Protein Targeting by Tyrosine- and Di-leucine–based Signals: Evidence for Distinct Saturable Components

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Abstract. Targeting of transmembrane proteins to lysosomes, endosomal compartments, or the trans-Golgi network is largely dependent upon cytoplasmically exposed sorting signals. Among the most widely used signals are those that conform to the tyrosine-based motif, YXXØ (where Y is tyrosine, X is any amino acid, and Ø is an amino acid with a bulky hydrophobic group), and to the di-leucine (or LL) motif. Signals conforming to both motifs have been implicated in protein localization to similar post-Golgi compartments. We have exploited the saturability of sorting to ask whether different YXXØ or LL signals use shared components of the targeting machinery. Chimeric proteins containing various cytoplasmic domains and/or targeting signals were overexpressed in HeLa cells by transient transfection. Endogenous transferrin receptor and lysosomal proteins accumulated at the cell surface upon overexpression of chimeric proteins containing functional YXXØ targeting signals, regardless of the compartmental destination imparted by the signal. Furthermore, overexpression of these chimeric proteins compromised YXXØ-mediated endocytosis and lysosomal delivery. These activities were ablated by mutating the signals or by appending sequences that conformed to the YXXØ motif but lacked targeting activity. Interestingly, overexpression of chimeric proteins containing cytoplasmic LL signals failed to induce surface displacement of endogenous YXXØ-containing proteins, but did displace other proteins containing LL motifs. Our data demonstrate that: (a) Protein targeting and internalization mediated by either YXXØ or LL motifs are saturable processes; (b) common saturable components are used in YXXØ-mediated protein internalization and targeting to different post-Golgi compartments; and (c) YXXØ- and LL-mediated targeting mechanisms use distinct saturable components.

Transport of newly synthesized transmembrane glycoproteins from the ER to the cell surface is thought to occur largely by default (50). Retention within compartments along this pathway or diversion to peripheral organelles requires some form of targeting or sorting information on the proteins themselves. This targeting information may be of two different types. One type relies on global physical–chemical properties of the protein, such as aggregation that contributes to protein retention within the ER (22, 59). Targeting via this mechanism depends on an intrinsic property of the protein and should therefore be unsaturable, i.e., unaffected by protein expression levels. The second type of sorting information consists of discrete signals on the target protein that are recognized by specific receptors. The receptors, in turn, actively mediate sorting of the target protein. An example of this type of signal is the sequence, KDEL1 present on the carboxy termini of several soluble ER resident proteins, which mediates ER localization by interaction with a membrane-bound receptor, ERD2 (30). Because the number of receptors may be limiting, targeting via this type of mechanism is expected to be saturable, as has been demonstrated for KDEL-dependent ER retention (29).

We have been studying the signals that target newly synthesized transmembrane glycoproteins for delivery to post-Golgi intracellular compartments, including the TGN, lysosomes, and endosomes. Sorting to these compartments

1. Abbreviations used in this paper: ITAMs, immunoreceptor tyrosine activation motifs; LL, di-leucine-based sorting signal; MHC, Major histocompatibility complex; TfR, human transferrin receptor.

The symbols used correspond to the one letter amino acid code as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; Ø, amino acid with a bulky hydrophobic side chain (L, I, M, F, V); X, any amino acid.
relies predominantly upon signals in the cytoplasmic domains of the proteins. The first well described cytoplasmic post-Golgi sorting signal, shown to be essential for internalization of the low density lipoprotein receptor, can be described by the amino acid sequence NPXY (9, 11), in which a tyrosine (Y) is preceded by an asparagine (N)-proline (P) dipeptide and a random amino acid (X). Since this initial description, many cytoplasmic sorting signals have been identified, most of which conform to one of several families of peptide sequence motifs. Two of these families have been extensively characterized. The first, like the NPXY motif, contains a critical tyrosine residue, but is described by the consensus motif, YXXΩ (for review see 73), in which the tyrosine (Y) is separated by two seemingly random amino acids (X) from an amino acid with a bulky hydrophobic side chain (Ω). This type of targeting signal has been shown to be responsible for the rapid internalization of endocytic receptors from the plasma membrane, as well as for protein targeting to various endosomal compartments, lysosomes, the basolateral surface of polarized epithelial cells, and the TGN (for review see 62, 73). The second type of signal is a so-called di-leucine-based (LL) signal, for which the only known requirement is the presence of two consecutive leucines or a leucine–isoleucine pair (23, 28); an acidic side chain 4 residues upstream seems to be beneficial, but not essential for activity of the motif (52). LL-based signals have been shown to mediate many of the same processes as tyrosine-based signals, including internalization and targeting to endosomal compartments and lysosomes (for review see 62) and to the basolateral surface of polarized epithelial cells (21, 39). Neither the mechanisms by which the tyrosine-based and LL signals function nor the distinguishing features of individual signals that are responsible for directing proteins to different subcellular compartments are currently well understood.

Since both types of signal are likely to be recognized by specific receptors, one would predict that YXXΩ and LL signal–dependent targeting to post-Golgi compartments would be saturable. Indeed, we and others have suggested saturability of protein targeting based on qualitative observations of the internalization or localization of specific YXXΩ-containing proteins (17, 20, 36). Here we demonstrate, using a quantitative flow cytometry assay, that targeting mediated by both YXXΩ and LL signals is indeed saturable. Overexpression of proteins containing these signals results in their accumulation at the cell surface, presumably by saturation of a component of the targeting machinery involved either in internalization from the plasma membrane and/or in sorting from the TGN. Overexpression of exogenous YXXΩ-containing proteins interferes with the YXXΩ signal–dependent sorting of endogenous proteins. Moreover, we show that common saturable components are used by the YXXΩ–dependent targeting machinery responsible for localization to several post-Golgi compartments. Finally, YXXΩ- and LL-mediated localization are shown to use distinct saturable components. These results suggest that: (a) Different YXXΩ signals may use a common mechanism to direct localization to distinct end compartments, and (b) YXXΩ and LL motifs may use different components in mediating localization to common end compartments.

