

A Lysosomal Targeting Signal in the Cytoplasmic Tail of the β Chain Directs HLA-DM to MHC Class II Compartments

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Abstract. In human B cells, class II molecules of the major histocompatibility complex (MHC-II) accumulate in an endosomal/lysosomal compartment, the MIIC, in which they may encounter and bind peptides. An additional molecule required for MHC-II peptide binding, HLA-DM (DM), has also been localized to the MIIC. Neither the relationship of the MIIC to the endosomal system nor the mechanisms by which DM localizes to the MIIC are understood. To address these issues, DM localization was analyzed in cells that do or do not express MHC-II. DM $\alpha\beta$ heterodimers were localized in transfected MHC-II-negative HeLa and NRK cells, in the absence of the MHC-II-associated invariant chain, to a prelysosomal/lysosomal compartment by immunofluorescence microscopy. To identify a potential targeting determinant, we analyzed the localization of a chimeric protein, T-T-Mb, in which the cytoplasmic tail of murine DM β (Mb) was appended to the luminal and transmembrane domains of a cell surface protein, Tac. Like intact DM, T-T-Mb was local-

ized to a lysosomal compartment in HeLa and NRK cells, as judged by immunofluorescence and immunoelectron microscopy. T-T-Mb was rapidly degraded in this compartment by a process that was blocked by inhibitors of lysosomal proteolysis. The DM β cytoplasmic tail also mediated internalization of anti-Tac antibody from the cell surface and delivery to lysosomes. Deletion from the DM β cytoplasmic tail of the tyrosine-based motif, YTPL, resulted in cell surface expression of T-T-Mb and a loss of both degradation and internalization; alanine scanning mutagenesis showed that the Y and L residues were critical for these functions. Similarly, mutation of the same Y residue within full-length DM β resulted in cell surface expression of DM $\alpha\beta$ heterodimers. Lastly, T-T-Mb was localized by immunoelectron microscopy to the MIIC in a human B lymphoblastoid cell line. Our results suggest that a motif, YTPL, in the cytoplasmic tail of the β chain of DM is sufficient for targeting either to lysosomes or to the MIIC.

ANTIGEN receptors on the surface of T lymphocytes recognize a complex formed between proteins of the major histocompatibility complex (MHC)¹ and peptides derived by intracellular proteolysis of foreign or endogenous proteins. MHC class II antigens (MHC-II) are heterodimeric cell surface glycoproteins that present, to CD4⁺ T cells, peptides generated predominantly from proteins encountered within the endosomal system. The pathway by which peptides are delivered and become

bound to MHC-II has been the subject of intensive investigation (22, 26).

MHC-II α and β chains assemble in the endoplasmic reticulum (ER) with a third polypeptide known as the invariant (I) chain (18, 39, 42, 51). I chain functions early by promoting $\alpha\beta$ assembly and ER egress (2, 6, 10, 45, 53, 85) and by blocking the peptide binding groove on assembled MHC-II molecules (69, 70, 81). Importantly, I chain also functions by directing MHC-II to the endosomal/lysosomal system by virtue of a targeting signal in its cytoplasmic tail (4, 44, 50, 61). Within endosomal/lysosomal compartments, I chain is proteolytically degraded (7, 59). Removal of an I chain-derived peptide, CLIP (class II-associated invariant chain peptide), from the MHC-II peptide binding cleft allows access of other peptide ligands to the binding site (3, 68, 72), including those derived from partially degraded and/or denatured internalized proteins. Ultimately, complexes consisting of MHC-II $\alpha\beta$ dimers bound to peptides are delivered to the cell surface.

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1. *Abbreviations used in this paper:* CLIP, class II-associated invariant chain peptide; I, invariant; LRSC, lissamine rhodamine sulfonyl chloride; MHC, major histocompatibility complex; MHC-II, major histocompatibility complex class II antigens; MIIC, MHC class II compartment; T-T-Mb, Tac luminal-Tac transmembrane-Mb cytoplasmic tail.

A great deal of attention has been given to the nature of the endosomal compartment(s) in which these antigen processing events occur. Immunoelectron microscopy of human B lymphoblastoid cell lines first demonstrated the existence of an endosomal/lysosomal compartment in which MHC-II accumulated (63). This compartment, dubbed MIIC (for MHC class II compartment), consists of electron-dense structures, characterized by either a multivesicular morphology or a multilaminar appearance with extensive membrane invaginations (63, 64). The MIIC is accessible to internalized proteins, such as soluble horseradish peroxidase, yet it contains only traces of recycling endocytic markers, such as the receptors for transferrin or mannose-6-phosphate (63, 64). In contrast, this compartment is enriched in lysosomal membrane proteins, such as CD63 and lamp-1, and in lysosomal hydrolases, such as cathepsin D and acid phosphatase (63). Additional studies have also implicated organelles with characteristics of lysosomes in the generation of MHC-II/peptide complexes (15, 29, 30, 60, 74), while other studies have implied that formation of such complexes and accumulation of MHC-II occur in earlier endocytic organelles (1, 83, 86) or in a series of sequential endosomal/lysosomal compartments (15, 64, 66). To understand the mechanisms by which peptides become associated with MHC-II, it will be essential to understand the position of the MIIC within the endosomal/lysosomal system and how components of the MHC-II antigen processing machinery are targeted to this compartment.

The recent identification of a specific component of the antigen-processing machinery, HLA-DM (referred to here as DM), offers the opportunity to better understand the nature of the MIIC. DM is expressed in cells that also express MHC-II and I chain, and consists of two type I transmembrane glycoprotein chains, α and β , that are homologous to both MHC class I and class II molecules (16, 17, 41). Defects in either or both of the genes that encode the DM α or β chains were shown to underlie the deficiency in MHC-II restricted antigen processing functions of mutant B lymphoblastoid and T/B hybrid cell lines (23, 24, 56), and DM expression reconstituted formation of MHC-II/peptide complexes in transfected HeLa cells expressing MHC-II and I chain (40). These data suggested that DM plays an essential supporting role in antigen processing by other MHC-II antigens. Indeed, it has recently been shown that DM facilitates the release of I chain derived CLIP and the subsequent binding of other peptides to MHC-II (22a, 79a, 79b). A better understanding of the localization, trafficking, and biochemical interactions of DM will provide insight into the mechanism and subcellular localization of the ultimate steps of DM-dependent MHC-II/peptide loading.

DM has been localized in human B lymphoblastoid cells and dendritic cells at steady state predominantly to the MIIC by immunoelectron microscopy (60, 77). Unlike MHC-II, DM does not appear to associate stoichiometrically with I chain (23); thus it must localize to the MIIC by virtue of a targeting signal other than that in the I chain cytoplasmic tail. We have analyzed the targeting properties and steady state localization of DM in non-MHC-II-expressing cells, and addressed how these properties relate to localization in MHC-II-expressing "professional" antigen presenting cells. We show that intact DM dimers are tar-

geted to lysosomes in a variety of nonlymphoid MHC-II-negative cells. Furthermore, by using chimeric proteins, we show that the cytoplasmic tail of the murine DM β chain is sufficient for targeting a heterologous protein both to lysosomes in nonlymphoid cells and to the MIIC in B lymphoblastoid cells. The tyrosine-based motif, YTPL, present in the DM β cytoplasmic tail, is an essential part of the lysosomal targeting determinant, and also mediates internalization from the cell surface. This determinant is unusual among tyrosine-based lysosomal targeting signals in that it is neither preceded by a glycine nor is it present at the COOH terminus. Our findings suggest that protein localization to the MIIC and to lysosomes may be mediated by similar cytoplasmic targeting events.

Materials and Methods

Cell Lines and Stable Transfections

NRK and HeLa cells were maintained as monolayers in DMEM supplemented with 7% (vol/vol) FBS, 50 μ g/ml gentamicin, 100 U/ml penicillin, and 100 μ g/ml streptomycin (complete medium). The B-lymphoblastoid cell line 721.45, generously provided by Dr. R. DeMars (University of Wisconsin, Madison, WI), was maintained in RPMI 1640 medium supplemented with 15% fetal bovine serum and the antibiotics mentioned above. Stably transfected clones of NRK and HeLa cells expressing selected transgenes were generated in order to (a) avoid overexpression in transiently transfected HeLa cells of proteins containing targeting signals, which resulted in accumulation at the cell surface and reduced rates of degradation and internalization, and (b) increase the fraction of NRK cells expressing the transgene of interest, because transfection efficiency was too low to analyze biochemically. To generate stable transfectants, cells were plated sparsely in 10-cm dishes containing 10 ml of complete medium. 10–20 μ g of the plasmid of interest (in some cases linearized) was combined with 1 μ g of pSV2Neo (80) or RSVNeo (27), expressing the Tn5 aminoglycoside 3' phosphotransferase II gene conferring resistance to G418, and used to transfect cells by the calcium phosphate precipitation method as described in reference 76. Selection with G418 (Geneticin, Gibco/BRL, Gaithersburg, MD; 1 mg/ml for HeLa, 500 μ g/ml for NRK) was initiated 2 d after transfection. G418-resistant colonies were expanded and assayed for expression by immunofluorescence microscopy in the absence and/or presence of 1 mg/ml leupeptin. Positive lines were maintained in medium containing G418 (500 μ g/ml for HeLa; 250 μ g/ml for NRK). For generation of stably transfected 721.45 cells, 25 μ g of the plasmid of interest was combined with 1 μ g of RSV.5 (hygro) (49) and used to transfect 10^7 cells by electroporation, using a Gene Pulser set at 960 μ F and 240 V (Biorad Labs., Hercules, CA). Cells were plated at 2,000–10,000 viable cells/well in 96-well plates, and selection with hygromycin (300 U/ml) was initiated 2 d after transfection. Hygromycin-resistant clones were assayed for expression by immunofluorescence microscopy on poly-L-lysine-coated coverslips as described (52) using cells treated or untreated with 50 mM NH₄Cl. Stable lines were maintained in medium containing 200 U/ml hygromycin.

Plasmids

All constructs were prepared in pCDM8.1 (8), a modification of pCDM8 (79), except DM α and DM β constructs. DM α - and DM β -encoding cDNAs were the gift of J. Trowsdale (Imperial Cancer Research Fund, London, UK), and were cloned into the XhoI site of pCDM8 by standard techniques (76). The plasmids pCDM8-Tac (75) and pCDM8-T-T-G (containing Tac luminal and transmembrane domains appended to the TGN38 cytoplasmic tail; reference 35) have been previously described. T-T-Mb and deletion constructs were prepared by the two-step polymerase chain reaction (PCR) procedure (32). Briefly, H-2Mb cytoplasmic tail sequences were amplified from RNA prepared from murine spleen (the generous gift of Dr. L. King; NCI, Bethesda, MD) by reverse transcription followed by PCR (Perkin Elmer, Norwalk, CT) using a back primer complementary to the end of the coding sequence and a primer incorporating sequences in both the beginning of the coding sequence for the Mb cytoplasmic tail and the end of the Tac transmembrane region

(forward primer). After amplification of the Tac transmembrane region from T-T-G using a back primer complementary to the previous forward primer, the PCR products were mixed and reamplified to generate Tac transmembrane/Mb cytoplasmic coding sequences with appropriate BglIII/XbaI restriction sites for replacing the corresponding region in the T-T-G plasmid by standard techniques. For deletion constructs, reverse strand back primers were made to introduce stop codons after the codons for the desired COOH-terminal amino acid. Alanine scanning mutants were generated by double PCR using the parent TTM.GSTYΔ as template and primers introducing point mutants encoding alanine replacements at the appropriate amino acid. The DMB.Y248A mutant was prepared in a similar fashion using the parent DMβ cDNA construct. pCDM8-derived plasmids containing HLA-DRA, HLA-DRB1*0101 (48), and human I chain p33 (70) cDNAs have been described.

