The Targeting of Lamp1 to Lysosomes Is Dependent on the Spacing of its Cytoplasmic Tail Tyrosine Sorting Motif Relative to the Membrane

Jack Rohrer, Anja Schweizer, David Russell,* and Stuart Kornfeld

Department of Medicine, *Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract. Lamp1 is a type I transmembrane glycoprotein that is localized primarily in lysosomes and late endosomes. Newly synthesized molecules are mostly transported from the *trans*-Golgi network directly to endosomes and then to lysosomes. A minor pathway involves transport via the plasma membrane. The 11–amino acid cytoplasmic tail of lamp1 contains a tyrosine-based motif that has been previously shown to mediate sorting in the *trans*-Golgi network and rapid internalization at the plasma membrane. We studied whether this motif also mediates sorting in endosomes. We found that mutant forms of lamp1 in which all the amino acids of the cytoplasmic tail were modified except for the RKR membrane anchor and the YXXI sorting motif still localized to dense lysosomes, indicating that the YXXI motif is sufficient to confer proper intracellular targeting. However, when the spacing of the YXXI motif relative to the membrane was changed by deleting one amino acid or adding five amino acids, lysosomal targeting was almost completely abolished. Kinetic studies showed that these mutants were trapped in a recycling pathway, involving trafficking between the plasma membrane and early endocytic compartments.

These findings indicate that the YXXI signal of lamp1 is recognized at several sorting sites, including the *trans*-Golgi network, the plasma membrane, and the early/sorting endosomes. Small changes in the spacing of this motif relative to the membrane dramatically impair sorting in the early/sorting endosomes but have only a modest effect on internalization at the plasma membrane. The spacing of sorting signals relative to the membrane may prove to be an important determinant in the functioning of these signals.

Lysosomes are acidic vacuoles involved in the degradation of extracellular and intracellular material (de Duve, 1963; Kornfeld and Mellman, 1989; Winchester, 1992). The limiting membrane of the lysosome is enriched in a family of highly glycosylated integral membrane proteins, termed lgps or lysosome-associated membrane protein (lamps)[1]. These proteins are also distributed in endosomes (Griffiths et al., 1988). The lamps have been divided into three different groups based on their amino acid sequences, but their overall domain structure is conserved and consists of a large lumenal domain containing 16–20 N-glycosylation sites, a single transmembrane-spanning region, and a short cytoplasmic tail of 10–11 amino acids (Fukuda, 1991).

Biosynthetic studies of various lamps have indicated that these molecules can be targeted to lysosomes by two routes. The first involves the transport of the lamp from the *trans*-Golgi network (TGN) directly to endosomes and then to lysosomes. In the second pathway, the lamps are initially delivered from the Golgi to the cell surface where they are internalized, and then transported along the endocytic pathway to lysosomes. Indirect transport via the plasma membrane has been suggested for Lep100 in chicken fibroblasts (Lippincott-Schwarz and Fambrough, 1987) and mouse L cells (Mathews et al., 1992), AC17 in MDCK cells (Nabi et al., 1991) and the precursor of lysosomal acid phosphatase (LAP) in stably transfected BHK cells (Braun et al., 1989), while direct transport from the TGN to the endosomal system has been shown for rat lgp120 in NRK, CHO, and MDCK cells (Green et al., 1987; Harter and Mellman, 1992; Höning and Hunziker, 1995), for LimpII in transfected COS cells (Vega et al., 1991; Sandoval et al., 1994; Ogata and Fukuda, 1994), for endogenous LAP in rat hepatocytes (Tanaka et al., 1990) and for lamp1 in 3T3 cells (D'Souza and August, 1986) and HL-60 cells (Carlsson and Fukuda, 1992).

Mutational analysis of various lamps has revealed that their cytoplasmic tails contain determinants that are both necessary and sufficient for their targeting to lysosomes (Williams and Fukuda, 1990; Harter and Mellman, 1992;

Please address all correspondence to S. Kornfeld, Washington University School of Medicine, Division of Hematology, Box 8125, 660 S. Euclid Avenue, St. Louis, MO 63110. Tel.: (314) 362-8803. Fax: (314) 362-8826.

1. *Abbreviations used in this paper*: CD-MPR, cation-dependent MPR; Lamp, lysosome-associated membrane protein; lgp, lysosomal glycoprotein; MPR, mannose 6-phosphate receptor; Man-6-P/IGFII receptor, mannose 6-phosphate/insulin-like growth factor II receptor.

Guarnieri et al., 1993; Höning and Hunziker, 1995). In the case of lamp1 and lgp120, the GYQTI motif has been shown to be necessary for the protein to be sorted in the TGN for delivery to endosomes (Williams and Fukuda, 1990; Harter and Mellman, 1992; Guarnieri et al., 1993; Höning and Hunziker, 1995). While this sorting step is quite efficient, a small percentage of lamp1 escapes to the plasma membrane where it is rapidly internalized due to the YQTI signal. The glycine residue, which is necessary for sorting in the TGN, is not required for efficient internalization at the plasma membrane. Another sorting step occurs in the early/sorting endosome where the lamps are selected for delivery to late endosomes whereas recycling receptors, such as the transferrin receptor (Tf-R) return to the cell surface by a default mechanism (Mayor et al., 1993). The cytoplasmic tail of lamp1 is capable of mediating this sorting step, as determined by the finding that the lamp1 cytoplasmic tail is sufficient to direct a chimeric protein to lysosomes (Williams and Fukuda, 1990; Höning and Hunziker, 1995). A fourth sorting step occurs in the late endosome where lamp1 is delivered to dense lysosomes whereas the mannose 6-phosphate receptors are excluded from that organelle (Rohrer et al., 1995). While it has been demonstrated that there is a default pathway from late endosomes to lysosomes (Rohrer et al., 1995), the existence of a signal mediated step that makes this process more efficient has also been suggested (Green et al., 1994).

Although the signals for the sorting of lamp1 in the TGN and at the plasma membrane have been extensively characterized, the nature of the signal in the cytoplasmic tail of lamp1 that directs sorting in the early/sorting endosome is unknown. Since lamp1 is sorted from the transferrin receptor at this site, and the latter protein also contains a tyrosine-based sorting signal, we considered three possibilities for how this segregation is achieved. One is that the particular sequence of the lamp1 tyrosine motif (YQTI) is recognized by a sorting protein(s) in the early/sorting endosome whereas the transferrin receptor signal (YTRF) fails to interact with this protein(s). Alternatively, the endosomal-sorting signal could require additional residues in the cytoplasmic tail, similar to the need for the glycine residue in Golgi sorting. A third possibility is that the spacing of the tyrosine-based signal from the membrane influences the interaction with the sorting protein(s) in the endosome. Differences in spacing of the signal, as it occurs in various proteins (Trowbridge et al., 1993), could potentially determine whether or not the signal is functional at a particular sorting station. To distinguish between these three possibilities, we generated a series of mouse L cells stably expressing mutant forms of human lamp1 and determined the subcellular distribution and trafficking of the transfected proteins. In one set of constructs various amino acid residues of the cytoplasmic tail were substituted with alanines and/or other amino acids, keeping the YXXI internalization motif intact to avoid mislocalization to the plasma membrane. None of these mutants had an altered lysosomal distribution demonstrating that the YXXI motif is sufficient for targeting lamp1 to lysosomes. In contrast, mutant forms of lamp1 in which the spacing of the YXXI motif relative to the transmembrane domain was changed by deleting one amino acid or adding five amino acids became trapped in a recycling pathway between early/sorting endosomes and the plasma membrane and failed to be delivered to lysosomes.