Materials and Methods

Cell Culture and Transfections

HeLa cells (American Type Culture Collection, Rockville, MD) were maintained in DME supplemented with 7% (vol/vol) FBS and antibiotics (50 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin). Jurkat cells (American Type Culture Collection) were maintained in DME supplemented with 10% (vol/vol) FBS and antibiotics. For transient transfections, confluent HeLa cells were split 1:8 to 1:12 into 10-cm dishes in 9 ml medium. In most experiments, one or two coverslips were placed in each dish before addition of cells for subsequent analyses by immunofluorescence microscopy. Calcium phosphate precipitates of DNA (1 μg) with a total of 10–20 μg of DNA, were added to the cells 4–6 h after plating, at which time the cells had adhered to the plastic dishes but had not yet extended (addition of the DNA/calcium phosphate mix at this time was found to improve transfection efficiency relative to its addition 20–24 h after plating). The next day, the medium was removed and fresh medium was added. Cells were generally assayed at ~48–60 h after transfection. Transfection efficiencies ranged from 20 to 70% as judged by immunofluorescence microscopy. For stable transfections, 104 Jurkat cells were incubated with 30 μg of plasmid DNA plus 3 μg of RSV.S(neo) (a gift of Dr. E. Long, National Institute of Allergy and Infectious Disease [NIAMD], National Institutes of Health [NIH], Bethesda, MD) in 0.8 ml of RPMI 1640 medium and transfected by electroporation in a 4-mm cuvette with a Gene Pulser (BioRad Labs, Hercules, CA) set at 290 V and 960 μF. After transfection, cells were distributed in a 96-well microtiter plate, and selection with 1 mg/ml G418 (Life Technologies Inc., Gaithersburg, MD) was initiated the following day. Surviving clones were tested for surface expression of the products of the transgenes by flow cytometry. Two clones with different surface expression levels for each construct were chosen for further analyses.

DNA Constructs

All constructs for transient transfection were prepared as described (36) in the mammalian expression vector pCDM8.1 or pCDM8, except for the TcT construct (27) and others used for stable transfection of Jurkat cells (see below), which were prepared in the expression vector pCDL-SRo (71); both vectors yielded similar protein expression levels upon transient transfection in HeLa cells. Sources of the chimeric constructs and markers are listed in Table I. The Tac-Lamp1 was constructed by the two-step PCR method (19). Briefly, a chimeric insert encoding the Tac transmembrane and cytoplasmic domains fused at the COOH terminus to the human Lamp1 cytoplasmic domain (6) and containing a 5' BglII site and a 3' XhoI site was constructed by PCR. The insert was ligated into a pCDM8.1-Tac construct that had been modified by addition of a BglII site inserted before the transmembrane domain (60). Tac-LSYTFRW was similarly constructed using PCR to generate a BglII/XbaI insert encoding the Tac transmembrane and cytoplasmic domains fused to the tyrosine-based signal from the human transferrin receptor (TIR), LSYTFRW, followed by a stop codon. The Tac-DKQTLL construct (originally called TT3,t3-t2; 28) was first prepared in pCDL-SRct and then transferred into pCDM8.1 as an EcoRI-XbaI fragment. The insert for Lamp1 was excised from the plasmid pSV-Lamp1 (8; a gift of Dr. M. Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA), using XhoI and BamHI, and cloned into Sall/BglII-digested pCDM8.1. The insert for CD4 was excised from pGEM4-C4D, provided by Dr. D. Litman (University of California, San Francisco, CA), by digestion with EcoRI and BamHI, and cloned into EcoRI/BglII-digested pCDM8.1. The sequence of all junctions and any PCR-generated inserts were verified by DNA sequencing using the Sequenase II kit (Amersham Corp., Arlington Heights, IL). Jurkat cells were transfected stably with pCDL-SRo expression constructs encoding Tac, TcT (27), or a TcT construct lacking the LL motif (TcT-ΔLL; 28). CD3 ε in pCDM8 (42) was the gift of Dr. P. Peters (University of Utrecht, The Netherlands). The human invariant chain Ip33 construct, lacking the first ATG (56), was in pCDM8 and was a gift of Dr. P. Roche (National Cancer Institute [NCI], NIH). The human TIR in pCDM8.1 was the gift of Dr. J. Harford (NCI, NIH).

Antibodies

The following monoclonal antibodies were used: 7G7.86 (anti-Tac; American Type Culture Collection), OKT4a (anti-CD4, a gift of Dr. L. Samelson, National Institute of Child Health and Human Development [NICHD]).
Table I. Cytoplasmic Tails and Constructs Used in This Study

