott-Schwartz, J., and Fambrough, D. M. (1986) J. Cell Biol. 102, 1593–1605
39. Guarnieri, F. G., Arterburn, L. M., Penno, M. B., Cha, Y., and August, J. T. (1993) J. Biol. Chem. 268, 1914–1914
40. Pytowski, B., Judge, T. W., and McGraw, T. E. (1995) J. Biol. Chem. 270, 5097–5103
41. Beaulame, B. D., Gibson, A., and Hopkins, C. R. (1996) J. Cell Biol. 111, 1811–1823