Antibodies

The anti-Tac monoclonal antibody 7G7.B6 (American Type, Culture Collection, Rockville, MD), anti-Tac polyclonal rabbit serum R3134 (gift of Dr. W. Leonard, NHLBI, Bethesda, MD), "anti-Tac" monoclonal antibody (84), anti-DRβ monoclonal antibody XD5.A11 (reference 67; gift of Dr. E. Long, NIAID, Rockville, MD), anti-human I chain antiserum Matilda (52), anti-DRα chain antiserum (reference 58; gift of Dr. H. Ploegh, Massachusetts Institute of Technology, Cambridge, MA) anti-lamp-1 polyclonal rabbit serum (reference 14; gift of Dr. M. Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA), anti-lgp120 monoclonal antibody Ly1C6 (reference 47; gift of Dr. I. Mellman, Yale University, New Haven, CT), anti-human transferrin receptor monoclonal antibody B3/25 (Boehringer Mannheim, Indianapolis, IN), anti-cation-independent mannose-6-phosphate receptor polyclonal rabbit antiserum (gift of Dr. B. Hoflack, EMBL, Heidelberg, Germany), anti-TGN38 polyclonal rabbit antiserum JH4 (12), and anti-human CD63 monoclonal antibody (AMAC Inc., Westbrook, ME) have been described. Anti-human DM antiserum was a gift of Drs. H. Zweerink (Merck, Sharp & Dohme, Rahway, NJ) and P. Cresswell (Yale University, New Haven, CT). An antibody recognizing the cytoplasmic tail of the HLA-DMβ chain was generated by immunization of a rabbit with a multiple antigenic peptide (MAP) corresponding in sequence to amino acids 228 to 241 of DMβ, as described (41). The resulting antiserum was affinity purified by adsorption to the MAP-DMβ peptide immobilized on Affigel 15 agarose (Biorad), extensive washing with 10 mM Tris, pH 7.5, followed by a brief wash in the same buffer containing 0.5 M NaCl, elution with 0.1 M glycine pH 2.8, neutralization with 0.1 M Tris, pH 8, and dialysis against phosphate buffered saline (PBS). The affinity purified antiserum (5 μg/ml) reacted specifically with DMβ chains, as judged by immunofluorescent staining of transiently transfected HeLa and NRK cells. Staining was competed by addition of the immunizing peptide, and no staining was observed in untransfected cells. The anti-DMβ serum had no reactivity towards the murine H-2Mb chain, as judged by immunofluorescence microscopy on transient and stable T-T-Mb transfectants. Fluorescein isothiocyanate-(FITC) and lissamine rhodamine sulfonil chloride-(LRSC) conjugated antibodies specific for mouse or rabbit immunoglobulins (Ig), used as secondary antibodies in immunofluorescence experiments, were obtained from Jackson ImmunoResearch (West Grove, PA). Phycoerythrin-conjugated anti-Tac (anti-CD25) antibody was obtained from AMAC.

Immunofluorescence Microscopy

Cells were plated sparsely on sterile coverslips in 6-well dishes. For transient transfections, cells were transfected the next day using the calcium phosphate precipitation method (76) and 3–5 μg of plasmid DNA, except where indicated. For cotransfection of DRα, DRβ, I chain, and T-T-Mb expressing cDNAs, 1 μg each of DRα and DRβ, 3 μg of T-T-Mb, and 5 μg of I chain plasmids were used. Medium was replaced the following day. Cells were subjected to fixation with 2% formaldehyde in PBS generally 36–42 h after transfection. For stably transfected cells, cells grown on coverslips were similarly fixed when they reached 50–70% confluence. In some cases, cells were pretreated for 4 h with medium containing 1 mg/ml leupeptin before fixation. For immunofluorescent internalization assays, cells were incubated in sterile 7G7.B6 supernatant supplemented with leupeptin for 4 h before fixation. Coverslips containing fixed cells were processed for indirect immunofluorescence microscopy as described (35), using various primary antibodies and fluorochrome-conjugated secondary antibodies. Cells were analyzed on a Zeiss photomicroscope using a 63× Planapo lens, and photography was with Kodak Tri-X-pan ASA 400 film.

Cryosectioning and Immunoelectron Microscopy

Stable transfectants that had been treated for 4 h with 1 mg/ml leupeptin (HeLa and NRK cells) or a combination of 1 mg/ml leupeptin, 0.1 mg/ml *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), and 0.1 mg/ml pepstatin A (721.45 cells) were fixed by adding an equal volume of fixative (4% [wt/vol] paraformaldehyde/0.4% [wt/vol] glutaraldehyde/0.1 M sodium phosphate, pH 7.4) to cells in tissue culture medium and incubating for 2 h at room temperature. Cells were rinsed in PBS containing 0.15 M glycine and embedded in 10% (wt/vol) gelatin. Gelatin blocks were infused with 2.3 M sucrose for 16 h at 4°C, and then frozen in liquid nitrogen. Thin cryosections (50 nm) were made with a cryo-ultramicrotome (Reichert Ultracut S) and Drukker Knives. For single labeling, sections were sequentially incubated at room temperature with polyclonal antibody to Tac and protein A conjugated to 10-nm gold particles. For double labeling, sections were stained first with antibody to Tac and protein A conjugated gold, followed by the indicated second antibody and protein A conjugated gold of a different size, as described (63). For quantitation, sections from at least 20 cells were analyzed for each cell type.

Flow Cytometry

For assaying cell surface expression of DM heterodimers containing either intact DMβ or DMβ with a cytoplasmic tail mutation, HeLa cells were transiently transfected with 2 μg each of pCDM8 plasmids encoding DMβ or DMB.Y248A, DMα, and Tac as a control, along with 4 μg of pCDM8.1 vector without an insert. After 36–40 h, a sample of cells was analyzed by immunofluorescence microscopy to determine transfection efficiency, and the remaining cells were released from culture dishes with 10 mM EDTA/PBS, counted, and washed. A fraction of the cells was pulse-labeled for quantitation of expressed DMαβ heterodimers by immunoprecipitation with anti-DM antiserum, SDS-PAGE analysis, and PhosphorImager analysis (see below). The remaining cells were bound to phycoerythrin-conjugated anti-Tac monoclonal antibody (AMAC, Westbrook, ME) and rabbit anti-DM antiserum followed by FITC-goat anti-rabbit Ig, and surface fluorescence was quantitated by flow cytometry on a FACScan (Becton Dickinson) using the CyQuest software. The analysis shown was gated for cells expressing moderate surface levels of Tac; immunofluorescence analysis showed that 95% of these cells coexpressed DM heterodimers.

Metabolic Labeling

For metabolic labeling of stable transformants of HeLa or NRK cells, $0.5-1 \times 10^7$ cells were trypsinized from dishes, and then incubated in suspension in methionine/cysteine-free DMEM containing 3–5% dialyzed fetal bovine serum for 30 min at 37°C. Cells were pelleted and pulse labeled by resuspension in the same medium containing 2 mCi/ml Tran³⁵S-label (ICN, Costa Mesa, CA) or EXPRE³⁵SS (New England Nuclear, Boston, MA) for 20 min at 37°C. Cells were pelleted again, resuspended in DMEM supplemented with 15× excess methionine and cysteine and 10% FBS, and chased for various periods of time. Addition of lysosomal inhibitors, as indicated in the text, was at the start of the chase period. Aliquots of cells and supernatants were collected at each time point. Cells were washed once in ice cold PBS, and cell pellets and supernatants were frozen on dry ice and stored at –70°C.

Immunoprecipitation

Metabolically labeled cell pellets were thawed and resuspended in ice cold lysis buffer (50 mM Tris, pH 7.4/300 mM NaCl/1% [wt/vol] Triton X-100/0.02% [wt/vol] Na₂S₂O₈/20 mM iodoacetamide) with a cocktail of protease inhibitors (0.25 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride, 0.1 mM N-*p*-tosyl-L-lysine-chloromethyl ketone, 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, 33 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml E-64, and 5 μg/ml pepstatin A) and incubated on ice for 20–30 min. Cell supernatants from each time point were adjusted to similar buffer conditions. Nuclei and insoluble debris were pelleted by centrifugation for 10–15 min in a microcentrifuge at 4°C. Lysates and supernatants were pre-cleared by incubation with 50 μl of a 50% slurry of protein A-Sepharose (Pharmacia, Piscataway, NJ) for 2 h at 4°C, and then subjected to immunoprecipitation with protein A-Sepharose beads coated with 7G7.B6 (anti-Tac) antibody for 2 h at 4°C. Sepharose pellets were washed 4× with ice cold wash buffer (50 mM Tris, pH 7.4/300 mM NaCl/0.1% [wt/vol] Triton X-100/0.02% Na₂S₂O₈) and once with ice cold PBS. Pellets were eluted by boiling with reducing SDS sample buffer and analyzed by SDS-PAGE (43) on Gel-Bond (FMC, Rockland, ME) bound gels made with 9% (wt/

vol) acrylamide and AcrylAide (FMC) as the crosslinker. After fixation, gels were treated for fluorography with 1 M salicylate, dried, and exposed to Kodak XAR-5 film. For quantitation, dried gels were analyzed by PhosphorImaging analysis on a Molecular Dynamics (Sunnyvale, CA) system. The sum of the radioactivity in all cell-associated Tac-specific bands as well as material shed into the medium was used when calculating degradation rates.

Quantitative Antibody Internalization Assay

The assay was performed essentially as described (46) using purified anti-Tac monoclonal antibody. Briefly, anti-Tac was radioiodinated using chloramine T (37). Untransfected and stably transfected HeLa cells expressing the constructs of interest were released from culture dishes with 10 mM EDTA/PBS, washed, and then bound to radioiodinated anti-Tac antibody on ice. After extensive washing at 4°C to remove unbound antibody, bound material was internalized for various periods of time at 37°C; at each time point, two aliquots were removed and added to ice cold medium. At the end of the incubation periods, cells were pelleted. One aliquot was resuspended in medium with and one without 1 mg/ml proteinase K, and cells were incubated on ice for 30 min. Cells were isolated by centrifugation through a cushion of fetal bovine serum, and cell pellets were cut from the bottom of frozen centrifuge tubes and counted in a Packard γ counter. Counts were adjusted for cell number, and the adjusted counts obtained using untransfected cells at each time point was subtracted from those obtained from experimental samples to give corrected counts. The final % internalization was then calculated as the corrected counts obtained with proteinase K treatment divided by those obtained without treatment at each time point. The results shown represent the pooled data from three separate experiments, each performed in duplicate.

Results

Localization of DM in Nonlymphoid Cell Lines

To characterize the compartment of nonlymphoid cells in which DM accumulates, two MHC-II-negative cell lines, HeLa and NRK, were transiently transfected with plasmids encoding human DM α and β chains. The cells were then analyzed by indirect immunofluorescence microscopy using an affinity purified antibody directed to the cytoplasmic tail of DM β . The resulting staining pattern in cells that expressed both DM α and β chains (Fig. 1, *left*) revealed vesicles of various sizes scattered throughout the cytoplasm. The vesicles stained by antibodies to DM β nearly completely overlapped with those stained by antibodies directed to endogenous lysosomal markers such as CD63 or Igp120 (Fig. 1, *right*). Localization to the vesicular compartment was dependent upon assembly of DM β with DM α chains, since expression of DM β alone resulted in a staining pattern consistent with ER retention (data not shown). These data establish that DM $\alpha\beta$ dimers are localized at steady state to a prelysosome/lysosome-like structure in NRK and HeLa cells, consistent with previous observations in MHC-II-negative cells (40) and with the presence of both DM and lysosomal markers in the MIIC of human B cell lines (63, 77). These data also demonstrate that, unlike for MHC-II, targeting of DM to this

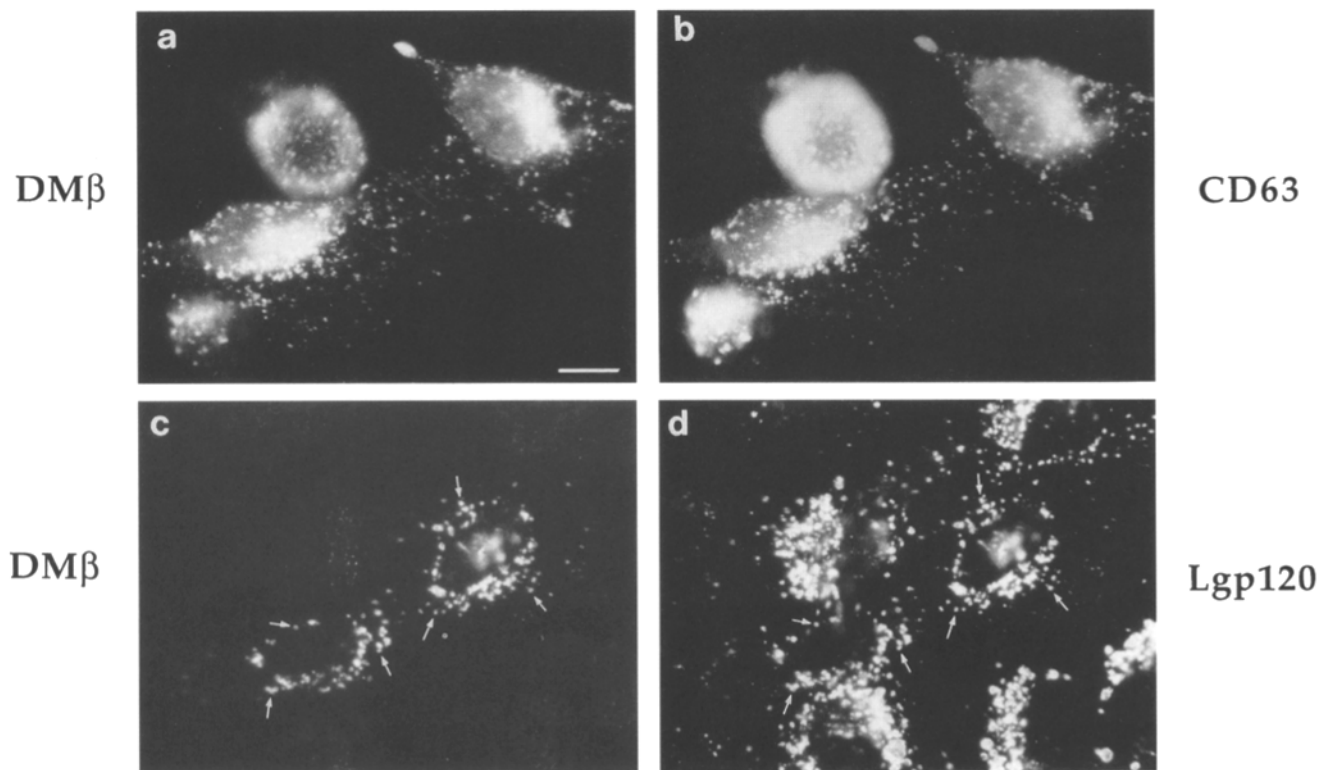


Figure 1. DM $\alpha\beta$ dimers colocalize with markers of lysosomal membranes. HeLa (*a* and *b*) or NRK (*c* and *d*) cells, grown on coverslips, were transiently transfected with plasmids encoding both human DM α and β chains. Cells were fixed with formaldehyde 40 h after transfection and processed for immunofluorescence microscopy, using an affinity-purified rabbit antibody to DM β (*a* and *c*) and mouse monoclonal antibodies to endogenous lysosomal proteins CD63 (*b*) and Igp120 (*d*) as primary antibodies, followed by LRSC-anti-rabbit Ig and FITC-anti-mouse Ig. Identical fields were photographed using filters for both fluorescein and rhodamine. Arrows in *c* and *d* point to examples of vesicles that are stained with both antibodies. Bar, 10 μ m.