| Construct name | Source of cytoplasmic tail | Sequence of cytoplasmic tail | Reference |
|----------------|---------------------------|-----------------------------|-----------|
| TTMb           | Murine DB3 (Mb)           | TMR-WRGRHSSYTPLSGTYSPGR-COOH | 36        |
| TTM.GSTYA     | ''                        | TMR-WRGRHSYSYTPLSGTY-S-COOH | 36        |
| TTM.GSTYA.Y248A| ''                        | TMR-WRGRHSSATPSGTY-S-COOH   | 36        |
| TTM.GSTYA.L251A| ''                        | TMR-WRGRHSSYTPASGTY-S- COOH | 36        |
| TTM.HSSSA     | ''                        | TMR-WRGRHSSSS- COOH         | 36        |
| Tac-Lamp1     | Tac/Human Lamp1           | TMR-RQRKSSRTTRKSHAGYQTI- COOH| This study|
| TTLa           | Human Lamp1               | TMR-RQRKSHAGYQTI- COOH      | This study|
| Tac-LSYTRF    | Tac/Human transferrin receptor | TMR-RQRKSSRTTRSYTRF- COOH  | This study|
| TT-TGN38 (T-T-G)| Rat TGN38                   | TM-HNKKIIAFLEKSKTRPKSADLYQNKL- COOH | 20        |
| TTγ            | Mouse TCRγ                | TM-RKFSRASSAANLDPQNYENLGLRREYDVLEKK | 27        |
| Lamp1          | Human Lamp1               | TM-KHHIAYEQE- COOH          | 42        |
| CD63          | Human CD63                | TM-KSIRSYGYPEY- COOH        | 8         |
| TR            | Human transferrin receptor | NH-MMDQARSFSNLFGGPESTYTRFLARQYDGNN    | VEMKLVDEEANADNKTAVKPRCSGSICY- TM |
| Lamp2          | Human Lamp2               | TM-KKHHAQEYEQ- COOH         | This study|
| CD4           | Human CD4                 | TM-RCRHRQQRERMSIQRLLSEKTCQCCHRFQKTC | 8         |
| Tac-DKQTL (TTγ3-2)| Tac/MouseCD3γ            | TMR-RQRKSSRTTRDKQTL- COOH   | 28        |
| TTγ-ALLγ17    | Tac/Mouse CD3γ            | TMR-RQRKSSRTTRDKQTQNEQLYQPLKREYDYQYSHEL- COOH | 28        |
| Ip33          | Human invariant chain     | NH-MDDQRDQLESENNEQPLMLGRRAGAPESCRG-SGM | 56        |

Construct name: The name used in this study; for two of the constructs, the corresponding name from the original reference is also included. Source of cytoplasmic tail: Chimeric proteins that include the Tac cytoplasmic domain are indicated by inclusion of "Tac." Sequence of cytoplasmic tail: Letters correspond to the single-letter amino acid code; TM, transmembrane region; -COOH, the carboxyl terminus of the protein; NH-, the amino terminus of the type II TR or invariant chain proteins; letters in italics denote cytoplasmic sequences derived from Tac. Letters in bold type indicate sequences with known or potential sorting activity. Reference indicates the original reference describing the use of the construct.

Flow Cytometry

For overexpression, HeLa cells were transfected transiently with 10–20 μg of the plasmid encoding the desired protein. For indicator protein expression, cells were transfected with 0.25–1 μg of plasmid. All DNA quantities were normalized within any given experiment by addition of pCMV8.1 expression vector without an insert. Generally, a sample of cells adhered to coverslips was removed at the time of harvesting and fixed for later analysis, as measured by immunofluorescence microscopy and metabolic pulse labeling/immunoprecipitation, respectively, described below. Cells were excluded from analysis by forward and side scatter measurements. The figures shown include only those experiments in which experimental and control samples had similar transfection efficiencies and levels of expression, as measured by immunofluorescence microscopy and metabolic pulse labeling/immunoprecipitation, respectively, described below.

Immunofluorescence Microscopy

Samples of transfected cells adhered to coverslips were fixed for 20–30 min with 2% formaldehyde in PBS. Cells were then processed for immunofluorescence microscopy as described (20). For quantitation of transfection efficiency, 200–400 random cells from each sample were counted and scored for expression of transgenes. In some cases, coverslips were counter-stained with an antibody to endogenous RXRβ to more easily visualize both transfected and untransfected cells.

Metabolic Labeling

Controls for Flow Cytometry. Aliquots consisting of equal cell numbers of each sample to be analyzed (generally 1–5 × 10⁶ cells) were taken at the time of EDTA release from the plates, pelleted, and resuspended in 0.25–0.5 ml of methionine- or methionine/cysteine-free DME supplemented with 3% (vol/vol) dialyzed FBS, antibiotics, and 25 mM Hepes, pH 7.4. Cells were incubated for 30–60 min at 37°C, pelleted, and then resuspended in 0.25 ml of the same medium containing 1–2 μCi/ml Tran35S-Label (ICN, Costa Mesa, CA) or EXPRE35S (DuPont/New England Nuclear, Boston, MA). Cells were incubated for 30 min at 37°C, pelleted, washed twice with PBS, and then stored at –70°C.

Pulse/Chase Analyses. Cells were trypsinized from a 10-cm dish, pelleted, and preincubated and labeled for 30 min as described above. At the end of the pulse, cells were placed on ice, pelleted, and resuspended in 5 ml complete medium supplemented with a 15-fold excess of methionine and cysteine and then incubated as indicated at 37°C. At the end of each time point, cells were pelleted, washed once with ice-cold PBS, and stored at –70°C.
**Immunoprecipitation and SDS-PAGE**

Metabolically labeled cell pellets were extracted, precleared, and subjected to immunoprecipitation with 7G7.B6 and/or other antibodies as described (36). Multiple rounds of immunoprecipitation were done sequentially on individual lysates in some cases. Immunoprecipitates were fractionated by SDS-PAGE on 9% acrylamide gels and analyzed both by fluorography/autoradiography and by PhosphorImager analysis on a scanner (Molecular Dynamics, Sunnyvale, CA) as described (36).