Materials and Methods

Materials

Enzymes used in molecular cloning were obtained from Boehringer Mannheim (Indianapolis, IN), New England Biolabs (Beverly, MA), or Promega (Madison, WI). α-minimal essential medium (α-MEM), Iscoves media and FBS as well as Lipofectin were from GIBCO BRL (Gaithersburg, MD); Percoll from Pharmacia (Uppsala, Sweden); nitrocellulose from Schleicher and Schuell (Keene, NH); protease inhibitors from Sigma Chem. Co. (St. Louis, MO); ECL Western blotting reagents from Amersham Corp. (Arlington Heights, IL); Expre^{35}S^{35}S label from New England Nuclear (DuPont Co., Wilmington, DE); NHS-SS-biotin from Pierce Chem. Co. (Rockford, IL); protein A–Sepharose beads from Repligen Corp. (Cambridge, MA); cell culture dishes from Falcon (Becton Dickinson Corp., Lincoln Park, NJ). Oligonucleotides were synthesized with a 380A Applied Biosystems solid phase synthesizer (Foster City, CA) by the Protein Chemistry Facility of Washington University.

Recombinant DNA

All basic DNA procedures were as described (Sambrook et al., 1989). For all the PCR reactions pBSK-Lamp1 (Rohrer et al., 1995) was used as a template and bp 998-1018 of lamp1 was used as a downstream primer. Point mutants within the cytoplasmic tail of lamp1 were created using standard PCR protocols. Appropriate oligonucleotides encoding the desired alanine replacements or alanine insertions as well as a MluI site at the 5′ end were used as upstream primers. The resulting PCR products were digested with Eco47III and MluI and the purified fragment was assembled with the EcoRI–Eco47III fragment of pBSK-Lamp1 and the EcoRI–MluI fragment of pSFFVneo (Fuhlbridgge et al., 1988) in a three part ligation.

The final plasmids were sequenced using the Sanger dideoxy chain termination method (Sanger et al., 1977) to verify that the mutant constructs were correct.

Cell Culture and Transfection

A Man-6-P/IGF-II receptor–deficient mouse L cell line designated D9 (LRec$^-$) was maintained in α-minimal essential medium (α-MEM) containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% humidified CO_2 atmosphere (Gabel et al., 1983). The cells were transfected with 20 μg of XbaI linearized DNA using Lipofectin (GIBCO BRL) according to the manufacturer's directions. Selection for resistance to neomycin (G418) was as described previously (Lobel et al., 1989) except that the final G418 concentration was 500 μg/ml. Resistant colonies were screened for human lamp1 expression by immunoblotting. Selected clones were expanded for further study and maintained in selective medium.

Antibodies

Monoclonal antibody (mAb) G1/139 against human lamp1 (Schweizer et al., 1988) and rabbit polyclonal antisera 931-A against human lamp1 (Carlsson et al., 1988) were kind gifts of Dr. H.-P. Hauri (Biozentrum, Basel, Switzerland) and Dr. M. Fukuda (Cancer Research Center, La Jolla, CA), respectively. Rat monoclonal antibodies against murine lamp1 (1D4B) were obtained from the Developmental Studies Hybridoma Bank (Baltimore, MD). HRP-conjugated anti–mouse secondary antibody was from Amersham Corp.

Percoll Gradient Fractionation

Confluent cells grown in a 100-mm petri dish were incubated for 12 h in growth medium supplemented with 100 μM each of pepstatin A and leupeptin. The cells were then harvested, ruptured with a ball bearing homogenizer, and fractionated on 18% Percoll density gradients as described previously (Rohrer et al., 1995).

Cell Surface Appearance of Lamp1

Confluent cells grown in six-well plates were rinsed twice with PBS and preincubated in methionine and cysteine-free growth medium containing 10% dialyzed FCS and 20 mM Hepes, pH 7.4, for 20 min. The cells were then incubated for 30 min with 200 μCi of EXPRE^{35}S^{35}S protein-labeling mixture in preincubation medium, and chased in normal culture medium supplemented with 10 mM unlabeled methionine and 20 mM Hepes, pH 7.4, for 0 min, 30 min, 1, 8, or 24 h. At the end of the chase the cells were chilled on ice and washed twice with ice cold PBS followed by five wash steps with PBS supplemented with 0.7 mM $CaCl_2$ and 0.25 mM $MgSO_2$ (PBS++) over a period of 30 min. NHS-SS-biotin was added at a concentration of 3 mg/ml in PBS++ for 45 min on ice. Biotinylation was stopped by washing four times with 50 mM glycine in PBS++. The cells were lysed in buffer II (100 mM sodium phosphate, pH 8.0, containing 1% Triton X-100 and a 1:500 dilution of a protease inhibitor cocktail [5 mg/ml benzamidine, and 1 mg/ml each of pepstatin A, leupeptin, antipain, and chymostatin in 40% dimethylsulfoxide-60% ethanol]) and centrifuged for 1 h at 100,000 *g* in a Ti 50 rotor (Beckman Instruments Inc., Palo Alto, CA). The resulting supernatants were collected and used for immunoprecipitation. For immunoprecipitation of lamp1 and mutant forms, 25 μl of protein A–Sepharose was washed once with 1 ml buffer I (100 mM sodium phosphate, 0.2% BSA, pH 8.0), and then incubated with 2 μl of a rabbit antiserum against human lamp1 in 500 μl of buffer I overnight at 4°C. The beads were then washed twice with buffer I and once with buffer II. The washed beads and the cleared supernatants from the cell lysates were combined and incubated as described previously (Rohrer et al., 1995). The immunocomplexes were released from the beads by boiling the samples for 10 min in 160 μl buffer III (100 mM sodium phosphate, pH 8.0) plus 0.5% SDS. One quarter of the supernatant was directly analyzed by SDS-PAGE whereas the other three quarters were transferred to a tube containing 80 μl of Streptavidin-agarose (Pierce Chem. Co.) and 1 ml buffer II. After an incubation of at least 3 h at 4°C the beads were pelleted, washed three times with buffer II, once with buffer III and once with buffer IV (10 mM sodium phosphate, pH 8.0). Biotinylated lamp1 molecules were eluted from the streptavidin-agarose beads by boiling the samples for 10 min with 40 μl 2× sample buffer (containing 0.2 M DTT) and analyzed by SDS-PAGE as described below.