compartment is independent of association with invariant chain, consistent with previous results (23), and imply that DM contains an autonomous targeting signal for a prelysosomal/lysosomal compartment.

The Cytoplasmic Tail of Murine DM β Is Sufficient for Targeting to a Prelysosomal/Lysosomal Compartment

Tyrosine- and di-leucine-based peptide sequence motifs in the cytoplasmic tails of many transmembrane proteins serve as endosomal and/or lysosomal targeting determinants (78, 82). While the cytoplasmic tail of the DM α chain is very short and has no recognizable targeting signal, the corresponding tail of the β chain is considerably longer and contains two conserved tyrosines that could potentially be part of targeting motifs (references 17, 41; see Fig. 5, *upper panel*). To determine whether targeting information was present in the β chain cytoplasmic tail, independent of other DM β or α chain sequences, a chimeric protein was created in which the mouse DM β (Mb) cytoplasmic tail was appended to the luminal and transmembrane domains of a prototypical type I cell surface glycoprotein, the IL-2 receptor α chain (Tac; see Fig. 2, *upper panel*). The localization of the chimeric protein, termed T-T-Mb (for Tac luminal-Tac transmembrane-Mb cytoplasmic tail), was compared with that of intact Tac by indirect immunofluorescence microscopy in stably transfected NRK or HeLa cells using antibodies directed to the Tac luminal domain.

The immunofluorescence pattern obtained upon staining NRK cells expressing the T-T-Mb chimera (Fig. 2 *a*) was distinct from that obtained with cells expressing Tac (Fig. 2 *c*). Whereas Tac was distributed mostly at the cell surface with very little in internal structures, T-T-Mb was present mostly internally in paranuclear structures, with sporadic vesicular staining in the cell periphery. Cell surface staining was apparent in only a fraction (10–20%) of transient transformants expressing high levels of T-T-Mb, and was undetectable by immunofluorescence in stable transformants (although low levels of cell surface staining could be detected by other assays; see Fig. 10). Similar results were obtained in HeLa cells stably expressing T-T-Mb and in several fibroblast cell lines (data not shown), showing that such localization was not unique to NRK cells. These data suggested that the DM β cytoplasmic tail caused intracellular localization of the Tac molecule.

It was surprising that the staining pattern obtained with T-T-Mb did not show more prominent staining of cytoplasmic vesicles, as seen for DM $\alpha\beta$ dimers. We reasoned that if the DM β tail were to direct the heterologous Tac protein to a lysosome-like compartment, the Tac luminal domain might be degraded and would no longer be detected by our antibodies. This would be in contrast to DM $\alpha\beta$ dimers which, like class II molecules, are expected to be more resistant to proteolysis (22). To test whether the T-T-Mb chimera was being degraded, we treated the transfectants with leupeptin, an inhibitor of a subset of lysosomal proteases, before fixation and indirect immunofluorescence analysis with the antibody to Tac. The resulting staining pattern for intact Tac (Fig. 2 *d*) was virtually unchanged, still displaying cell surface labeling. In contrast, the staining pattern for T-T-Mb now consisted of

multiple bright cytoplasmic vesicles (Fig. 2 *b*), much as had been seen for DM $\alpha\beta$ transfectants (Fig. 1). Identical staining patterns were obtained with HeLa cells stably expressing T-T-Mb (e.g., see Fig. 3) and a variety of transiently transfected fibroblasts (data not shown). An identical pattern was also obtained using a chimera containing the cytoplasmic tail of the β_2 form of murine DM β (data not shown), which has a single amino acid change (serine₂₅₂ \rightarrow proline; reference 40). These results demonstrate that the cytoplasmic tail of DM β contains a targeting determinant for a vesicular compartment that contains leupeptin-sensitive proteolytic enzymes in nonlymphoid cell lines.

To further characterize the compartment to which the DM β tail targeted the Tac antigen, double staining by indirect immunofluorescence analysis was used to colocalize T-T-Mb with various endogenous and transfected molecules. As shown in Fig. 3, *a* and *b*, T-T-Mb was colocalized in leupeptin-treated stable HeLa cell transfectants with transiently expressed human DM $\alpha\beta$ heterodimers. Identical results were obtained in NRK cells (data not shown). This demonstrates that the cytoplasmic tail of murine DM β is sufficient to effect localization of DM in nonlymphoid cells. Note that leupeptin treatment was necessary, even in cells coexpressing DM, to observe vesicular staining of T-T-Mb.

To determine whether T-T-Mb was delivered to the same compartment as that in which MHC-II molecules accumulated, HeLa cells were cotransfected with plasmids encoding human class II chains (HLA-DR α and β), as well as invariant (I) chain and T-T-Mb, and then treated with leupeptin before indirect immunofluorescence using antibodies to both DR β and Tac. The results, corroborated by a similar analysis in M1 fibroblasts (not shown), revealed nearly complete colocalization of DR β and T-T-Mb (Fig. 3, *e* and *f*). Thus, T-T-Mb is targeted in nonlymphoid cells to a compartment in which class II antigens accumulate, as expected for a compartment with similarity to the B cell MIIC. Finally, T-T-Mb showed nearly perfect colocalization in stably transfected, leupeptin-treated HeLa cells with an endogenous marker of lysosomes, lamp-1 (Fig. 3, *c* and *d*); similar results were observed in NRK cells (data not shown). In contrast, the immunofluorescence profiles of markers for early endosomes (transferrin receptor; HeLa cells), the TGN (TGN38; NRK cells), and pan-endosomes (cation-independent mannose-6-phosphate receptor; NRK cells), were quite distinct from that of T-T-Mb in leupeptin-treated cells (data not shown). Taken together, these data suggest that T-T-Mb is localized to a MIIC-like compartment in nonlymphoid cells, and that this compartment corresponds to prelysosomes and/or lysosomes.

Immunoelectron Microscopy Localization of T-T-Mb in Stably Transfected HeLa Cells

The T-T-Mb-accumulating compartment was further characterized in stably transfected HeLa cells by immunoelectron microscopy. After cryosectioning, leupeptin-treated cells were stained first with an antiserum to Tac followed by protein A conjugated to 10-nm gold particles, and then with an antiserum to lamp-1 followed by protein A conjugated to 15-nm gold particles. T-T-Mb was found to accu-

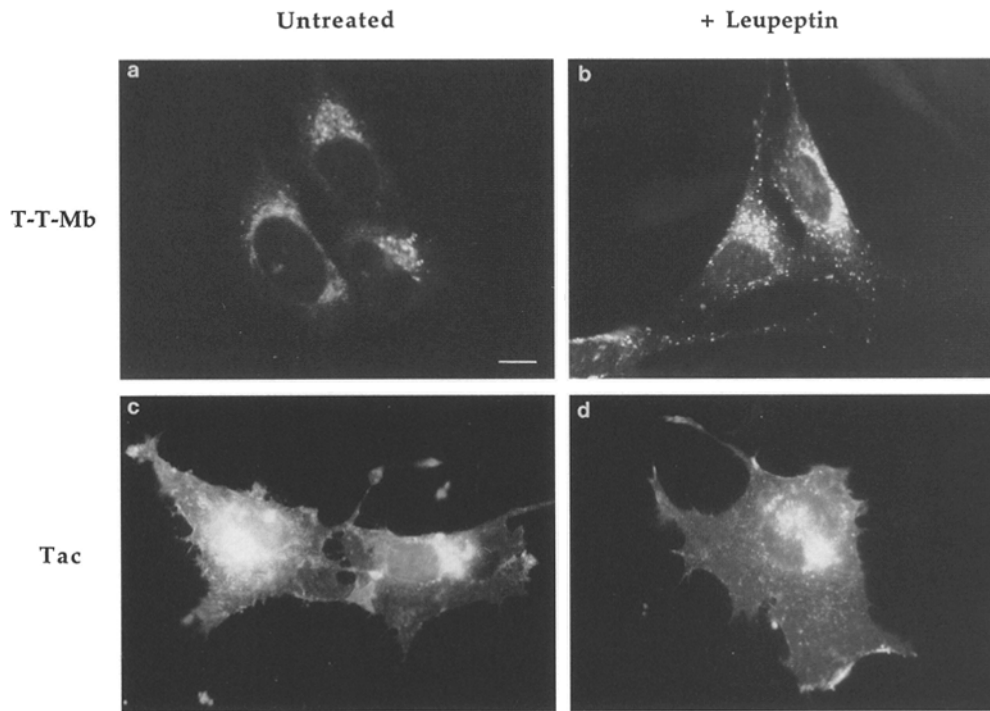
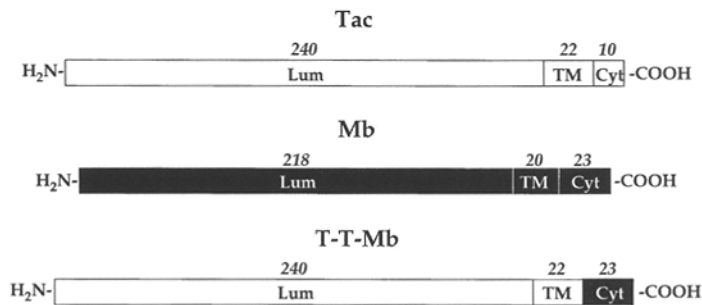


Figure 2. The cytoplasmic tail of DM β is sufficient for targeting to a degradative, vesicular compartment. (*Upper panel*) Schematic diagram of Tac/Mb chimeras. Tac-derived amino acid sequences are represented in white, and mouse DM β (Mb) sequences are represented in black. Numbers represent the number of amino acids in the luminal (*Lum*), transmembrane (*TM*), and cytoplasmic (*Cyt*) domains of each protein; the signal peptide is included for the luminal domains. Amino (H_2N) and carboxyl ($COOH$) termini of each construct are indicated. (*Lower panel*) Immunolocalization of T-T-Mb and Tac in NRK cells. NRK cells grown on coverslips were transiently transfected with plasmids encoding T-T-Mb (*a* and *b*) or Tac (*c* and *d*). After 36–40 h, cells were left untreated (*a* and *c*) or treated with 1 mg/ml leupeptin (*b* and *d*) for 4 h. Cells were then fixed and processed for immunofluorescence microscopy using a monoclonal antibody to Tac and LRSC-anti-mouse Ig. Bar, 10 μ m.

mulate predominantly in the membranes of dense, multivesicular structures characteristic of lysosomes (Fig. 4 *a*). Lamp-1 was also found in these round, nontubular structures, confirming their identity as lysosomes. Gold particles corresponding to T-T-Mb were found less frequently in tubulo-vesicular structures characteristic of the TGN, as well as in Golgi and ER structures. Only rarely was staining observed on the cell surface. The data confirm that the predominant steady state distribution of T-T-Mb is in internal structures of HeLa cells, consistent with the conclusion that the DM β tail confers lysosomal targeting.