**Radioiodinated Internalization Assay**

Internalization of $^{125}$I-labeled 7G7.B6 (anti-Tac) antibody was done as previously described (36). Briefly, transfected cells were released from plates by EDTA treatment (see above), washed with DME containing 0.5% (wt/vol) BSA, and incubated with $^{125}$I-labeled 7G7.B6 on ice for 30–60 min. Cells were washed and resuspended in DME/10% PBS and then warmed to 37°C. At the indicated times, cells were diluted into ice-cold PBS and pelleted after all samples were taken. Half of the cell samples were resuspended in PBS alone and the other half in PBS containing 1 mg/ml proteinase K, and all cells were incubated on ice for 30 min. Cells were pelleted through a cushion of FBS to remove degraded label, and cell pellets were counted in a Packard $^\gamma$ counter. Results are expressed as the mean ± SD of three experiments each performed in duplicate, except for the TTM.HSSS$\Delta$ sample used as a negative control for internalization, which was analyzed once in duplicate.

**Flow Cytometric Internalization Assay**

Jurkat clones stably expressing Tac, TTF, or TTyDLL7$\gamma$ were harvested, resuspended in ice-cold 7G7.B6 supernatant, and incubated on ice for 30 min. One sample of each clone was resuspended in PBS containing 0.2% (wt/vol) BSA and 0.02% NaN$_3$ (buffer A) for determination of background staining with secondary antibody alone. Cells were washed three times with ice-cold DME/10% (vol/vol) FBS, and one sample of each clone was removed at time 0. The remaining cells were divided into four aliquots and incubated at 37°C. Cells were harvested after 1, 2, or 4 h and stained with FITC-goat anti-mouse Ig in buffer A for 30 min. One sample at 4 h was restained with 7G7.B6 before addition of secondary antibody to ensure equivalent surface staining relative to the 0 time point. Cells were analyzed by flow cytometry as described above, and dead cells were excluded using propidium iodide staining. To calculate internalization, the mean fluorescence intensity of each sample was calculated, and that obtained with background staining (No. 7G7.B6 antibody) was subtracted from that of each sample. The resulting numbers were divided by the mean fluorescence intensity at time 0 to obtain the percentage remaining at the cell surface.

**Results**

**Overexpression of a Chimeric Protein Containing the Cytoplasmic Tyrosine-based Signal from H-2Mβ Results in Its Accumulation at the Cell Surface**

We and others have previously characterized a sorting signal in the cytoplasmic tail of the β chain of HLA-DM/H-2M that conforms to the YXXØ consensus motif for post-Golgi sorting and mediates delivery of a reporter protein to lysosome-like antigen processing compartments (31, 36; Table 1). During our analysis of the chimeric protein TTMb, containing the cytoplasmic tail of the H-2Mβ chain (Mb) appended to the luminal and transmembrane domains of the Tac antigen reporter protein, we noticed that targeting was inefficient at high expression levels, often resulting in localization to the cell surface. To address this issue quantitatively, we used flow cytometry to measure the level of surface expression of the Tac reporter in HeLa cells transiently transfected with increasing amounts of DNA encoding either TTMb, containing the full-length Mb cytoplasmic tail, or TTM.HSSS$\Delta$, containing a truncated form of the Mb tail lacking the YXXØ motif. The latter chimeric protein localizes exclusively to the cell surface (36). Cells were cotransfected with equal amounts of DNA encoding a cell surface marker, CD4, to mark the transfected cells during analysis.

The levels of Tac surface expression in cells that expressed CD4 are shown in Fig. 1. Transfection with even the lowest amount of DNA encoding the TTM.HSSS$\Delta$ chimera, 0.5 μg, resulted in relatively high surface expression levels. Increasing the amount of transfected DNA to 2 or 5 μg increased the median expression level by three- or eightfold, respectively. In contrast, transfection with 0.5 μg of DNA encoding the TTMb chimera resulted in very low levels of surface expression, due to the predominant localization of this protein to lysosomes (36). Increasing the amount of transfected DNA to 2 or 5 μg, however, resulted in the emergence of a population of cells expressing high levels of the protein at the cell surface (10- and 50-fold increases, respectively). The disproportionately large increase in TTMb expression upon transfection of modestly increased amounts of DNA most likely reflected saturation of the lysosomal targeting machinery and default localization to the plasma membrane.

**Generalized Saturation of Lysosomal Targeting Mediated by YXXØ Signals**

The apparent saturation of TTMb lysosomal targeting observed above could have been either specific only for the chimeric protein or more generalized for YXXØ-dependent lysosomal targeting. If the latter was true, then overexpression of TTMb should result in the cell surface accumulation of other YXXØ-containing lysosomal proteins. We therefore assayed for the cell surface displacement of endogenous lysosomal proteins upon overexpression of TTMb. Cells transfected transiently with large amounts (10–20 μg) of DNA encoding TTMb or Tac were stained with antibodies to the luminal domains of Tac and of CD63, an endogenous lysosomal protein with a cytoplasmic YXXØ targeting signal (GYEV; 42), and analyzed by flow cytometry.

The results of two-color flow cytometric analyses are shown in Fig. 2. Those cells that were not transfected, i.e., those expressing no Tac or TTMb on the cell surface (lower left and lower right quadrants), expressed very little cell surface CD63, comparable to cells that had not been exposed to DNA (see Fig. 3). In transfected cells, there was a broad range of surface expression of the transgenes, with some cells expressing very low and others expressing very high levels. In the case of Tac (Fig. 2 a), regardless of its level of expression, the level of CD63 surface expression remained the same; even in those cells expressing the most Tac, the pattern of surface CD63 expression was similar to that of the untransfected cells. In contrast, in cells transfected with TTMb (Fig. 2 b), the level of TTMb surface expression correlated with that of endogenous CD63; increased levels of TTMb expression resulted in higher CD63 expression at the cell surface.