Internalization Assay

The internalization of lamp1 was measured using NHS-SS-biotin (Pierce Chemical Co.) as previously described (Bretscher and Lutter, 1988). Cells grown in six-well plates were rinsed twice with ice cold PBS, twice with PBS++, and then incubated with 3 mg/ml NHS-SS-biotin in PBS++ for 15 min to biotinylate cell surface proteins. Biotinylation was stopped by washing once with 50 mM glycine in PBS++ and twice with PBS++. Some of the cells were then incubated at 37°C with prewarmed growth media containing 10% FCS and 20 mM Hepes, pH 7.4, for different periods of time (1 min, 5 min, 15 min, and 45 min). The cells were returned to 4°C to stop protein internalization, and then incubated on ice twice for 20 min in a freshly prepared glutathione solution (50 mM glutathione, 75 mM sodium chloride, 1 mM EDTA, pH 8.0, 1% BSA, 75 mM sodium hydroxide) to remove the biotin from proteins that were present on the cell surface. After reduction, the excess glutathione was quenched with a 5-min incubation in PBS++ containing 5 mg/ml iodacetamide. The cells were then washed twice with PBS++, lysed in 1 ml buffer II, and passed five times through a 25-gauge needle connected to a 1-ml syringe. The resulting homogenates were centrifuged for 1 h at 100,000 *g* in a Ti50 rotor and the supernatant subjected to immunoprecipitation using a polyclonal antibody against human lamp1 as described above. The immunocomplexes were released from the protein A–Sepharose beads by boiling for 3 min in electrophoresis sample buffer (containing 62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol and 0.001% bromophenol blue). The samples were further analyzed by SDS-PAGE and immunoblotting with a streptavidin-HRP conjugate as described below.

Steady-State Surface Distribution

Cells were grown in six-well plates and biotinylated as described above for the cell surface appearance assay. After the incubation with 50 mM glycine and two wash steps with PBS++, the cells were lysed in buffer II and passed five times through a 25-gauge needle connected to a 1-ml syringe. The samples were then immunoprecipitated using a polyclonal antibody against human lamp1 as described above. The immunocomplexes were eluted from the protein A–Sepharose beads by boiling the samples for 10 min in 120 μl buffer III containing 0.5% SDS. The eluate was transferred to a tube containing 80 μl of streptavidin-agarose and 400 μl buffer II. After an incubation of at least 3 h at 4°C, the beads were pelleted and the supernatants containing lamp1 molecules that were not biotinylated were carefully collected. The proteins in these supernatants were precipitated by the method of Wessel and Flügge (1984). The precipitates were solubilized in 50 μl of 2× sample buffer containing 0.2 M DTT. The beads were washed twice with buffer II, and the biotinylated lamp1 molecules were released by boiling the beads for 10 min in 50 μl of 2× sample buffer (containing 0.2 M DTT). The samples were further analyzed by SDS-PAGE and immunoblotting as described below.

SDS-PAGE, Fluorography, and Immunoblotting

Proteins were separated on 8% SDS-polyacrylamide mini-gels (BioRad Laboratories) using the Laemmli (1970) system. After electrophoresis gels were either treated with Amplify, dried and exposed to film (XOmatAR, Eastman Kodak Co., Rochester, NY), or transferred onto nitrocellulose membranes according to the method of Towbin et al. (1979). The nitrocellulose sheets were blocked with 3% nonfat dry milk powder (Schnuck Markets Inc., St. Louis, MO) in PBS. For the Percoll density fractionation experiments and the experiments to determine the cell surface distribution of the lamps at steady state, the blots were subsequently incubated with mAb G1/139 against lamp1 (diluted 1:1,000 in PBS-3% powdered milk) followed by an HRP-conjugated anti–mouse secondary antibody (Amersham Corp.). For internalization assays, the nitrocellulose membranes were washed two additional times in PBS and then incubated with a streptavidin-HRP conjugate. Immunoreactive proteins were visualized using the enhanced chemiluminescence detection system according to the manufacturer's directions (Amersham Corp.). The autoradiographs were quantitated using a personal densitometer (Molecular Dynamics Inc., Sunnyvale, CA).

Immunoelectron Microscopy

Immunoelectron microscopy was performed on frozen sections from murine L cells fixed in 1% glutaraldehyde in Hepes saline and infiltrated in PVP/sucrose as described previously (Russell et al., 1992; Russell, 1994). Sections were probed with rat anti-murine lamp1 (1D4B) (Chen et al., 1986) and murine anti-human lamp1 (G1/139) (Schweizer et al., 1988), followed by goat anti–rat IgG conjugated to 6 nm gold and goat anti–mouse IgG conjugated to 18 nm gold (both from Jackson Immunoresearch Laboratories, West Grove, PA). Controls were conducted with nontransfected L cells (for G1/139) and HepG2 cells (for 1D4B). In both instances under the conditions used background labeling remained below 5% of the specific label.

Surface biotinylation of L cells was performed on ice for 20 min with 1 mg/ml sulfo-NHS-biotin (Pierce) in PBS, pH 7.6, as described (Russell, 1994). After biotinylation, the cells were washed in two changes of cold medium before the addition of DMEM (no bicarbonate) with 20 mM Hepes, pH 7.2, and 10% FBS at 37°C. The culture was placed on a warmed block at 37°C for 7.5 min, and then fixed and processed for immunoelectron microscopy as described above. Biotinylated cell proteins, and human lamp1, were detected by reacting the sections with streptavidin (1 μg/ml), and then affinity-purified rabbit anti-streptavidin (1 μg/ml) and mouse anti-human lamp1 (G1/139), followed by goat anti–rabbit IgG conjugated to 6 nm gold and goat anti–mouse IgG conjugated to 18 nm gold (Russell, 1994). This method is markedly more sensitive than detection with streptavidin gold.

The relative distribution of gold particles was scored by counting all gold particles in eight negatives taken at 26,000 magnification for each experimental condition. Colocalization of human lamp1 label with either 1D4B (murine lamp1) or biotinylated cell plasma membrane components was only scored positive if the vesicles contained two or more gold particles corresponding to murine lamp1 or biotin. The standard deviations shown in Fig. 8 were calculated by treating each individual negative as an independent data set.

Results

The YXXI Motif in the Cytoplasmic Tail of Lamp1 Is Sufficient for Targeting to Lysosomes

To define features of the lamp1 cytoplasmic tail required for sorting at the different stations enroute to lysosomes,

we initially generated three constructs in which various portions of the wild-type sequence were replaced with either alanines and/or serine-lysine (Fig. 1 *A*). In one construct (Lamp 382-83A) Ser^{382} and His^{383} in the stem portion of the cytoplasmic tail were changed to alanines. In a second construct (Lamp/SK), Gln^{387} and Thr^{388}, which form part of the tyrosine sorting motif, were changed to Ser and Lys, respectively, to mimic the trafficking signal of the Man-6-P/IGF-II receptor (Canfield et al., 1991; Jadot et al., 1992). The third construct (Lamp 382-85A/SK) had amino acids 382-385 replaced by alanines as well as Gln^{387} and Thr^{388} changed to Ser and Lys, respectively. The YXXI motif was preserved in all the mutants to avoid mislocalization to the plasma membrane (Williams and Fukuda, 1990; Harter and Mellman, 1992; Guarnieri et al., 1993; Höning and Hunziker, 1995). The constructs were stably transfected into mouse L cells and the distribution of the expressed proteins analyzed by isosmotic Percoll density gradients which separate dense lysosomes from other organelles (Green et al., 1987). The fractions from the gradients were combined into three pools: pool I contains the bulk of the dense lysosomes (70–80% of β-hexosaminidase activity), pool II contains intermediate density membranes and pool III contains low density membranes, including the endoplasmic reticulum, Golgi complex, plasma membrane, and endosomes (Rohrer et al., 1995). For the 12 h before harvesting the cells, the monolayers were grown in media supplemented with pepstatin A and leupeptin to prevent potential degradation of mutant lamps in lysosomes.