Identification of a Targeting Motif in the DM β Cytoplasmic Tail

A subset of tyrosine-based cytoplasmic tail targeting determinants for post-Golgi compartments consists of the motif YXX \emptyset , in which Y corresponds to tyrosine, X corresponds to any amino acid and \emptyset corresponds to a bulky hydrophobic residue, such as leucine, isoleucine, phenylalanine, or methionine (82). Many lysosomal transmem-

brane proteins have such a YXX \emptyset sequence at their COOH-termini, most often preceded by a small side chain residue, such as glycine or alanine. The cytoplasmic tails of both murine and human DM β contain a YXX \emptyset motif, YTPL, but it resides in the middle of the sequence and is preceded by a series of serine residues (see Fig. 5, *upper panel*). To determine if this or other signals was responsible for targeting T-T-Mb to lysosomes in nonlymphoid cells, a number of truncations in the tail were constructed, and the resulting immunofluorescence profiles were analyzed in transiently transfected NRK cells, both before and after treatment with leupeptin.

As seen in Fig. 5, *a–f*, deletion of five residues (TTM.GSTY Δ ; “GSTY Δ ”) or 10 residues (TTM.YTPL Δ ; “YTPL Δ ”) from the COOH terminus of the DM β cytoplasmic tail had no detectable effects on the immunofluorescence pattern of T-T-Mb. In both cases, the chimera was retained intracellularly, and vesicles were observed upon treatment with leupeptin. Localization of TTM.GSTY Δ to lamp-1-positive lysosomes was confirmed by immunoelectron microscopy (Fig. 4 *b*). Both of these constructs re-

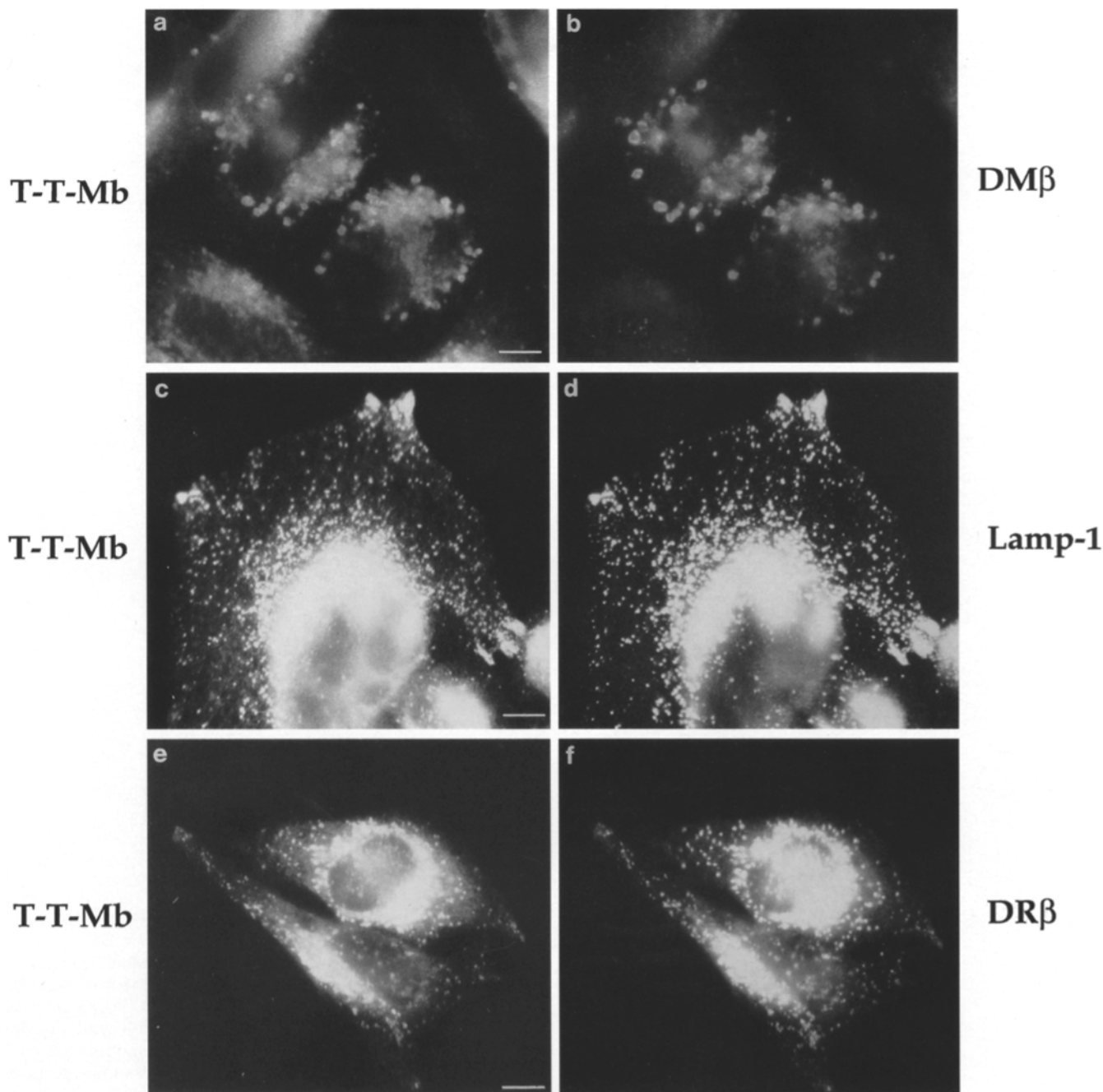


Figure 3. Molecules that colocalize with T-T-Mb in HeLa cells. (*a–d*) HeLa cells stably transfected with T-T-Mb and grown on coverslips were either untreated (*c* and *d*) or transiently transfected with plasmids encoding human DM α and DM β chains (*a* and *b*). After 36 h, cells were treated with 1 mg/ml leupeptin for 4 h, and then fixed and processed for immunofluorescence microscopy. (*e* and *f*) HeLa cells grown on coverslips were transiently transfected with plasmids encoding T-T-Mb, human MHC-II chains DR α and DR β , and I chain before treatment with leupeptin, fixation, and processing as above. Cells were costained with anti-Tac antibodies (*a*, *c*, and *e*) and antibodies to either the transfected DM β chain (*b*), the endogenous late endosomal/lysosomal transmembrane protein lamp-1 (*d*), or the transfected DR β chain (*f*), followed by appropriate FITC- and LRSC-conjugated secondary antibodies. Bars: (*a* and *b*) 5 μ m; (*c–f*) 10 μ m.

tained the YTPL motif; it was present at the COOH terminus of the TTM.YTPL Δ construct. Removal of YTPL (TTM.HSSSA Δ ; “HSSSA”) resulted in a complete loss of the vesicular staining pattern (Fig. 5, *g* and *h*). Like intact Tac, the TTM.HSSSA chimera was expressed predominantly at the cell surface, both in the absence and presence of leupeptin. Immunoelectron microscopy confirmed that the TTM.HSSSA chimera was localized mostly to the cell

surface (average 387 gold particles per 100 μ m, compared with 8 and 5 gold particles per 100 μ m of cell surface for T-T-Mb and TTM.GSTY Δ , respectively), with significant staining in secretory compartments (ER, Golgi, transport vesicles), but not in lysosomes (Fig. 4 *c*). These data demonstrate that the YTPL motif is an essential component of the lysosomal targeting signal in the DM β cytoplasmic tail.

To further delineate those features of the YTPL motif

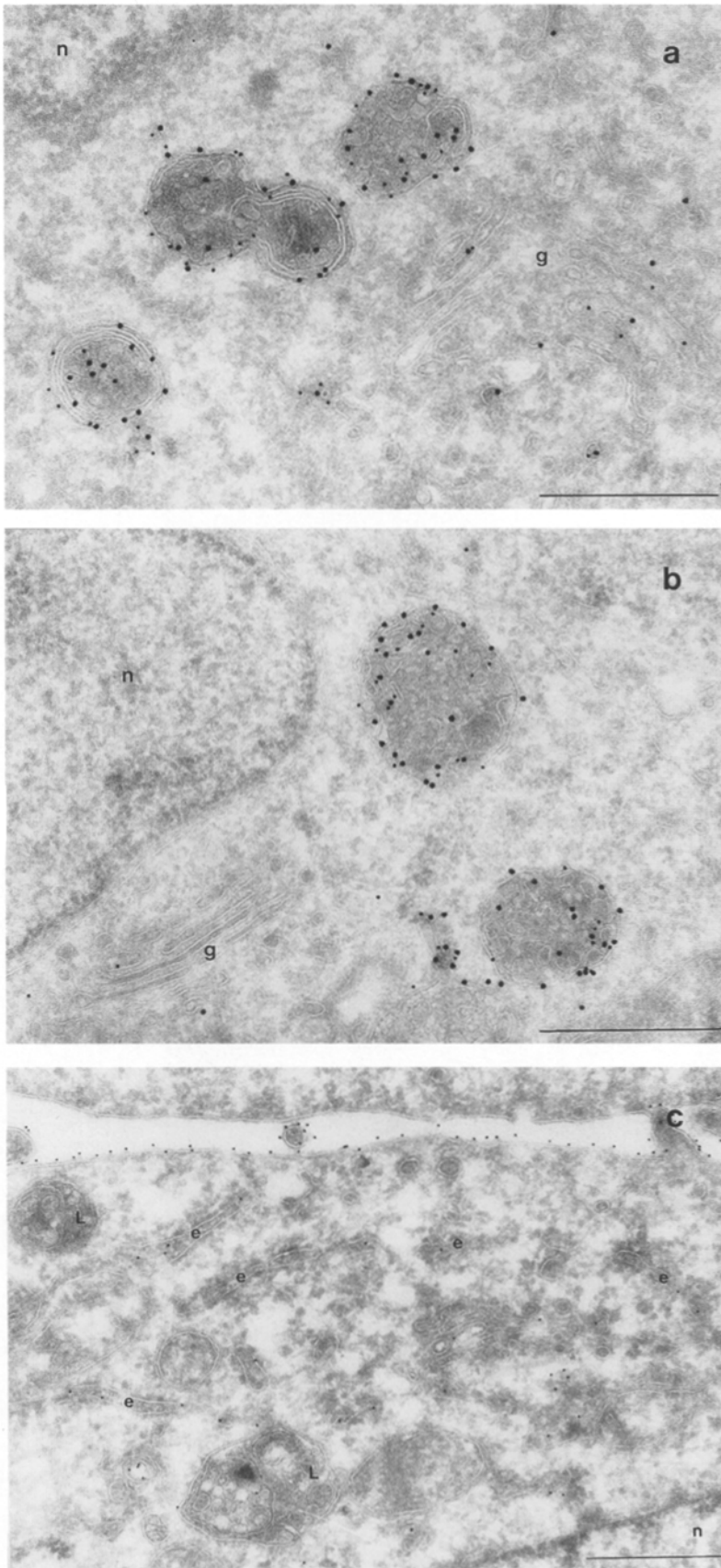


Figure 4. Localization of T-T-Mb in HeLa cells by immunoelectron microscopy. Stably transfected HeLa cells expressing either T-T-Mb (*a*), TTM.GS-TY Δ (*b*; see below), or the TTM.HSSSA chimera (*c*, see below) were treated for 4 h with 1 mg/ml leupeptin before fixation with glutaraldehyde and paraformaldehyde. (*a* and *b*) Frozen thin sections were prepared and stained first with a polyclonal rabbit antiserum to Tac followed by protein A-gold conjugates (10-nm particles), and then, after refixation, with an antiserum to lamp-1 followed by protein A-gold conjugates (15-nm particles). Samples were analyzed by immunoelectron microscopy. Dense lysosomal structures are labeled with gold particles of both sizes. The nucleus (*n*) and Golgi apparatus (*g*) of the cells shown are indicated. Bars, 500 nm. (*c*) Frozen thin sections were stained with a polyclonal rabbit antiserum to Tac followed by protein A-gold conjugates (10-nm particles), and then analyzed by immunoelectron microscopy. The nucleus (*n*), ER (*e*), and lysosomes (*L*) are indicated. Bar, 200 nm.

that are critical for lysosomal targeting, residues within and around the motif were individually mutated to alanine. Mutagenesis was done in the context of the TTM.GSTY Δ construct, since its immunolocalization pattern was identical to that of the intact T-T-Mb chimera, and was limited to those residues constituting and preceding the YTPL motif; distal residues were deemed uncritical, since the TTM.YTPL Δ construct retained lysosomal localization. Mutated constructs were expressed by transient transfection in NRK cells, and leupeptin-treated cells were analyzed by indirect immunofluorescence. As seen in Fig. 6, substitution of alanine for either the tyrosine or leucine residues within the YTPL motif completely ablated lysosomal targeting, and resulted in a protein localized predominantly to the plasma membrane (Fig. 6, *e* and *h*). In contrast, there were no noticeable effects on localization by substitution of alanine for either the threonine or proline residues (Fig. 6, *f* and *g*) or for any of the three serine residues preceding the YTPL motif (Fig. 6, *b–d*). These results suggest that the tyrosine and leucine residues are critical for lysosomal localization, but that other positions are more permissive of alanine substitution.