To simplify the analyses, the data from Fig. 2 were replotted by gating on surface Tac- or TTMb-expressing cells and analyzing the surface expression of CD63 in one dimension. In those cells expressing Tac at the cell surface, CD63 surface expression was unaltered relative to un-
Targeting mediated by the Mb cytoplasmic tail is saturable. HeLa cells were transfected with 2 μg of pCDM8.1-CD4 and 0.5, 2, or 5 μg of pCDM8.1 plasmids encoding either TTMb (upper panels) or TTM.HSSSA (lower panels). The total amount of DNA was brought to 10 μg by the addition of pCDM8.1 with no insert. At 40 h after transfection, cells were harvested and stained with FITC-conjugated anti-CD4 and PE-conjugated anti-Tac. Fixed ceils were then analyzed by flow cytometry. Shown is the intensity of Tac surface staining (x-axis) measured for cells gated for positive CD4 surface expression. Cell number is plotted on the y-axis. The dotted line represents the highest level of Tac surface staining intensity in cells transfected with CD4 only. The solid curve indicates the experimental sample. The efficiencies of cotransfection, measured as the fraction of CD4+ cells that also expressed the TTMb or TTM.HSSSA construct, were monitored by indirect immunofluorescence microscopy on detergent-permeabilized samples of each culture, and were as follows: TTMb, 0.5 μg, 78%; 2 μg, 95.8%; 5 μg, 94.8%. TTM.HSSSA, 0.5 μg, 68%; 2 μg, 93.6%; 5 μg, 82.2%.

transfected cells (Fig. 3 a). However, in those cells expressing TTMb at the cell surface (Fig. 3 b), there was a significant accumulation of CD63 at the surface relative to untransfected cells, resulting in a 2.5–3.5-fold increase in the median fluorescence intensity. Similar shifts upon overexpression of TTMb, but not of Tac, were observed in the surface expression of two other endogenous lysosomal proteins having YXXO signals, Lamp1 (Fig. 3, c and d), and Lamp2 (Fig. 3, e and f). In contrast, the level of surface expression of major histocompatibility complex (MHC) class I molecules, which normally localize predominantly to the cell surface, was unchanged by overexpression of either Tac or TTMb (data not shown). These results demonstrate that several proteins that use YXXO signals for localization to lysosomes can compete for a common saturable component(s) of the targeting machinery.

**Dependence of Competition on the YXXO Motif**

To determine whether saturation of the lysosomal targeting machinery was indeed dependent on the YXXO motif, we compared the ability of truncation and alanine scanning mutants of TTMb (see Table I) to displace endogenous CD63 or Lamp1 to the cell surface (Fig. 4). Truncation of distal regions of the cytoplasmic tail, as in the TTM.GSTYA chimeric protein, did not affect the ability to displace either CD63 (Fig. 4 b) or Lamp1 (not shown). However, no cell surface displacement of either CD63 or Lamp1 was observed upon overexpression of chimeras in which the YTPL motif was removed (TTM.HSSSA; Fig. 4 c) or in which the tyrosine (TTM.GSTYA.Y248A; Fig. 4 d) or leucine (TTM.GSTYA.L251A; Fig. 4 e) residues were replaced by alanine. These mutations have also been shown to abolish lysosomal targeting at low levels of expression (31, 36). Thus, the ability of the Mb cytoplasmic tail to effect displacement of lysosomal proteins to the cell surface correlated with its ability to mediate localization to lysosomes and was dependent on an intact YTPL signal.

**Competition by YXXO Motifs that Direct Targeting to Distinct Compartments**

Competition among lysosomal proteins for a common sat-
meric proteins consisting of Tac appended to a variety of protein (Fig. 5 b), TfR, a constitutively recycling tails and/or YXXO targeting signals from Lampl, a lysosomal cytoplasmic tails with YXXO targeting signals (see Table I for the sequences of the cytoplasmic tails and YXXO motifs). As shown in Fig. 5, CD63 was displaced to the cell surface by chimeric proteins containing either the Mb cytoplasmic tail or the internalization signal from the TfR. Overexpression of the signal-deficient TTMb.HSSS chimera had no effect on surface TfR levels. Thus, transmembrane proteins that use YXXO motifs for localization to several post-Golgi compartments are susceptible to competition for a common saturable component.

Factors Compete for the Sorting Machinery

To rule out that lysosomal proteins were more sensitive to displacement than proteins destined for other compartments, we tested the ability of the chimeric proteins to displace endogenous TfR to the cell surface. As shown in Fig. 5 e, surface TfR levels were doubled, relative to those in untransfected cells, upon overexpression of Tac chimeric proteins containing either the Mb cytoplasmic tail or the internalization signal from the TfR. Overexpression of the saturable component.

Only YXXO Sequences that Function as Targeting Signals Compete for the Sorting Machinery

If displacement of lysosomal or endosomal proteins to the cell surface was truly due to saturation of the YXXO-dependent sorting machinery, then one would predict that overexpression of structurally similar YXXO sequences that fail to act as sorting signals would also fail to compete for the saturable components. To test this prediction, we analyzed the endocytic signaling capacity of immunoreceptor tyrosine activation motifs (ITAMs). ITAMs consist of two discontinuous tetrapeptides that conform to the YXXO motif and serve as docking sites for SH2 domains upon phosphorylation of the tyrosine residues (80). The cytoplasmic domain of the TCRζ chain, an important signaling component of the T cell receptor complex, contains three ITAMs, and hence six copies of the YXXO consensus motif (76). To address whether these ITAMs can also serve as endocytic signals, we first analyzed the ability of the cytoplasmic domain of TCRζ to mediate internalization.