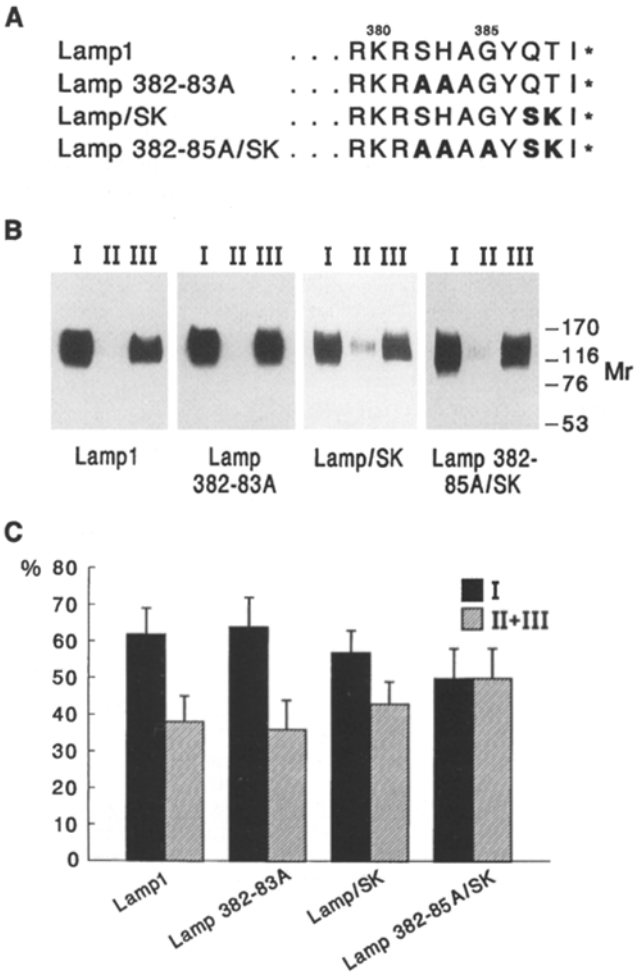

Figure 1. Point mutations within the cytoplasmic tail of lamp1 do not significantly affect lysosomal targeting. (*A*) Sequence of the cytoplasmic tail of lamp1. Amino acids replacing the wild-type sequence are shown in bold letters. (*B*) Mouse L cells stably expressing lamp1, lamp 382-83A, lamp/SK, and lamp 382-85A/SK were preincubated with pepstatin A and leupeptin for 12 h. The cells were then homogenized with a ball bearing homogenizer and postnuclear supernatants were subjected to Percoll density gradient centrifugation (18% Percoll). The collected fractions were combined into pool I, II, and III (three each) and further analyzed by SDS-PAGE and immunoblotting with mAB G1/139. (*C*) Immunoblots shown in *B* and those from additional experiments were quantitated. For each construct the value of pool I (dense lysosomes, *filled bars*) and the values of pool II and III combined (*striated bars*) were expressed as their percentage of the sum of all three pools.

As shown in Fig. 1 *B*, all three mutant proteins were recovered in dense lysosomes to about the same extent as wild-type lamp1. When the results of several experiments were quantitated, it was determined that 64 ± 10% of lamp 382-83A, 54 ± 6% of lamp/SK and 50 ± 8% of lamp 382-85A/SK was distributed in dense lysosomes vs 62 ± 11% of lamp1 (Fig. 1 *C*).

Taken together, these results show that YXXI is the only possible signal in the cytoplasmic tail of lamp1 for sorting to dense lysosomes.

Spacing of the YQTI Sequence Is Crucial for Lysosomal Targeting of Lamp1

We next analyzed the importance of the spacing between the YQTI sequence and the transmembrane domain for lysosomal targeting. A series of constructs were prepared in which Ala^{384} was either removed (Lamp d384) or alanine spacers of one, three, or five residues were introduced between amino acids His^{383} and Ala^{384} (Lamp S1, Lamp S3, and Lamp S5) (Fig. 2 *A*). When postnuclear supernatants of cells expressing these constructs were analyzed on Percoll density gradients, Lamp S1 and Lamp S3 had essentially normal distributions (Fig. 2 *B*). In contrast, Lamp d384 and Lamp S5 were almost completely excluded from the dense lysosomes. Quantitation of several experiments showed that just 3 ± 3% of Lamp d384 and 2 ± 1% of Lamp S5 were present in dense lysosomes whereas 59 ± 6% of Lamp S1 and 46 ± 7% of Lamp S3 were recovered in that organelle (Fig. 2 *C*).

These data show that the spacing of the YQTI sequence relative to the transmembrane domain is crucial for delivery of lamp1 to dense lysosomes.

Newly Synthesized Lamp d384 and Lamp S5 Appear on the Cell Surface

To determine which step(s) in the delivery of Lamp d384 and Lamp S5 to dense lysosomes is blocked, biosynthetic studies were performed in conjunction with surface biotinylation to detect those molecules that appear at the plasma membrane. Cell lines expressing either the wild-type lamp1 or the various spacing mutants were labeled for 30 min with [^{35}S]methionine/cysteine and chased for up to