Lysosomal Degradation of T-T-Mb

The morphological data presented above show that T-T-Mb accumulates in a lysosomal compartment in the presence of an inhibitor of lysosomal proteases. To confirm that T-T-Mb is targeted to a degradative compartment in the absence of inhibitors, trafficking was analyzed biochemically by pulse/chase analyses. Stably transfected HeLa cells expressing T-T-Mb, TTM.GSTY Δ (retaining YTPL), TTM.HSSSA Δ (lacking YTPL), or intact Tac were metabolically pulse labeled with [³⁵S]methionine/cysteine, and then chased for up to 8 h with excess unlabeled methionine/cysteine. Lysates and supernatants from each time point were analyzed by immunoprecipitation with antibodies to Tac and subsequent SDS-PAGE, followed by autoradiography and PhosphorImager analysis. As seen in Fig. 7 *A*, both intact Tac and TTM.HSSSA Δ were quite stable over the 8 h of the experiment. In contrast, both the T-T-Mb and TTM.GSTY Δ chimeras rapidly disappeared from the cells. The loss could not be attributed to shedding of the Tac luminal domain, as shown by immunoprecipitation from the cell supernatants at each time point (Fig. 7 *A*), suggesting that the loss was due to intracellular degradation. The results of pulse/chase analyses for both stably transfected HeLa and NRK cells are quantitated in Fig. 7 *B*. T-T-Mb had a half-life of ~2.5–3 h in HeLa cells, compared with a half-life of greater than 8 h for Tac or TTM.HSSSA Δ . Degradation of T-T-Mb was even more rapid in NRK cells, in which the half-life was only 1–1.5 h. These data imply efficient lysosomal delivery of the T-T-Mb chimera that is dependent on the YTPL motif.

To demonstrate that the degradation observed above was due to proteolysis in a lysosomal compartment and to correlate the morphologic and biochemical results, the effect on degradation of various lysosomal inhibitors was analyzed in a pulse/chase experiment. As seen in Fig. 8 *A* and quantitated in Fig. 8 *B*, the nearly complete loss of T-T-Mb from lysates of stably transfected HeLa cells observed after 8 h of chase was at least partially blocked by all lyso-

somal inhibitors tested, including leupeptin (Leu), ammonium chloride (NH₄Cl), methionine methyl ester (MME), and a combination of inhibitors (leupeptin, pepstatin A, E-64, and methionine methyl ester; LPEM). The data firmly establish that T-T-Mb is proteolytically degraded in a lysosomal compartment.

The YTPL Motif Is Necessary for Lysosomal Delivery of DM $\alpha\beta$ Dimers

The experiments outlined above show that the YTPL motif in the DM β cytoplasmic tail is sufficient to confer lysosomal targeting to a heterologous protein. To determine whether this motif is also necessary for lysosomal targeting of the intact DM complex, the tyrosine residue within the YTPL motif in the cytoplasmic tail of the full-length DM β molecule was mutagenized to alanine (resulting in DM β .Y248A), and the localization of DM $\alpha\beta$ dimers containing wild-type DM β or DM β .Y248A was analyzed by indirect immunofluorescence in HeLa cells. The results show that while DM dimers containing wild-type DM β chains were localized predominantly to lysosomes (Fig. 9 *a*), the majority of dimers containing DM β .Y248A were present at the cell surface (Fig. 9 *b*, although a low level of vesicular staining was still visible). Analysis of the transfectants by flow cytometry (Fig. 9 *c*) convincingly illustrated the effect of the tyrosine mutation on cell surface expression of DM; transfectants expressing DM dimers with wild-type DM β displayed only low levels of surface expression at best, whereas those with mutated DM β .Y248A exhibited much higher levels of surface expression, comparable to that of a *bona fide* cell surface marker, CD4 (not shown). Thus, the tyrosine within the YTPL motif is necessary for efficient lysosomal delivery of the intact DM molecule.

The DM β Cytoplasmic Tail Acts as an Internalization Signal

Many of the tyrosine-based signals that cause localization of proteins to lysosomes also act as internalization signals (82). To determine whether the DM β cytoplasmic tail also contained a signal for internalization from the cell surface, antibody uptake experiments were performed. Radioiodinated antibodies to Tac were bound on ice to the surface of HeLa cells stably expressing T-T-Mb, TTM.GSTY Δ , TTM.HSSSA Δ , or Tac, and then bound antibody was allowed to internalize for various times at 37°C. Internalization was measured as a function of inaccessibility of cell-associated antibody to proteinase K added to intact cells on ice after the 37°C incubation. As shown in Fig. 10 *A*, while both T-T-Mb and TTM.GSTY Δ transfected cells initially bound much less anti-Tac antibody at the cell surface (see figure legend), the bound antibody was rapidly internalized. For T-T-Mb, nearly 70% of the antibody initially bound at 0°C was internalized by 5 min. The TTM.YTPL Δ chimera also mediated rapid internalization (data not shown), consistent with an essential role for the YTPL motif. In contrast, Tac and TTM.HSSSA Δ , in which the YTPL motif was deleted, internalized anti-Tac antibody at a much slower rate (15–20% by 20 min). When continuous antibody uptake was analyzed in leupeptin-treated T-T-

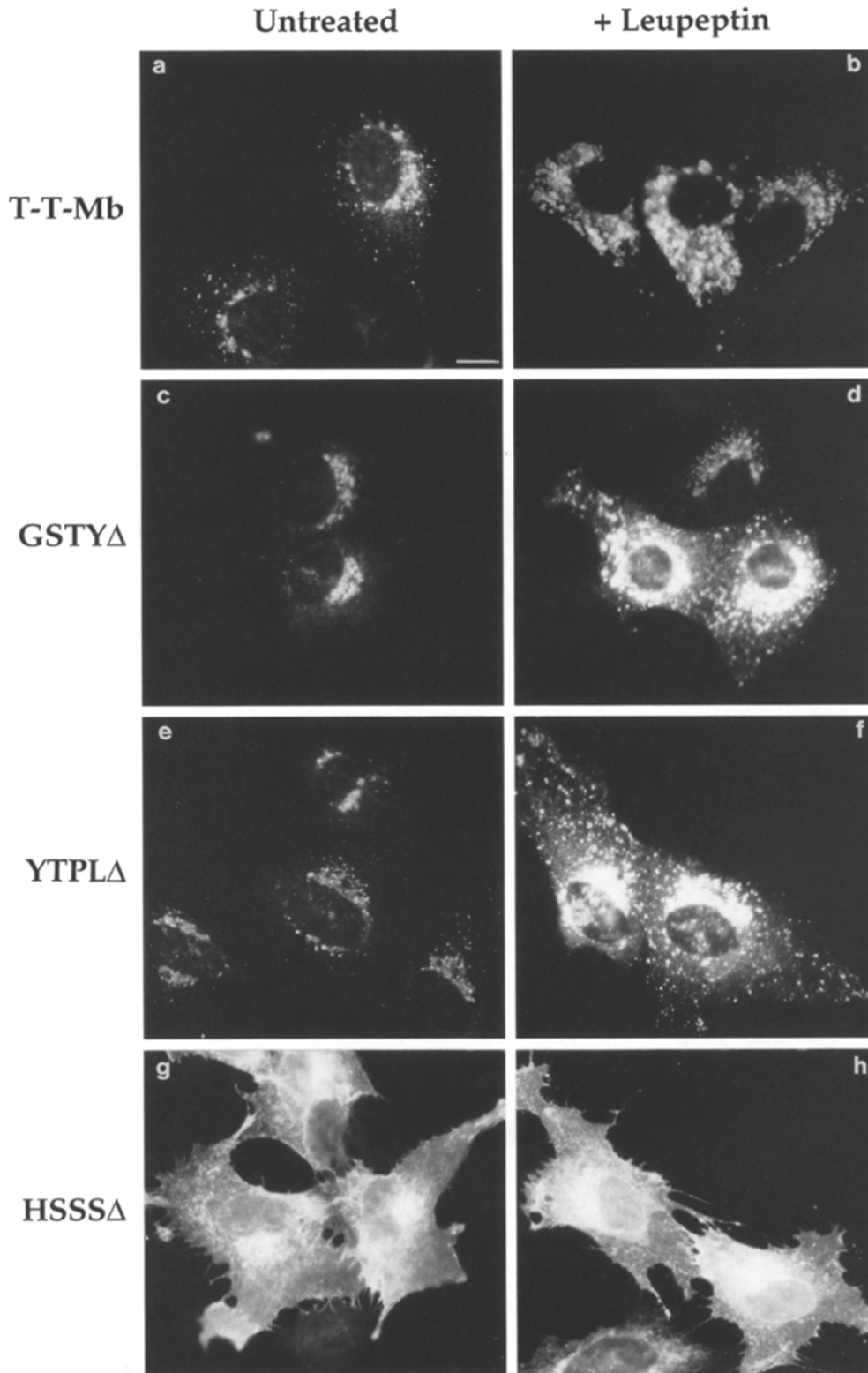
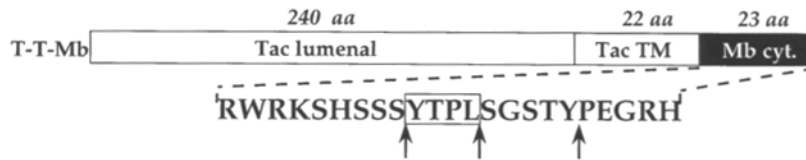


Figure 5. The YTPL motif in the cytoplasmic tail of DMβ is an essential component of the targeting determinant. T-T-Mb (a and b) or T-T-Mb-derived chimeras with progressive COOH-terminal truncations (c and d, TTM.GSTYΔ; e and f, TTM.YTPLΔ; g and h, TTM.HSSSA) were transiently transfected into NRK cells grown on coverslips. Cells were untreated or treated with 1 mg/ml leupeptin as in Figs. 2 and 3, and then processed for immunofluorescence using a monoclonal antibody to Tac and LRSC-conjugated anti-mouse Ig. The constructs are named for the remaining COOH-terminal sequence in the cytoplasmic tail as indicated in the upper panel; e.g., TTM.GSTYΔ (*GSTYΔ*) is truncated after the sequence GSTY, etc. Bar, 10 μm.