Jurkat, a human T-leukemic cell line, was transfected with plasmids encoding either Tac or the chimeric proteins TTγ, consisting of the cytoplasmic domain of the TCRζ chain appended to the luminal and transmembrane domains of Tac (27), or TTγALL7, a chimera containing a tyrosine-based internalization signal from CD3-γ (28; see Table I). Cells stably expressing similar levels of each protein were assayed for their ability to mediate uptake of anti-Tac antibody by flow cytometry. As shown in Fig. 6 a, TTγALL7 mediated efficient internalization of anti-Tac antibody, with ~65–80% internalized by 30 min. In contrast, the uptake of anti-Tac by TTγ was much slower and comparable to that of the negative control Tac. Thus, despite having six consensus YXXO motifs, the cytoplasmic tail of the TCRζ chain is innately incapable of directing internalization. This indicates that the mere presence of a YXXO motif is insufficient to mediate post-Golgi targeting and internalization.

We next tested whether overexpression of the TCRζ cytoplasmic tail could saturate the YXXO-dependent target-
Endogenous CD63 (Fig. 6 c) and Lampl (not shown) failed to accumulate significantly over background at the cell surface upon overexpression of TTMb, even at levels of biosynthetic expression at which TTMb induced significant CD63 accumulation (Fig. 6 b). Taken together, these data suggest that the YXXΦ motif must possess internalization or targeting activity to compete for the saturating component(s) of the targeting machinery.

Overexpression Results in Decreased Rates of Internalization and Lysosomal Targeting

A cotransfection assay was developed to examine whether overexpression-induced cell surface displacement was due to inhibition of endocytosis and/or lysosomal targeting. For this purpose, we made use of the observation documented in Fig. 1 that transfection of low levels of DNA encoding TTMb results in nonsaturating protein expression levels. HeLa cells were cotransfected with low amounts (0.5 μg) of TTMb DNA and saturating amounts (10–20 μg) of either empty expression vector (pCDM8.1), to monitor internalization without overexpressed proteins, or saturating levels of Lamp1, TfR, or CD4. The results (Fig. 8) show that the rate of internalization of TTMb was consistently lower (by ~50%) in cells overexpressing Lamp1 or TfR relative to cells overexpressing CD4 or cotransfected with empty expression vector. Note that internalization of TTMb was never reduced to the level observed for the negative control TTM.HSSSΔ construct, which lacks an internalization signal (Fig. 8, triangles). These results suggest that overexpression of YXXΦ-containing proteins indeed resulted in competition for sorting at the plasma membrane.

We next sought to address whether overexpression also blocked the bulk of protein sorting by analyzing the rate of lysosomal delivery of TTMb. We made use of the observation that TTMb is rapidly degraded upon delivery to lysosomes (36); thus, if TTMb sorting to lysosomes was inhibited, its degradation should be delayed. Pulse–chase analyses (Fig. 9 a) showed that overexpression of Lamp1,
Figure 5. Cell surface accumulation of CD63 and TIR by overexpression of distinct YXXO signals. (a–d) HeLa cells were transiently transfected with 20 μg of pCDM8.1 plasmids encoding Tac (a–d), TTMb (a), Tac-Lampl (b), Tac-LSYTRF (c), or TT-TGN38 (d), then stained with FITC anti-CD63 and PE anti-Tac, fixed, and analyzed by flow cytometry as described above. Intensity of CD63 staining (x-axis) was measured for cells gated for positive surface expression of Tac. The thick, unfilled curve represents staining of cells transfected with Tac, and the filled curve represents staining of cells transfected with the chimeric constructs. Comparable levels of protein expression and transfection efficiency were monitored as before. (e) Cells transiently transfected with 20 μg of plasmids encoding either Tac (a–d) or TTMb (a) were stained with FITC anti-Tac and biotinylated transferrin, followed by rhodamine-streptavidin. Samples were analyzed by flow cytometry, and the mean fluorescence intensity of rhodamine labeling was measured for triplicate samples of cells gated for positive Tac surface expression.

but not of CD4, caused a decrease in the overall degradation rate for TTMb. The decrease in degradation was likely a consequence of decreased TTMb targeting rather than an indirect effect on lysosomal function, since overexpression of Lamp1 did not affect the degradation rate of the LL-containing protein, Tac-DKQTLL (Fig. 9 b; see below). These results show that lysosomal targeting was generally inhibited by competition among YXXO targeting signals, regardless of whether recognition of the signals occurred at the plasma membrane or the TGN.

Targeting via Di-leucine Motifs Uses a Distinct Saturable Factor

A second type of signal, the LL motif, is also responsible for targeting proteins to lysosomal or endosomal compartments. It was therefore of interest to determine whether targeting mediated by LL signals used the same saturable component of the sorting machinery as that mediated by YXXO motifs. HeLa cells were transiently transfected with saturating levels of either TTMb or a chimeric protein in which Tac was fused to the LL signal from the CD3γ chain, DKQTLL (28). The displacement of CD63 was assessed by flow cytometry. While overexpression of TTMb induced the characteristic increase in CD63 surface expression (Fig. 10 a), overexpression of Tac-DKQTLL was much less effective (Fig. 10 b). Surface levels of endogenous TIR were also not significantly affected by overexpression of Tac-DKQTLL (Fig. 10 c). Lastly, whereas overexpression of Lamp1 reduced the rate of lysosomal delivery and degradation of TTMb, it had no effect on the degradation of Tac-DKQTLL (Fig. 9 b). In contrast, overexpression of CD4, which has a weak LL signal in its cytoplasmic domain (1), slightly reduced the rate of lysosomal delivery of Tac-DKQTLL but not TTMb (Fig. 9, a and b). These results demonstrate that cytoplasmically exposed LL motifs are unable to compete with YXXO motifs for sorting to endosomal or lysosomal compartments. Thus, targeting of proteins via LL motifs does not seem to use the same saturable component as that used in targeting of proteins via YXXO motifs.
Figure 7. Effects of overexpression of various proteins on the surface expression of TTMb. HeLa cells were transiently transfected with 0.5 μg of pCDM8.1-TTMb and either 10 μg of pCDM8.1 (a) or 2 μg of pCDM8.1-CD4 and 10 μg of pCDM8 or pCDM8.1 plasmids encoding CD63 (b), Lamp-1 (c), or TIR (d), or 12 μg of pCDM8.1-CD4 (e). Cells were stained with FITC anti-CD4 and PE anti-Tac and analyzed by flow cytometry. The PE staining intensity was plotted for cells gated for positive surface expression of CD4, except for a, in which the intensity was plotted for ungated cells (filled curve). The thick, unfilled curve represents the fluorescence intensity of unstained cells. Cotransfection efficiencies of CD4 and TTMb were comparable among all samples as judged by indirect immunofluorescence microscopy.