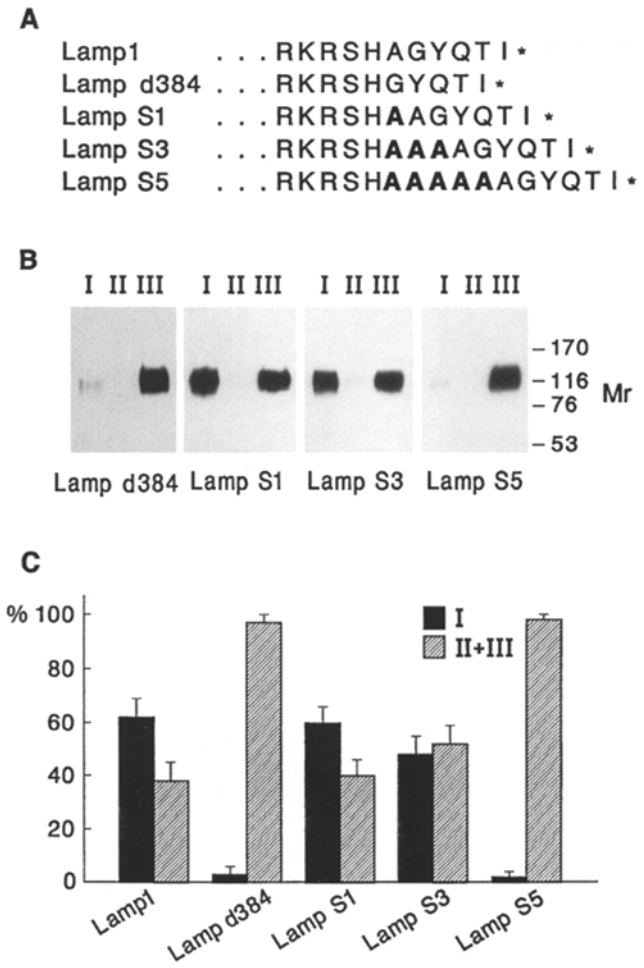

Figure 2. Effect of spacing of the YQTI signal on lysosomal targeting of lamp1. (*A*) Sequence of the cytoplasmic tail of lamp1. Alanines inserted into the wild-type sequences are shown in bold letters. (*B*) Mouse L cells stably expressing Lamp d384, Lamp S1, Lamp S3, and Lamp S5 were preincubated with pepstatin A and leupeptin for 12 h and then fractionated as described in Fig. 1. Proteins of pool I, II, and III were subjected to SDS-PAGE and immunoblotting with mAb G1/139. (*C*) Immunoblots shown in *B* and those from additional experiments were quantitated. For each construct the value of pool I (dense lysosomes, *filled bars*) and the values of pool II and III combined (*striated bars*) were expressed as their percentage of the sum of all three pools.

24 h. At various times during the chase, cells were chilled to 4°C and incubated with the membrane impermeable probe NHS-SS-biotin to biotinylate lamp molecules that were present on the cell surface. The lamps were then immunoprecipitated and one quarter of the material was used to determine the total amount of labeled protein while the other three quarters were incubated with streptavidin-agarose beads to determine the content of biotinylated (surface) molecules. The two samples were analyzed by SDS-PAGE, autoradiography, and scanning densitometry to determine values for the total and surface molecules. The quantitative data from several experiments is shown in Fig. 3. As previously reported, only 3% of lamp1 was detected at the cell surface after 1 h of chase and by 3 h of chase surface lamp1 was undetectable (Harter and Mellman, 1992; Rohrer et al., 1995). Somewhat more, Lamp S1 and Lamp S3 reached the cell surface (maximum of 13% and 6% after 1 h, respectively), but by 24 h only 3% of Lamp S1 and 1% of Lamp S3 remained at the cell surface. In contrast to these findings, 49% of Lamp d384 and 54% of Lamp S5 was detected on the cell surface at 3 h of chase and 39% of each protein was still present on the cell surface after 24 h of chase. As shown in Fig. 3, while the appearance of wild-type lamp1 at the cell surface was maximal at 1 h of chase, the peak level of Lamp d384 and Lamp S5 on the cell surface did not occur until 3 h of chase. The autoradiograph from this experiment also revealed that all of the mutant lamps exited the ER and underwent N-linked oligosaccharide processing in the Golgi with the same kinetics as the wild-type lamp1 (data not shown).

The pulse chase experiments indicated that a significant proportion of Lamp d384 and Lamp S5 was present on the cell surface at steady-state. This was confirmed in an independent manner. Cells expressing the various constructs were chilled on ice and incubated with NHS-SS-biotin to label the surface proteins. The cells were then solubilized and the lamps were immunoprecipitated with rabbit anti-human lamp1 antibody. The resultant immunocomplexes were dissociated and incubated with streptavidin-agarose beads to bind the biotinylated (surface) molecules. The supernatant fluids, containing the nonbiotinylated (intracellular) lamp molecules, were carefully collected and the two fractions were analyzed by SDS-PAGE and immuno-

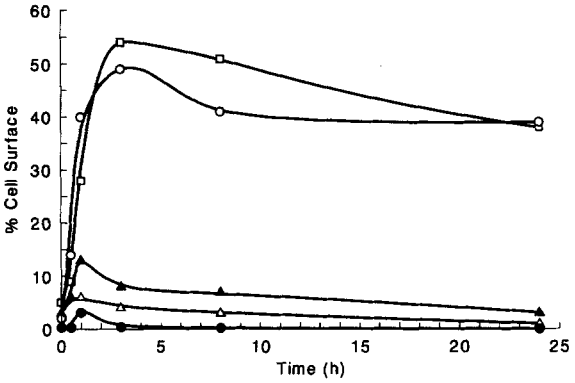

Figure 3. Surface appearance of newly synthesized lamp1 and mutant forms. Mouse L cells stably expressing lamp1, Lamp d384, Lamp S1, lamp S3, or Lamp S5 were labeled with [^{35}S]methionine/cysteine for 30 min and then chased for the indicated times. At each time point labeled cell surface proteins were derivatized using NHS-SS-biotin. The cells were lysed, and the surface-biotinylated and internal lamp1 molecules were immunoprecipitated using a polyclonal anti-Lamp1 antibody (931-A). After solubilization of the first immunoprecipitate, three quarters of each sample was incubated with streptavidin agarose beads to precipitate surface-biotinylated molecules. All precipitates were analyzed by SDS-PAGE and autoradiography and the autoradiographs of several experiments were quantitated by scanning densitometry. The values presented in the figure represent the percentage of lamp1 (●), Lamp d384 (○), Lamp S1 (▲), Lamp S3 (△), and Lamp S5 (□) detected at the cell surface relative to the total amount detected at a given time point.