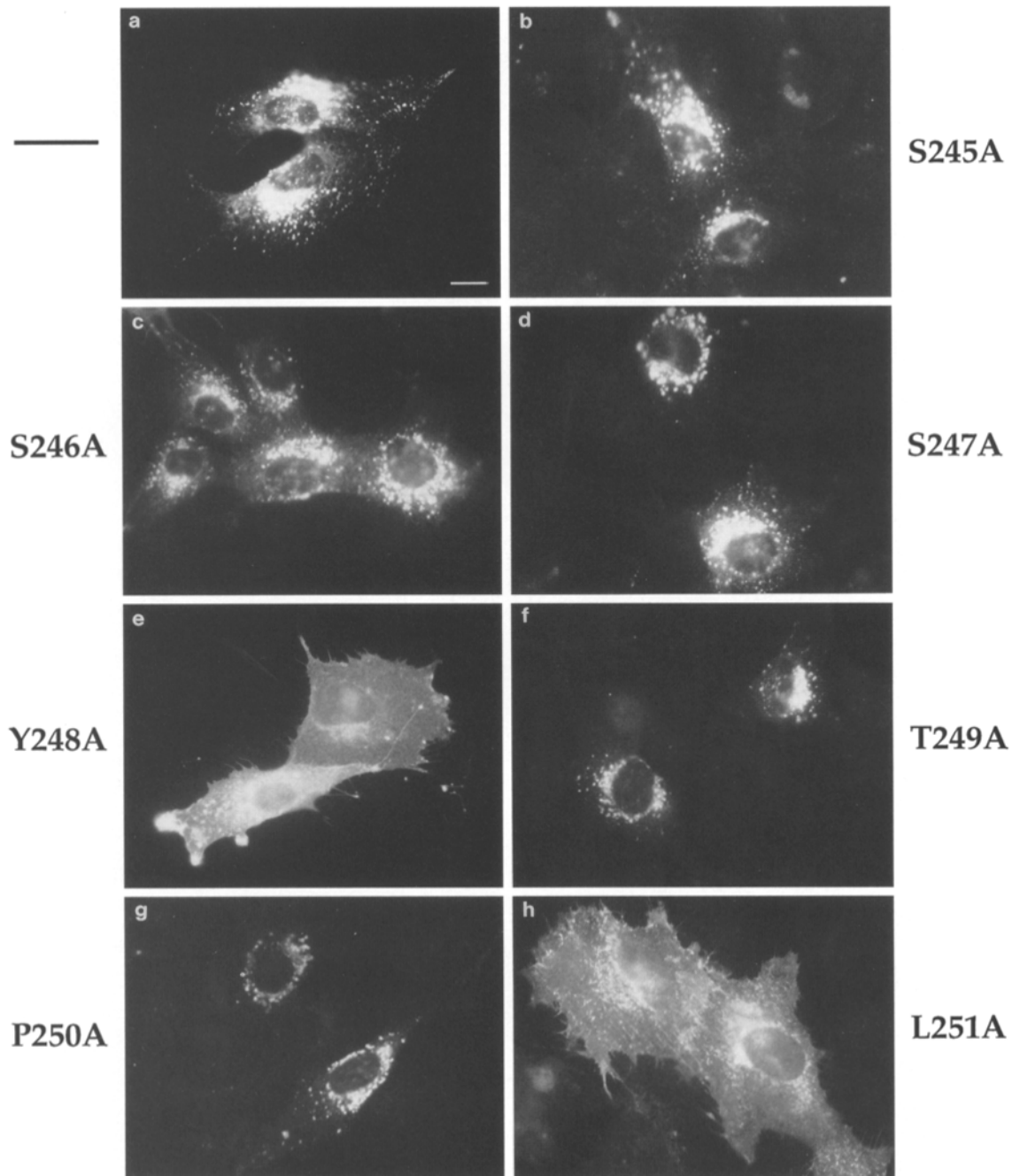


Figure 6. The Y and L residues are the most critical components of the DMB targeting determinant. As indicated in the top panel, point mutations were separately introduced into codons #245-251 (relative to full-length H-2Mb) of the Mb sequence in the TTM.GSTY Δ chimera, converting each codon individually into an alanine codon. Plasmids encoding individual alanine scanning mutants were transiently transfected into NRK cells, and leupeptin-treated cells were processed for immunofluorescence microscopy as described in the previous figures using a monoclonal antibody to Tac and LRSC-conjugated anti-mouse Ig. Each mutant is named for the codon that is mutated to alanine; e.g., S245A (*b*) has serine #245 converted to alanine. *a* (-) shows the immunofluorescence profile with nonmutated TTM.GSTY Δ . Bar, 10 μ m.

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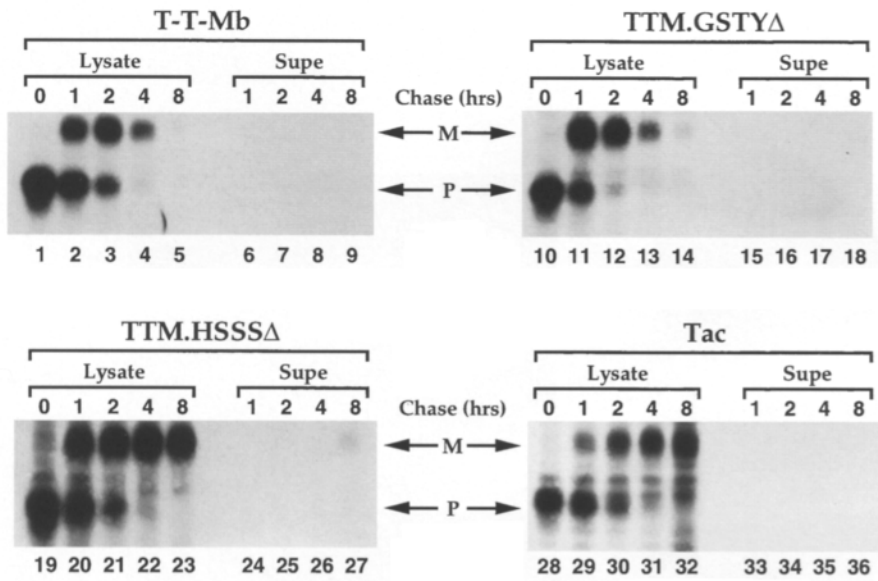
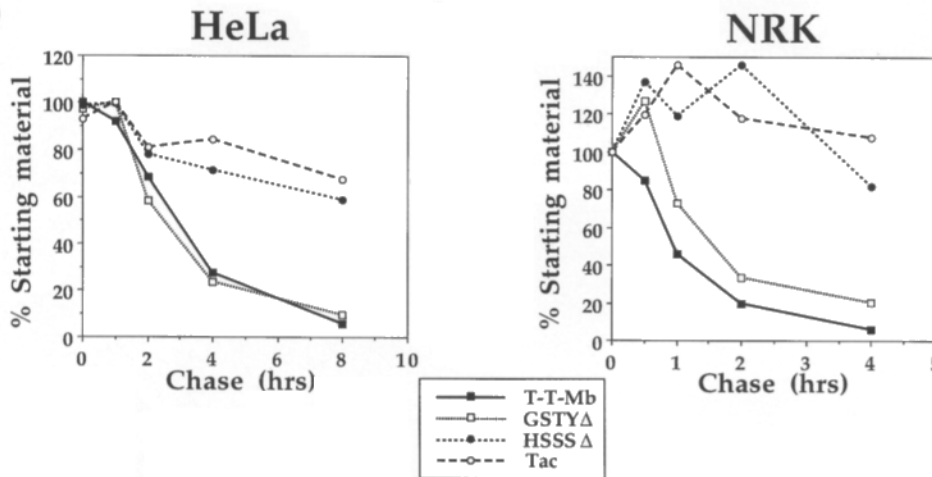


Figure 7. Pulse/chase analysis of T-T-Mb degradation. (A) Stably transfected HeLa cells expressing T-T-Mb (lanes 1–9), TTM.GSTYΔ (lanes 10–18), TTM.HSSSA (lanes 19–27; see Fig. 5, top panel for description) or Tac (lanes 28–36) were metabolically labeled with Tran³⁵S-label for 20 min, and then chased for the indicated times in the presence of excess unlabeled methionine and cysteine. Cells and supernatants were collected at each time point, and both cell lysates and supernatants (Supe) were immunoprecipitated with a monoclonal antibody to Tac. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. The positions of the unprocessed glycosylated ER precursor of Tac (P) and the fully processed, mature form (M) are indicated. (B) Gels in A and gels of similar pulse/chase experiments using stably transfected NRK cells expressing T-T-Mb, TTM.GSTYΔ, TTM.HSSSA, or Tac were analyzed by PhosphorImaging analysis, and bands corresponding to Tac or the various Tac chimeric proteins were quantitated at each time point. Quantitation represents the sum of relative intensities of all specific bands in precipitates from both lysates and supernatants at each time point, divided by the sum of all specific bands in the lysates after the pulse. It should

B



be noted that in NRK cells, a large fraction of both TTM.HSSSA and Tac was shed into the medium at later time points (up to 75% of starting material by 4 h of chase; data not shown). A smaller but still significant fraction of these molecules was shed by HeLa cells (observable in longer exposures of the gels). Very little of the T-T-Mb or TTM.GSTYΔ molecules were shed by either cell line.

Mb-expressing cells by immunofluorescence microscopy (Fig. 10 B), the internalized antibody colocalized with both the endogenous lysosomal marker, lamp-1 (HeLa cells) and the total pool of synthesized T-T-Mb (not shown). In contrast, untransfected cells or cells expressing TTM.HSSSA showed no uptake by this assay (data not shown). This suggests that those T-T-Mb molecules that appeared transiently on the cell surface and that were internalized were targeted to lysosomes.

The DMβ Tail Mediates Localization of Tac to the MIIC in Human EBV-transformed B Cells

The previous experiments demonstrate that both DMαβ dimers and a chimeric protein containing the murine DMβ

cytoplasmic tail are targeted to a lysosomal compartment in nonlymphoid MHC-II-negative cells. To address whether the DMβ cytoplasmic tail YTPL motif alone was sufficient for targeting to the MIIC in antigen-presenting cells, T-T-Mb and TTM.HSSSA were localized by immunoelectron microscopy in stable transfectants of a human B lymphoblastoid cell line, 721.45. As seen in Fig. 11 a, T-T-Mb was localized in this cell line predominantly to internal structures with a dense, multivesicular and multilaminar morphology. As in HeLa cells, only very little T-T-Mb staining was observed at the cell surface. In contrast, TTM.HSSSA was localized almost exclusively to the cell surface (240 gold particles per 100 μm, compared with 8 gold particles per 100 μm for T-T-Mb), with additional staining evident in early secretory structures (Fig. 11 b). Only very few gold

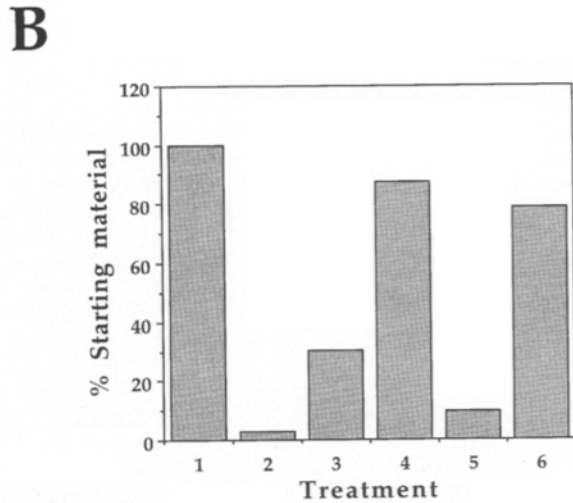
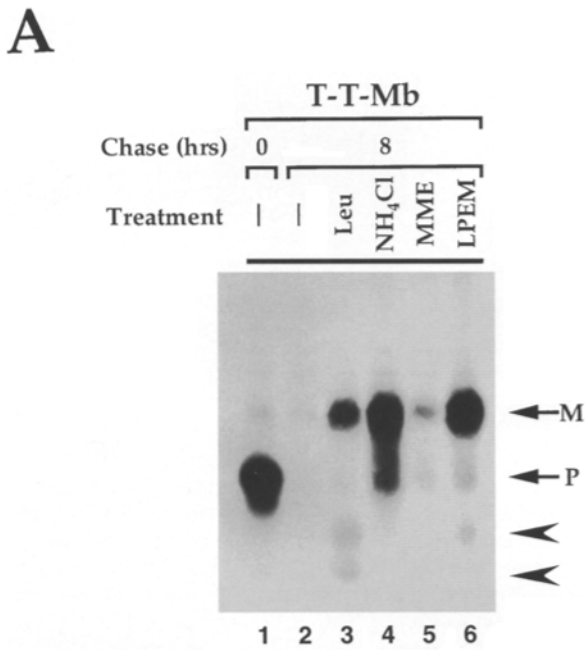


Figure 8. The degradation of T-T-Mb during the chase occurs in lysosomal compartments. HeLa cells stably transfected with T-T-Mb were metabolically labeled for 20 min (0 chase; lane 1) as in Fig. 7, and then chased for 8 h in the absence (–; lane 2) or presence of the following inhibitors of lysosomal proteolysis, trafficking, or function: 1 mg/ml leupeptin (Leu; lane 3); 50 mM NH_4Cl (NH_4Cl ; lane 4); 20 mM methionine methyl ester (MME; lane 5); and a combination of 100 $\mu\text{g}/\text{ml}$ each of leupeptin, pepstatin A, and E-64 with 20 mM MME (LPEM; lane 6). Cell lysates from each time point were immunoprecipitated with a monoclonal antibody to Tac, and immunoprecipitates were fractionated by SDS-PAGE. Gels were analyzed by (A) fluorography and (B) PhosphorImaging analysis and band quantitation. Arrows in A point to the ER precursor form of T-T-Mb (P) and the processed, mature form (M), and arrowheads point to probable cleavage intermediates of T-T-Mb that remain intact after inhibition of some proteolytic events. The quantitation in B is a measure of the sum of the intensity of all T-T-Mb-related bands with each treatment at each time point divided by the sum of the intensity of all bands present at the pulse time point.

particles staining TTM.HSSSD were found in lysosomal or endocytic structures. These data indicate that the YTPL motif in the DM β cytoplasmic tail confers localization to an endosomal/lysosomal structure in a “professional” antigen-presenting cell, such as a B-lymphoblastoid cell line.

To determine whether T-T-Mb was localized to the MIIC in these cells, cryosections were immunolabeled with antibodies to Tac and to either MHC-II or the lysosomal/MIIC marker lamp-1. As seen in Fig. 12 a, T-T-Mb could be colocalized with lamp-1 in dense, multivesicular structures in these cells, much as had been seen in HeLa cells. T-T-Mb and CD63 were also colocalized in these structures (data not shown). Those multivesicular and multilaminar structures that contained MHC-II molecules were also stained with antibodies to T-T-Mb (Fig. 12 b). This demonstrates that the DM β cytoplasmic tail can confer localization to the MIIC. Interestingly, there were some, albeit few, lysosomal structures that contained T-T-Mb but did not contain MHC-II molecules (see Fig. 12 b). Similar localization to both lysosomes and MHC-II-rich structures has been seen for lamp-1 and for CD63 (63, 64). Thus, while the targeting signal in the cytoplasmic tail of DM β is sufficient to confer localization to the MIIC, some other region of DM β or the DM $\alpha\beta$ dimer may be necessary for exclusive partitioning from classical lysosomes.