could have resulted if the LL-dependent sorting machinery was not itself saturable. To address the saturability of LL-mediated localization, we measured the overexpression-induced cell surface displacement of the MHC class II–associated invariant chain. Invariant chain has two well-characterized LL-like motifs present in its NH2-terminal cytoplasmic tail (4, 46, 51); we used a mutated form of the human invariant chain, Ip33, that is relatively efficiently transported out of the ER (2). HeLa cells were transfected with low levels of Ip33 and saturating levels of chimeric proteins consisting of Tac fused to various targeting signals. As shown in Fig. 10, d–g, overexpression of TTMb, TTLamp1, or Tac-LSYTRF, all of which contain YXXΦ motifs, had very little effect on the levels of surface expression of Ip33; parallel staining showed the expected displacement of CD63 to the cell surface in these cells (data not shown). In contrast, overexpression of Tac-DKQTLL resulted in marked displacement of Ip33 (Fig. 10g) despite having no effect on CD63 (not shown). These data show clearly that Tac-DKQTLL and Ip33 share a common saturable component of their targeting machinery. Furthermore, they confirm the inability of LL and YXXΦ motifs to compete for common saturable components.

Discussion

Saturation of the endocytosis or sorting of particular internalized surface receptors and proteins localized to post-Golgi compartments has been previously described (3, 6, 16, 17, 20, 36, 39, 74, 78), but the signals serving as the target of the saturable component(s) and the extent of saturability to a broad range of receptors were not addressed. In this manuscript, we have shown that the YXXΦ-signal-dependent mechanisms by which proteins are targeted to lysosomes, endosomes, or the TGN are saturable. Furthermore, we have extended this analysis to show that LL signal–dependent localization is also saturable. We have used this analysis to show that LL signal–dependent localization is also saturable. Furthermore, we have shown that a saturable component(s) of the targeting machinery is shared by proteins using similar types of signals to localize to multiple distinct compartments.

We have not yet identified the saturable component(s) involved in YXXΦ–mediated protein localization in vivo. However, among the potential candidates are two that...
components, what then, determines localization to different compartments? The answer to this question may be complex. First, there may be multiple receptors that bind with weak avidity to all YXXQ motifs, but with higher avidity to particular motifs or to ones in particular conformations or contexts. Overexpression of any single motif might thus overwhelm the system such that all of the receptors are saturated. For example, both AP-2 and AP-1 bind to multiple YXXQ motifs, but they may bind with differing affinity to lysosomal vs. endosomal signals (47), and additional related coat proteins may bind to TGN signals. Second, the saturable component highlighted by our study may not be the receptor(s), but rather a regulator or accessory protein common to all of the receptors. An example might be the small GTP binding protein, ARF1, which has been shown to be necessary for the recruitment of AP-1 complexes to the membrane (55, 68, 72, 79). The involvement of a saturable regulatory component would still allow differential recognition of the motifs by distinct receptors that may not be limiting. Third, YXXQ motifs could mediate internalization or localization to a common intermediate compartment, such as the sorting endosome, from which other signals then implement localization to distinct end compartments (38, 69, 77). In this instance, overexpression would saturate the first step of a two-step process of localization. One potential example of such a secondary targeting signal may be a cysteine-containing sequence shown to be responsible for diverting mannose 6-phosphate receptors from a “default” lysosomal targeting pathway (58, 64). Additionally, whereas most of the described lysosomal proteins contain short cytoplasmic tails in which the YXXQ motifs constitute a large fraction of the total peptide length, proteins targeted to other post-Golgi compartments generally contain substantially larger cytoplasmic domains (73). Careful dissection of these cytoplasmic domains may reveal novel targeting determinants.

We have shown here that overexpression of YXXQ signal-containing proteins can result in surface accumulation at least partially by decreasing the rate of internalization. Furthermore, the decrease in internalization of TTMb paralleled the decrease in the rate of bulk delivery to lysosomes. The data are consistent with the notion that some lysosomally targeted proteins cycle through the cell surface before lysosomal delivery. This pathway for lysosomal delivery has been proposed for a number of lysosomal proteins (7, 37, 43), in addition to the LL-dependent in-
Saturable Targeting by Tyrosine, Di-leucine Signals

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Table II. Relationship of Surface Accumulation and the Inhibition of the Rate of Internalization of TTMb

| Condition          | TTMb surface expression | TTMb rate of internalization | Lampl rate of biosynthesis |
|--------------------|-------------------------|-------------------------------|---------------------------|
| Control            | 9.25, 27                | 51.3 ± 3.5                    | 1                         |
| Lampl overexpression | 47, 129, 277           | 25.8 ± 4.9                    | 6-15                      |

*Expressed as arbitrary units of mean fluorescence intensity in three separate experiments.

†Expressed as the percentage of internalized TTMb after 20 min at 37°C relative to the amount at the surface at time 0.