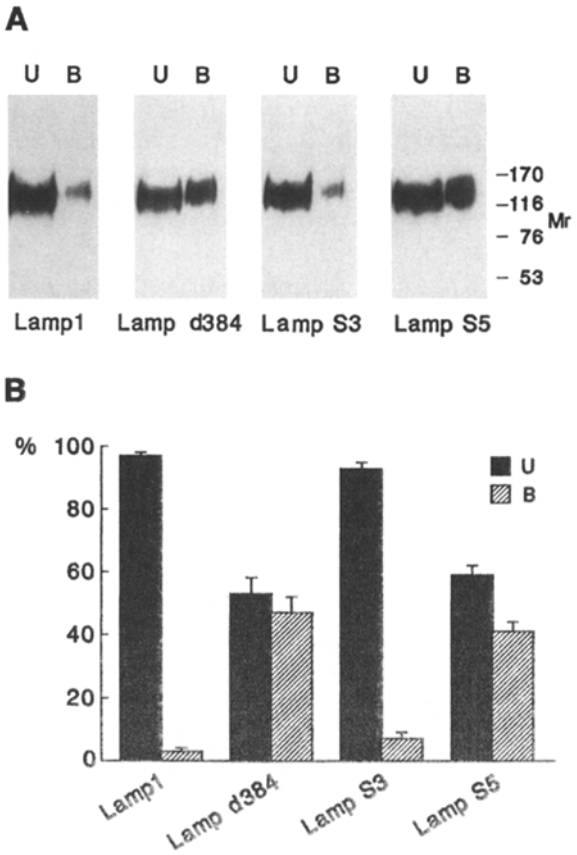

Figure 4. Steady state cell surface distribution of lamp1 and mutant forms. (*A*) Cell surface proteins of mouse L cells stably expressing lamp1, Lamp d384, Lamp S3, and Lamp S5 were derivatized using NHS-SS-biotin. The cells were lysed and the surface-biotinylated and internal Lamp1 molecules were immunoprecipitated using a polyclonal anti-Lamp1 antibody. After solubilization of the first immunoprecipitate the samples were incubated with streptavidin-agarose beads to precipitate surface-biotinylated molecules. The supernatant containing proteins that did not bind to streptavidin (unbound, *U*) and the precipitate containing proteins that did bind to streptavidin (bound, *B*) were analyzed by SDS-PAGE and immunoblotting. (*B*) Autoradiographs shown in *A* and those from additional experiments were quantitated by scanning densitometry. For each construct the value of bound (*B*, *filled bars*) and the value of unbound (*U*, *striated bars*) proteins were expressed as their percentage of the total value of the two fractions combined.

blotting. The results of a typical experiment are shown in Fig. 4 *A* and the quantitation of multiple experiments is given in Fig. 4 *B*. As expected, very low amounts of lamp1 and Lamp S3 were present on the cell surface at steady-state (3 ± 1% and 7 ± 2%, respectively) whereas Lamp d384 and Lamp S5 were greatly enriched at the plasma membrane (47 ± 5% and 41 ± 3%, respectively). The latter two values are in good agreement with the 24-h time point of the pulse chase experiment.

Lamp d384 and Lamp S5 Recycle to the Cell Surface

The increased amount of Lamp d384 and Lamp S5 on the plasma membrane at steady-state relative to the wild-type lamp1 could reflect either impaired internalization of these mutant proteins or increased recycling of internalized molecules from endosomes back to the plasma membrane, or a combination of both of these processes.

To address this issue more directly, the rate of internalization of lamp1 and the various mutants was determined. To do this, cells expressing the different lamps were grown in six-well plates, chilled on ice, and incubated with NHS-

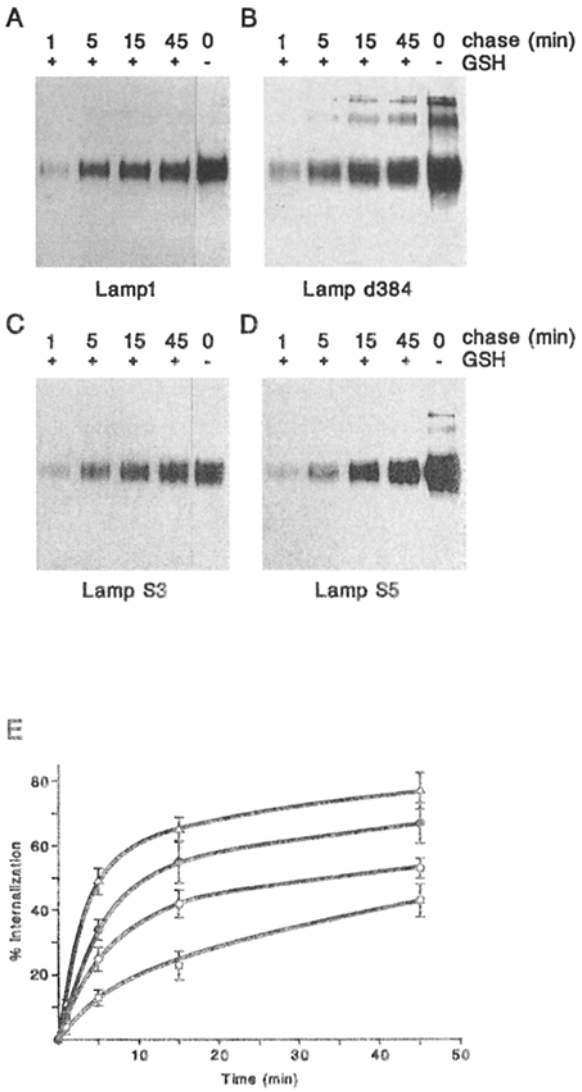

Figure 5. Internalization of lamp1 and mutant forms. Cell surface proteins of mouse L cells stably expressing lamp1 (*A*), Lamp d384 (*B*), Lamp S3 (*C*), and Lamp S5 (*D*) were derivatized using NHS-SS-biotin. The cells were then incubated at 37°C for the indicated time and subsequently chilled on ice. The biotin groups remaining at the cell surface were removed by incubation in a glutathione solution. The cells were lysed and lamp1 and mutant forms were immunoprecipitated using a polyclonal anti-Lamp1 antibody. Immunoprecipitates were resolved by SDS-PAGE and subjected to immunoblotting using a streptavidin-HRP conjugate. The higher molecular weight bands probably represent lamp oligomers. (*E*) The immunoblots shown in *A–D* and those from additional experiments were quantitated. For each construct, the values were expressed as their percentage of the sample that was kept at 4°C and not treated with glutathione. Lamp1 (●), Lamp d384 (○), Lamp S3 (△), and lamp S5 (□).

SS-biotin to label the cell surface proteins as described above. The cells were then warmed to 37°C for various times up to 45 min to allow for protein internalization, and subsequently chilled on ice again to stop any further membrane trafficking. Glutathione, a nonmembrane permeable reducing agent, was then added to the media to strip the biotin from proteins remaining at the cell surface. After the glutathione treatment, the cells were solubilized and the various lamps immunoprecipitated. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with streptavidin-HRP to detect biotinylated lamp molecules. The results of typical experiments are shown in Fig. 5. It is apparent in all instances that as the cells are incubated at 37°C for increasing periods of time, the biotinylated lamp molecules are progressively protected from the glutathione. The quantification of this and additional experiments is shown in Fig. 5 *E*. These data show that the mutant Lamp S3 is internalized slightly faster than the wild-type lamp1 whereas Lamp d384 and Lamp S5 are internalized at ~60% and 40% the rate of lamp1.

Given that both Lamp d384 and Lamp S5 are internalized at about half the rate of wild-type lamp1, we conclude that these proteins must be recycling from an endosomal compartment back to the plasma membrane in order for ~40% of the molecules to be present on the cell surface at steady-state vs only 3% of the wild-type lamp1. Therefore the major sorting defect is most likely at the level of the endosome.

Immunoelectron Microscopy Confirms the Altered Intracellular Localization of Lamp d384 and Lamp S5

Fig. 6 shows the localization of the expressed human lamp1, Lamp d384, and Lamp S5 in mouse L cells compared to that of endogenous murine lamp1 as determined by immunoelectron microscopy. For these experiments, ultrathin cryosections were probed with a rat anti–murine lamp1 antibody that is specific for the endogenous lamp1 and a mouse anti–human lamp1 antibody that is specific for transfected lamps. It is apparent that the transfected wild-type lamp1 exhibits a strong colocalization with the murine equivalent whereas the overlap between the mutant lamp molecules that are present in intracellular vesicles and the endogenous lamp is not well maintained. A statistical analysis of these data is shown in Fig. 8 *A*. The overlap between the wild-type lamp1 and the endogenous lamp was 90% whereas the overlap of internal Lamp d384 and Lamp S5 with the endogenous lamp1 was only 44% and 36%, respectively. In addition, Lamp d384 and Lamp S5 were present at the cell surface to a much greater extent than wild-type lamp1 (46% and 25% vs 2%, respectively).