Discussion

Lysosomal Targeting of DM in Nonlymphoid Cells

We have shown that assembled DM $\alpha\beta$ dimers are localized at steady state to the same compartment as endogenous lysosomal proteins, such as CD63 and Igp120, in nonlymphoid class II-negative cells. In multiple experiments using transiently transfected HeLa or NRK cells, virtually all vesicles containing lysosomal markers contained coexpressed DM molecules (see Fig. 1). The lysosomal targeting of DM in fibroblasts was dependent on assembly of α chains with β chains. The immunofluorescent staining pattern for DM β in transfected cells that did not coexpress DM α chains suggested localization to the ER (data not shown). This demonstrates the requirement for assembly of the DM $\alpha\beta$ complex in order to exit the ER, consistent with observations for other multi-subunit complexes (36). It is worth noting that, unlike for MHC-II molecules, efficient ER egress (2, 6, 45, 85) and lysosomal targeting (4, 44, 50) of DM were independent of expression of the I chain in all cell types examined. Our results therefore indicate that DM must possess its own lysosomal targeting signal.

A Cytoplasmic Targeting Signal Directs DM to Lysosomes

Using a chimeric protein approach, we have shown that the cytoplasmic tail of the murine DM β chain (Mb) is sufficient to mediate lysosomal targeting of a heterologous protein in nonlymphoid cells (Figs. 2–4). The localization of the T-T-Mb chimera to the same structures as assembled DM complexes (Fig. 3) and the cell surface localization of DM dimers lacking the DM β cytoplasmic tail targeting signal (Fig. 9) make it unlikely that information

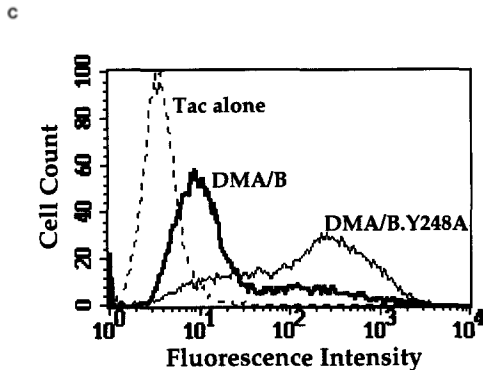
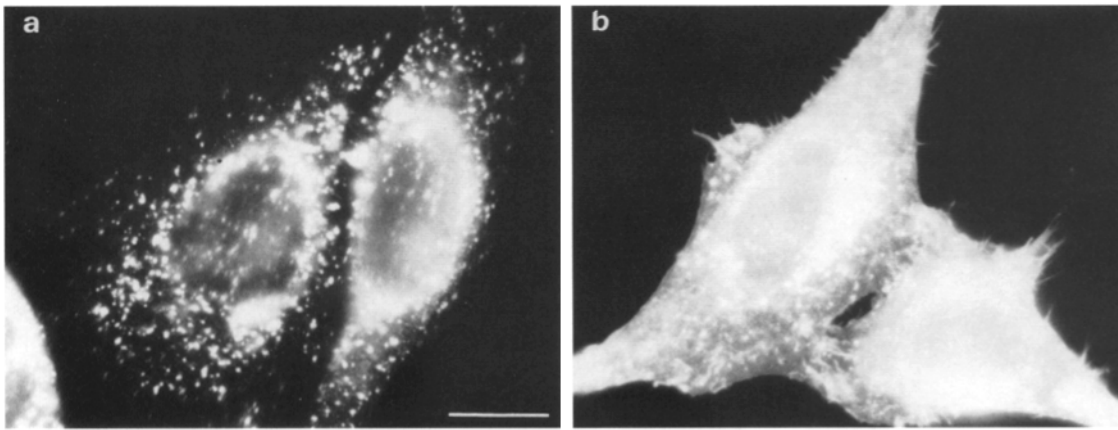


Figure 9. The cytoplasmic tail of DM β is necessary for efficient localization of DM $\alpha\beta$ dimers. (*a* and *b*) HeLa cells grown on coverslips were transiently transfected with 0.5 μg of plasmids encoding DM α and either wild-type DM β (DMB; *a*) or DMB.Y248A (*b*). Cells were fixed with formaldehyde 36 h after transfection, and then analyzed by indirect immunofluorescence microscopy using a rabbit antiserum to purified DM $\alpha\beta$ complexes and LRSC-anti-rabbit Ig. (*c*) HeLa cells were transfected with plasmids encoding Tac, DM α , and either DM β or DMB.Y248A in 10-cm dishes, harvested 36 h after transfection, and analyzed by flow cytometry using the rabbit antiserum to DM $\alpha\beta$ with FITC-anti-rabbit Ig and phycoerythrin-conjugated anti-Tac. The histogram shown represents surface DM staining for cells that were gated for expression of moderate surface levels of Tac (a range of 10–400 fluorescence units). The negative control (–) shows DM staining for cells transfected with Tac alone. Dot plot profiles showed a linear relationship at all expression levels between Tac and DM staining for cells transfected

with Tac, DM α , and DMB.Y248A, as well as for Tac and a control surface-expressed protein, CD4 (not shown). In the experiment shown, an equivalent fraction of Tac-expressing cells coexpressed DM $\alpha\beta$ and DM α /B.Y248A dimers (96.5% and 96.2%, respectively, measured by indirect immunofluorescence of a sample of the transfected cells). Protein expression levels were higher (1.6- and 2.2-fold for DM α and β , respectively) in cells transfected with wild-type DM β relative to those transfected with DMB.Y248A (measured by pulse-labeling, immunoprecipitation, and PhosphorImaging analysis of a fraction of the transfected cells), ruling out overexpression of DM α /B.Y248A as the cause of the increased surface expression. Bar, 20 μm .

other than the β chain cytoplasmic tail is necessary for targeting DM molecules in MHC-II–negative cells. Furthermore, the T-T-Mb chimera colocalized with MHC-II molecules expressed with I chain in transfected HeLa (Fig. 3) and M1 cells. These data imply that the DM β cytoplasmic tail serves a targeting function for DM complexes similar to that for which the I chain cytoplasmic tail serves for MHC-II, and explains the ability of HLA-DM to reach lysosomal/endosomal structures in the absence of I chain (40, 77).

The compartment to which the DM β tail targeted the Tac chimera was identified as lysosomes by the following criteria: (*a*) it colocalized by both immunofluorescence and immunoelectron microscopy with endogenous lysosomal markers (Figs. 3 and 4); (*b*) it appeared as an electron-dense compartment with typical lysosomal morphology by immunoelectron microscopy (Fig. 4); (*c*) it had proteolytic activity (Fig. 7); and (*d*) proteolysis was inhibited by various pharmacological modifiers of lysosomal protease activity or pH (Figs. 2 and 8). Taken together with the colocalization of T-T-Mb with transfected DM $\alpha\beta$ dimers in leupeptin-treated cells, these data confirm the notion that intact DM molecules are localized to a lysosomal compartment in nonlymphoid cells by virtue of the cytoplasmic tail of the β chain.

Characteristics of the Lysosomal Targeting Signal in DM

The critical component of the DM β targeting signal was determined from deletion analysis to be a tyrosine-based amino acid sequence, YTPL (Fig. 5), that is completely conserved between human and murine forms of the DM β chain (17, 41). A similar motif is absent from all other known MHC class Ia and class II cytoplasmic tails, suggesting that DM may be unique among known MHC molecules regarding its ability to localize to lysosomal compartments in the absence of I chain. On the other hand, similar tyrosine-based YXX \emptyset motifs are found in many lysosomal proteins, such as lamp-1 (GYQTI; reference 25), lamp-2 (GYEQF; reference 25), CD63 (GYEVM; reference 55) and CD68 (AYQAL; reference 33); for lamp-1, this sequence has been shown to mediate lysosomal targeting (28, 31, 34, 88). Unlike the majority of these lysosomal targeting signals, however, the YXX \emptyset motif in DM β is neither located at the COOH terminus of the cytoplasmic tail nor preceded by a glycine or alanine, both of which are characteristics that have been suggested to be critical for lysosomal targeting (31, 34, 88). The YTPL motif also resembles other tyrosine-based motifs implicated in targeting to endosomes or to the TGN (11, 35, 82, 89).

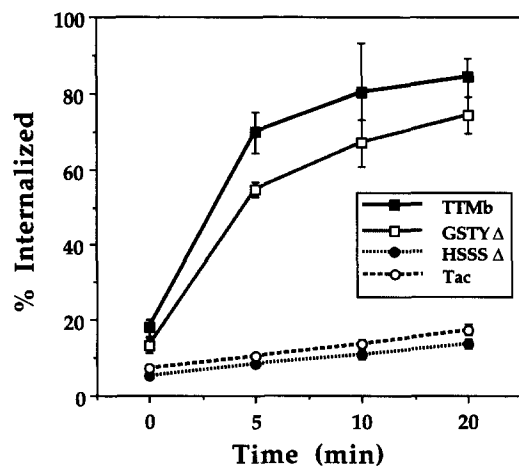
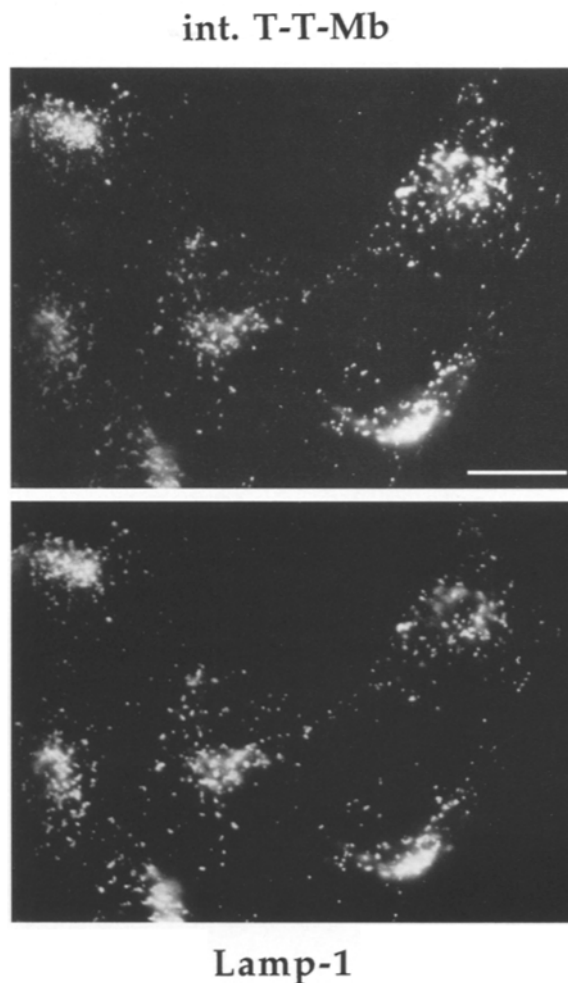
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Figure 10. The cytoplasmic tail of DM β contains an internalization signal. (A) Quantitative antibody internalization assay. Radioiodinated anti-Tac antibody was bound to the surface of HeLa cells stably transfected with T-T-Mb, TTM.GSTY Δ , TTM.HSSSA Δ , or Tac on ice, and washed cells were then allowed to internalize bound antibody at 37°C for the indicated amount of time. Antibody remaining at the cell surface after the 37°C incubation was cleaved by digestion with proteinase K on ice, and cell associated radiolabel before and after proteinase digestions was quantitated at each time point. The % internalization represents the fraction of total cell associated radiolabel at each time point that was proteinase K-resistant. The amounts of radioactive antibody initially bound (CPM per 10⁷ cells) for each transfectant were as follows: T-T-Mb, 5918 \pm 1428; TTM.GSTY Δ , 6540 \pm 369; TTM.HSSSA Δ , 29923 \pm 2033; Tac, 15171 \pm 861. Note that the amount of bound antibody for the Tac transfectant should be considered in light of the lower frequency of cells expressing the transfected gene relative to cells expressing the chimeras (~50%, relative to ~98% for other cells). (B) HeLa cells stably transfected with T-T-Mb and grown on coverslips were exposed to a monoclonal antibody to Tac in the presence of 1 mg/ml leupeptin at 37°C for 4 h. Cells were then fixed and processed for immunofluorescence microscopy using LRSC anti-mouse Ig to detect internalized antibody (*int. T-T-Mb*; upper panel) and rabbit anti-lamp-1 antiserum followed by FITC anti-rabbit Ig to detect the endogenous lysosomal marker lamp-1 (*Lamp-1*; lower panel). When HeLa cells stably transfected with TTM.HSSSA Δ or Tac were similarly incubated with anti-Tac antibody at 37°C, only the cell surface was stained, and untransfected cells showed no subsequent staining with LRSC anti-mouse Ig (data not shown).