‡Expressed as arbitrary units, calculated from phosphorimaging scans of anti-Lampl immunoprecipitates from metabolically pulse-labeled transfected cells, divided by the fraction of cells expressing transgenes monitored by indirect intracellular immunofluorescence microscopy.

variant chain (57). However, it is also possible, indeed likely, that a similar saturation-induced block occurs at the level of sorting from the TGN (5, 17, 49) or even from endosomes (38). Such a block would not necessarily result in accumulation at the TGN; it may rather permit default delivery to the plasma membrane. The observed accumulation at the cell surface could then be due to the added load of targeting signals localizing to the plasma membrane, exacerbated by the subsequent block in internalization. Since we cannot assess from our data the relative amount of protein trafficking through the cell surface before lysosomal delivery, our observations do not predicate for or against either pathway of lysosomal targeting.

Our data regarding the surface accumulation and rate of internalization for TTMb upon overexpression of Lampl bear further discussion on this point. From metabolic labeling and immunoprecipitation analyses, we calculate that similar levels of TTMb are expressed in cells cotransfected with either empty expression vector or the Lampl expression construct, and that Lampl overexpression ranges from ~6-15-fold relative to untransfected cells (data not shown; see Table II). We have shown here that under these conditions, the rate of TTMb internalization is reduced by approximately twofold (see Fig. 8 and Table II), and steady state surface levels of TTMb are increased by 5-10-fold, as quantitated by the mean fluorescence intensity in flow cytometric analyses (see Fig. 7 and Table II). At steady state, the amount of intracellular TTMb, \([I]\), can be related to the amount of surface expressed TTMb, \([E]\), by the equation

\[
\frac{[I]}{[E]} = \frac{k_i}{k_e}
\]

in which \(k_i\) and \(k_e\) are the rate constants for internalization and externalization, respectively (12, 41). However, since the redistribution of TTMb to the cell surface is due to competition mediated by the YXXO motif, the value \(k_e\) must reflect the bulk rate constant of externalization for all proteins containing such motifs. Thus, the twofold decrease in the rate of internalization of TTMb can alone account for the 5-10-fold increase in cell surface expression only if up to fivefold more YXXO motifs are externalized when Lampl is expressed up to 15-fold over control levels. Considering that Lampl is one of the major lysosomal proteins synthesized in most cells (15), it is plausible that the increase in Lampl synthesis may account for the fivefold increase in total YXXO delivery to the surface with-
out invoking a saturable transport step at the TGN. However, it is perhaps more likely that this increased rate of YXXO externalization is partially the result of diversion of a higher fraction of YXXO-containing proteins to the cell surface due to saturation of a targeting event at the TGN.

We have shown that, as for YXXO motifs, common saturable components are shared among LL motif targeting signals, from the invariant chain and CD3γ, that mediate delivery to late endosomal/lysosomal compartments. This is the first demonstration that different LL signals can use similar targeting mechanisms and that targeting by these mechanisms is saturable. The results also demonstrate the utility of the flow cytometric surface displacement assay in defining and grouping signals into functional categories. For example, the targeting signals on the invariant chain are rather divergent from other LL motifs (see Table I) and may have been categorized separately; our data show that they are recognized in a similar manner to other LL motifs. Similarly, it will be of interest to determine whether NPXY-type tyrosine-based signals (9) or the acidic cluster/casein kinase II site signals described in furin and the mannose-6-phosphate receptors (40, 63, 70, 75) compete for the YXXO or LL sorting machinery; preliminary data suggest that the acidic cluster/casein kinase II site in furin does not use the same saturable component as that used by the YXXO signals. The in vivo competition assay could also be used to identify unknown YXXO or LL targeting motifs in transmembrane proteins and to identify new categories of signals. Furthermore, the assay could be used to address the functionality of individual motifs within large cytoplasmic domains that contain multiple potential signals, such as the receptors for epidermal growth factor or mannose 6-phosphate.

Probably the most intriguing finding in this study was that targeting through LL and YXXO motifs use distinct saturable components. Minimally, these data suggest that there are distinct binding sites for each motif. Interestingly, while many YXXO targeting motifs tested were shown to bind to either or both of the μ1 or μ2 chains of the AP-1 and AP-2 complexes, respectively, LL motifs failed to bind to either chain in a yeast two-hybrid assay system (47). Our data would still be consistent with binding of LL motifs to a distinct site elsewhere on the AP-1 and AP-2 complexes; indeed, recent evidence has been obtained for binding of a LL-like motif to AP-1 and AP-2 in vitro (18).

Our data could also be explained if LL motifs bind in vivo to coat proteins or receptors distinct from the AP-1 or AP-2 complex. In fact, internalization of the epidermal growth factor receptor has been shown to occur via an AP-2-independent mechanism, despite its ability to bind to AP-2 (45). It is tempting to speculate that there may be different internalization pathways specific for LL or for YXXO motifs, perhaps using distinct trafficking routes to arrive at similar end compartments. The existence of more than one pathway of internalization has recently been demonstrated both in HeLa cells and in dendritic cells (10, 61), and a distinction between clathrin-mediated endocytosis and potocytosis, mediated through caveolae, has been documented (33). This might explain why at least two distinct types of targeting signal have evolved; it may be important, under certain circumstances, to cycle cellular components through distinct vesicular intermediates. Multiple targeting pathways may be particularly relevant to the process of MHC class II-restricted antigen presentation. Whereas class II molecules are targeted to a late endosomal/lysosomal antigen processing compartment by association with the LL-containing invariant chain (4, 26, 32), HLA-DM can be targeted to the same compartment by virtue of the YXXO motif on the β chain (31, 36). The use of distinct pathways by each complex may be critical to segregate MHC class II molecules from HLA-DM before arrival at the antigen processing compartments to prevent nonproductive interactions until their interaction is beneficial. Perhaps the two components use divergent pathways to parallel the pathways taken by internalized antigens. Identification of components of the sorting machinery operative on both motifs will be critical to address these issues.

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