To further analyze the nature of the vesicles that contained label for the different human lamp1 constructs, cells were surface-labeled with NHS-sulfobiotin on ice, and then the cells were warmed to 37°C for 7.5 min to allow endocytosis of cell surface molecules and labeling of early endocytic compartments. The distribution of the different lamps relative to the internalized biotinylated cell surface proteins is shown in Fig. 7 and analyzed statistically in Fig. 8 *B*. There was minimal overlap (12% of total label) between the transfected wild-type lamp1 and the intracellular vesicles containing the internalized, biotinylated cell surface proteins. In contrast, more than half of the intracellular Lamp d384 (57%) and Lamp S5 (61%) colocalized with vesicles containing biotinylated proteins. As expected, significant amounts of these mutant lamps were detected on the cell surface.

Taken together, these data indicate that the transfected wild-type lamp1 is mostly distributed in late endocytic compartments and lysosomes, whereas Lamp d384 and Lamp S5 are localized mainly in early endocytic compartments and at the plasma membrane.

Discussion

The data presented in this study demonstrate that small changes in the spacing of the tyrosine-based signal (YXXI) of the cytoplasmic tail of lamp1 relative to the lipid bilayer results in an almost complete block in the trafficking of this membrane protein to lysosomes. Instead, the mutant proteins enter a recycling pathway primarily involving trafficking between the plasma membrane and early endosomal compartments. These findings are best discussed in terms of our current understanding of the targeting of lysosomal membrane glycoproteins to lysosomes. As shown in Fig. 9 *A*, two routes have been described for the trafficking of these molecules to lysosomes. One subset of the lysosomal membrane glycoproteins including lamp1, is believed to be delivered directly from the TGN to endosomal compartments and then to the lysosomes without appearing on the plasma membrane (Fig. 9, steps 1, 2, and 3) (D'Souza and August, 1986; Green et al., 1987; Vega et al., 1991; Harter and Mellman, 1992; Carlsson and Fukuda, 1992). In the simplest case, the lamp1 molecules would be transported to early endosomal compartments, and then progress to late endosomes and lysosomes as occurs with lysosomal enzymes (Ludwig et al., 1994). However, it is possible that some of the lamp1 molecules are delivered directly to later endosomal structures. Other lysosomal membrane glycoproteins appear at the cell surface before their arrival in lysosomes (Lippincott-Schwarz and Fambrough, 1987; Braun et al., 1989; Nabi et al., 1991; Mathews et al., 1992). These proteins may go directly from the TGN to the plasma membrane where they are internalized and pass through the endosomal system to gain access to the lysosomes (Fig. 9, steps 4, 5, 2, and 3). Alternatively, the proteins may be transported from the TGN to early endosomal compartments, and then either continue on to the lysosome or cycle back to the plasma membrane. This situation would occur when the trafficking signal in the cytoplasmic tail does not mediate efficient sorting at step 2. When the protein reaches the cell surface, it can be reinternalized and either return to the plasma membrane, or go to the lysosome. Assuming that a fraction of the pool is siphoned off to the lysosome during each cycle, eventually the majority of the molecules will end up in that organelle. The best described recycling pathway that includes the plasma membrane involves internalization into the early/sorting endosome followed by transport to the recycling endosome and finally return to the plasma membrane (Mayor et al., 1993). This is believed to be a default pathway whereas retention in the endosomal compart-