The critical components of this signal were shown by alanine scanning mutagenesis to be the tyrosine and leucine (Fig. 6). The tyrosine was also shown to be critical for lysosomal localization of intact DM $\alpha\beta$ dimers (Fig. 9). Surprisingly, mutagenesis of other residues within and around the YTPL sequence or deletion of residues COOH-terminal to the motif had no detectable effect on lysosomal localization (Fig. 6). What features, then, distinguish a lysosomal YXX ϕ targeting signal from a TGN or endosomal signal? The distance of the YXX ϕ signal from the membrane (which is similar among DM β and other lysosomal proteins, such as CD63 [55] and lamp-1 [25], but greater in constitutively recycling receptors [82]), the global conformation of the tyrosine and leucine side chains (19, 87), and sequence determinants that are not revealed by alanine scanning mutagenesis may all play a role in distinguishing precise localization properties of a given signal. On the other hand, the YXX ϕ motifs may be necessary only for gaining access to the sorting machinery by

B

internalization from the cell surface and/or partitioning at the level of the TGN; having accessed the sorting machinery, additional determinants may specify compartmental localization. Defining additional elements in the DM β tail that determine lysosomal targeting will require more extensive mutagenesis and structural approaches.

Implications of Internalization for the Targeting Pathway of T-T-Mb

The DM β cytoplasmic tail contained information not only for lysosomal targeting in NRK and HeLa cells, but also for internalization from the cell surface (Fig. 10). Internalization was extremely rapid in stably transfected HeLa cells, resulting in uptake of 70% of surface-bound antibody after 5 min at 37°C. Deletion analysis showed that, as with lysosomal targeting, the critical feature for directing internalization was the YTPL motif. Furthermore, it was observed by immunofluorescence microscopy that consti-

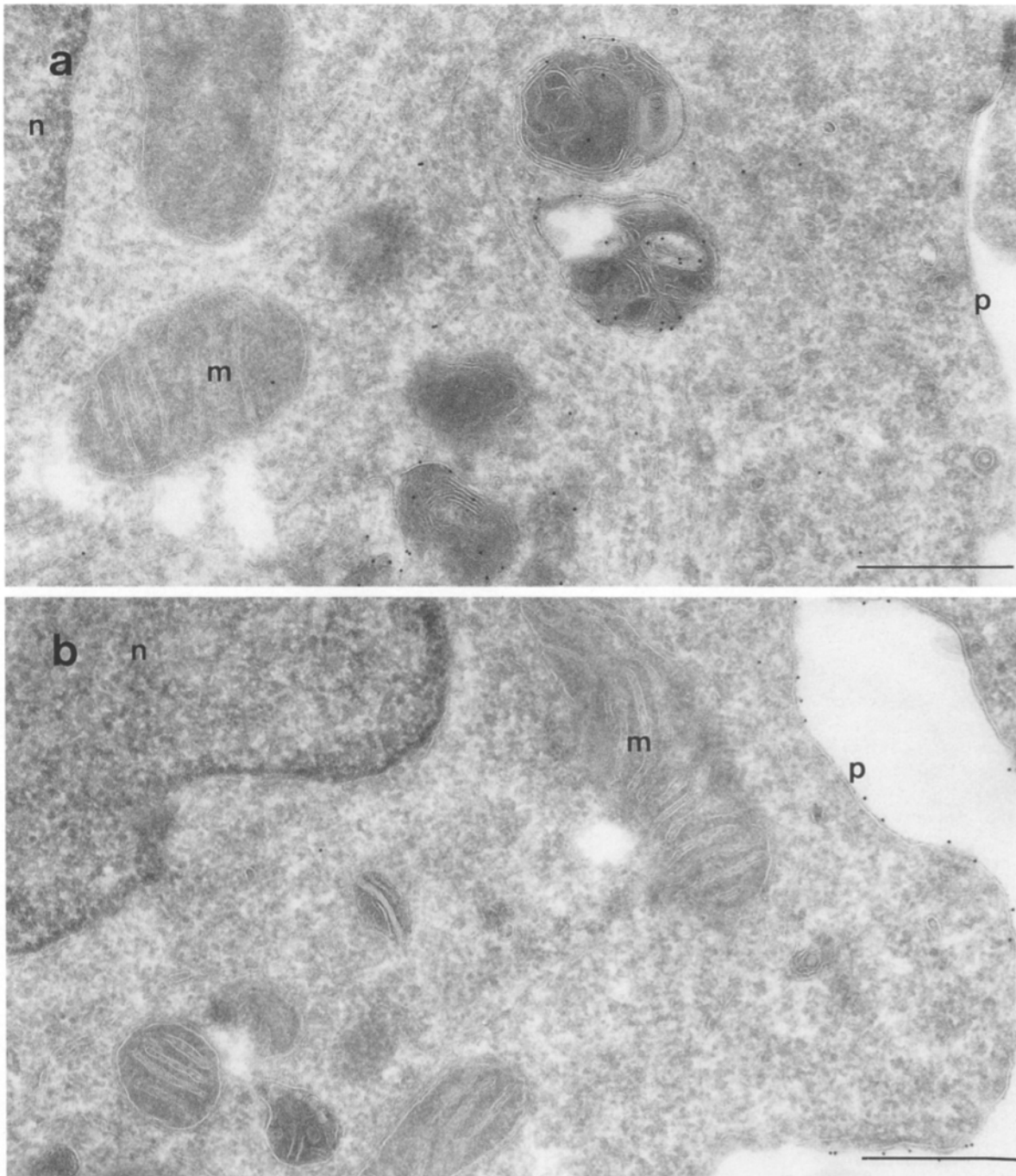


Figure 11. Localization of T-T-Mb and TTM.HSSSA in a B-lymphoblastoid cell line by immunoelectron microscopy. Stable transfectants of the human B-lymphoblastoid cell line, 721.45, expressing T-T-Mb (a) or TTM.HSSSA (b) were treated for 4 h with 1 mg/ml leupeptin, and then fixed with glutaraldehyde and paraformaldehyde. Frozen thin sections were stained with anti-Tac antiserum and protein A conjugated to 10-nm gold particles. Shown are electron micrograph images of representative cell samples. The nucleus (*n*), plasma membrane (*p*), and a mitochondrion (*m*) of each cell are indicated. Note the staining of dense multilaminar and multivesicular structures in *a* and the absence of cell surface staining. Bar, 500 nm.

tutively internalized antibody accumulated in lysosomal structures, colocalizing with the steady state pool of T-T-Mb (Fig. 10 B). The inability to detect internalized antibody in cells expressing the TTM.HSSSA construct or in untransfected cells suggests that the antibody accompanied internalized chimeric protein to the lysosomal structures. We thus conclude that at least a portion of T-T-Mb

molecules present in lysosomes are trafficked first through the cell surface.

The trafficking of a lysosomally targeted transmembrane protein through the cell surface is not unprecedented. Similar indirect modes of delivery to late secretory compartments have been suggested and/or demonstrated for a number of lysosomal proteins (13, 54, 57) as well as

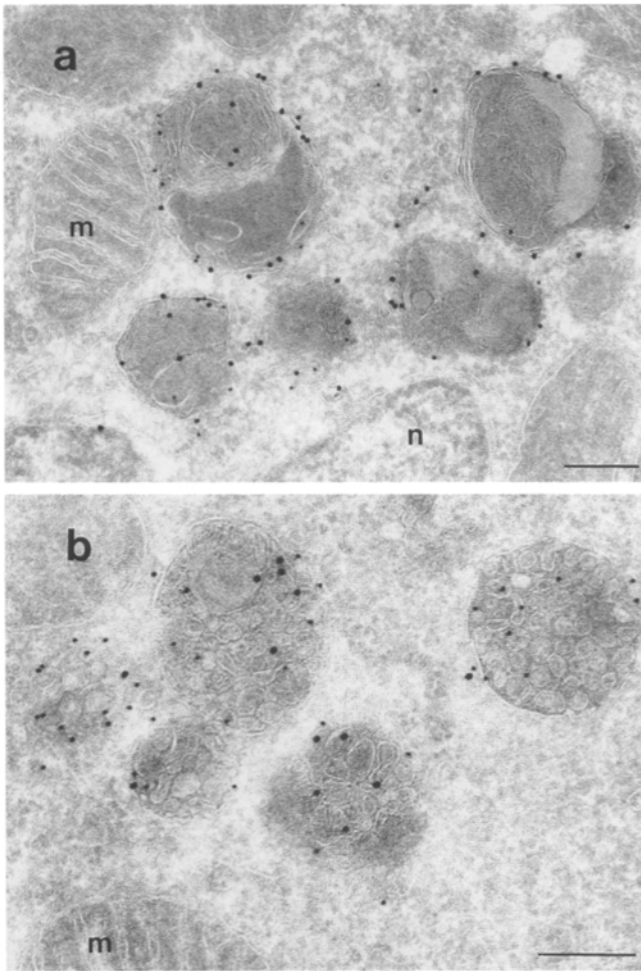


Figure 12. Presence of T-T-Mb in the MIIC and lysosomes of B-lymphoblastoid cells. A stable transfectant of 721.45 cells expressing T-T-Mb was treated for 4 h with 1 mg/ml leupeptin, and then fixed and processed for cryosectioning and immunolabeling. Cells were labeled first with an antiserum to Tac followed by 10-nm gold particle-conjugated protein A, and then antisera to either lamp-1 (a) or to MHC-II DR α molecules (b) followed by 15-nm gold particle-conjugated protein A. A mitochondrion (m) and the nucleus (n) in the cells are indicated. Note the absence of MHC-II staining (15 nm gold) in some of the T-T-Mb-stained structures in b. Bar, 200 nm.

MHC-II/I chain complexes (5, 15, 71, 73). If intact DM molecules were similarly trafficked through the cell surface, they might be accessible to a cohort of MHC-II molecules and endocytosed antigens present in earlier endocytic compartments, as suggested by others (1, 15, 21, 58, 66, 83). This would provide a full complement of antigen processing molecules throughout the endocytic pathway. On the other hand, evidence also exists for direct transport of lysosomal proteins (31) and MHC-II molecules (5, 63, 64) from the TGN without prior cell surface delivery, suggesting that the indirect pathway may be a retrieval mechanism for missorted proteins. Accumulation of T-T-Mb at the cell surface was clearly exacerbated by overexpression in transiently transfected HeLa and COS-1 cells (data not shown), and some degree of overexpression and consequent missorting to the cell surface cannot be

ruled out even in our stable transfectants. Lastly, an intriguing explanation for the observed internalization is that it may represent a physiological salvage mechanism to retrieve DM molecules that had been delivered from the MIIC to the cell surface together with peptide-loaded MHC-II molecules. Such a mechanism would allow for the recycling of DM molecules for use in additional antigen processing events, whereas peptide-bound MHC-II molecules, now lacking a strong internalization signal, would remain at the cell surface.

The Lysosome as an Alternative Destination for MIIC-resident Proteins

Endogenous DM has been shown to localize in human B lymphoblastoid cell lines and dendritic cells to the MIIC (60, 77). We have now shown that the cytoplasmic tail of DM β is sufficient to target Tac to the MIIC when transfected into the B cell line, 721.45 (Figs. 11 and 12). This demonstrates that MIIC localization is mediated by tail-associated mechanisms similar or identical to those that mediate localization to other endosomal compartments and lysosomes.

Besides tyrosine motifs, some lysosomal and internalized proteins rely on a second type of targeting determinant, a di-leucine motif, to effect their localization (20, 38, 46). The I chain contains two modified di-leucine motifs, and it is believed that both of these signals are responsible for targeting I chain, and hence MHC-II molecules, to the MIIC or related compartments (4, 61, 65). Thus, taken together with the results shown here, it is likely that targeting to the MIIC, like to other post-Golgi compartments, can be mediated by both types of cytoplasmic signal. It remains to be seen, however, whether the I chain di-leucine motifs direct newly synthesized MHC-II to the identical compartment as that to which DM is directed, or to a separate subdomain of the MIIC or subcompartment of the endosomal system.

The fact that the DM β cytoplasmic tail sequence mediates localization to lysosomes in nonlymphoid cells and to the MIIC in B lymphoid lines suggests that lysosomes may serve as the “default” destination for DM and perhaps other proteins in cells that lack a specialized antigen processing compartment. Delivery of proteins to other cell type-specific organelles, such as the cytolytic granules of cytotoxic T lymphocytes and NK cells (62) and secretory granules of rat basophilic leukemia cells (9), may also have a component that overlaps with lysosomal delivery. Segregation of these organelles from classical lysosomes may depend upon either cell type-specific targeting and/or fusion machinery components, or interactions between luminal and/or transmembrane domains of cell type-specific cargo molecules. An understanding of the ultrastructural localization of DM and T-T-Mb in different cell types and the biochemical interactions among MIIC-resident proteins will contribute further insights into the biogenesis of this late endosomal organelle.

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