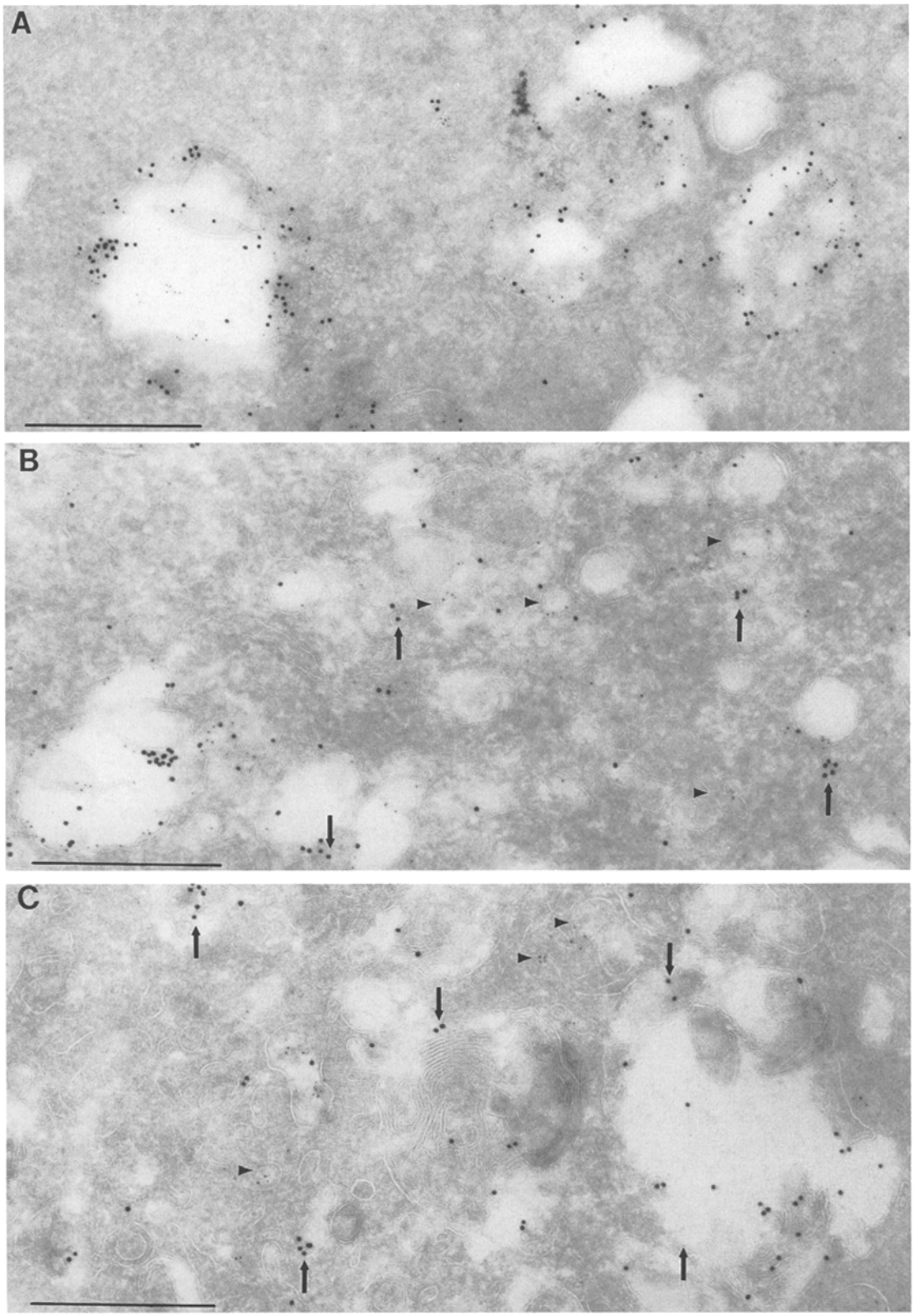

Figure 6. Cellular distribution of various lamps as determined by immunocytochemistry. Electron micrographs of frozen thin sections prepared from murine L cells transfected with wild-type human lamp1 and constructs encoding human Lamp1 with altered cytoplasmic domains. All sections were probed with 1D4B rat anti-murine lamp1 (5-nm gold goat anti–rat IgG) and G1/139 mouse anti-human lamp1 (18-nm gold goat anti–mouse IgG). All micrographs are of internal areas of the cells. (*A*) L cells expressing wild-type human lamp1. There is a clear overlap in the distribution of the murine and wild-type human lamp1, and the majority of vesicles shown contain gold particles corresponding to both lamp1's. (*B* and *C*) L cells expressing the Lamp S5 construct (*B*) and the lamp d384 construct (*C*). In these cells the overlap in localization between the mutant lamp1's and the murine lamp1 is less marked, and a significant fraction of the labeled intracellular mutant lamp1's is in vacuoles that are negative for murine lamp1 (see Fig. 8 *A* for quantitation). Vesicles uniquely labeled for the mutant lamp1's are marked with arrows and for murine lamp1 with arrowheads. 21% of Lamp S5 and 42% of Lamp d384 were detected on the cell surface vs 2% of the wild-type lamp1. Bars: 0.5 μm.

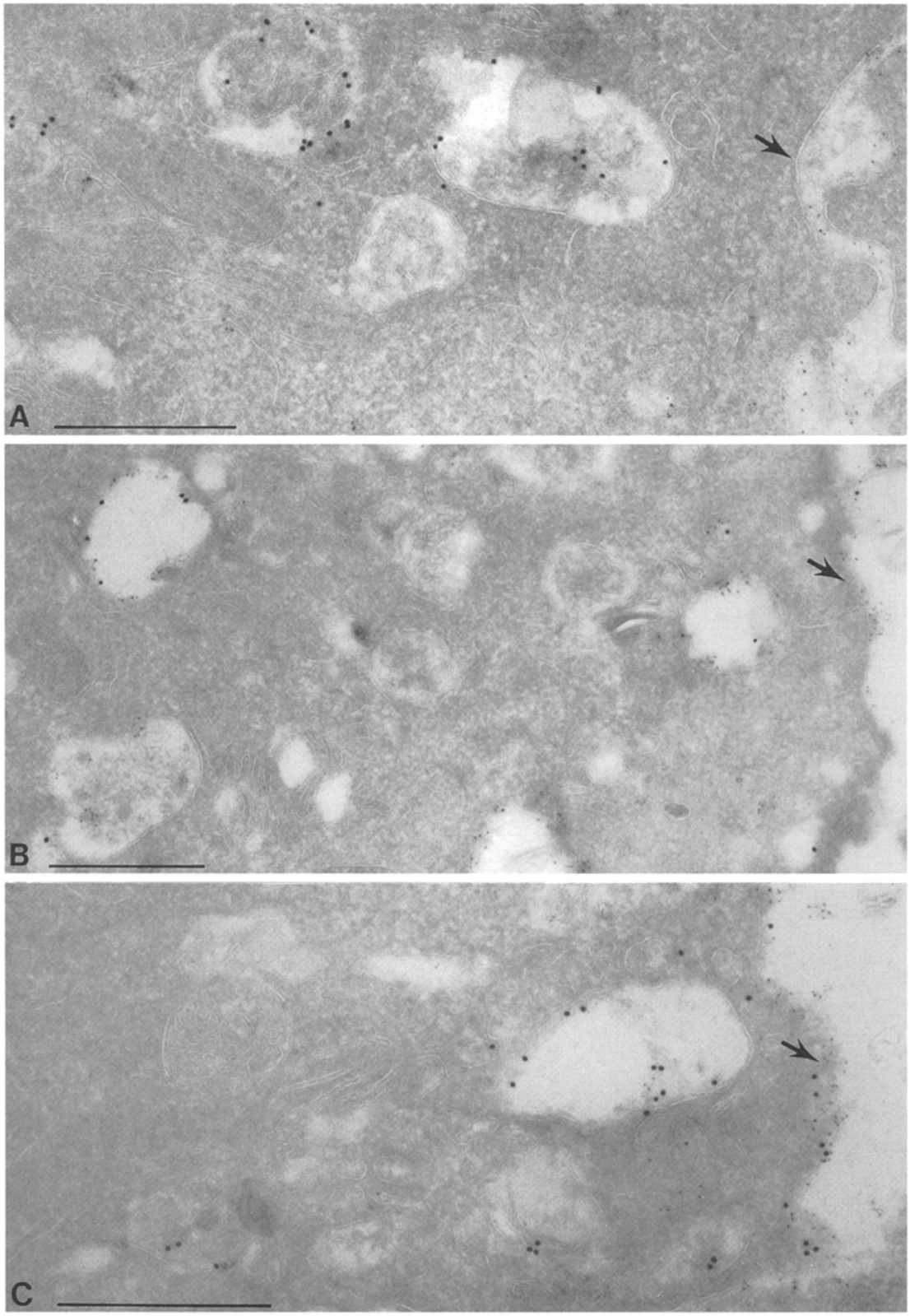

Figure 7. Lamp d384 and Lamp S5 but not lamp1 are enriched in early endosomes. Electron micrographs of frozen thin sections from murine L cells transfected with wild-type and mutant human lamp1. These cells were surface-labeled with sulfo-NHS-biotin and incubated at 37°C for 7.5 min to label early endocytic compartments. All sections were probed with streptavidin/rabbit anti-streptavidin (5-nm gold goat anti–rabbit IgG) and G1/139 mouse anti-human lamp1 (18-nm gold goat anti–mouse IgG). (*A*) L cells expressing wild-type human lamp1. There is minimal colocalization in the distribution of biotinylated plasma membrane proteins and wild-type human lamp1. The arrow shows a region of plasma membrane. (*B*) L cells expressing the Lamp S5 construct. In these cells Lamp S5 was observed on the cell surface (*arrow*) and in vesicles containing biotinylated proteins, in addition to other intracellular vesicles. (*C*) L cells expressing the Lamp d384 construct of human lamp1. There is a strong overlap in the distribution of both markers at the cell surface (*arrow*) and in vesicles at the cell periphery. The statistical analysis of the distribution of human lamp1 with respect to biotinylated plasma membrane proteins is shown in the bar graph in Fig. 8 *B*. Bars: 0.5 μm.

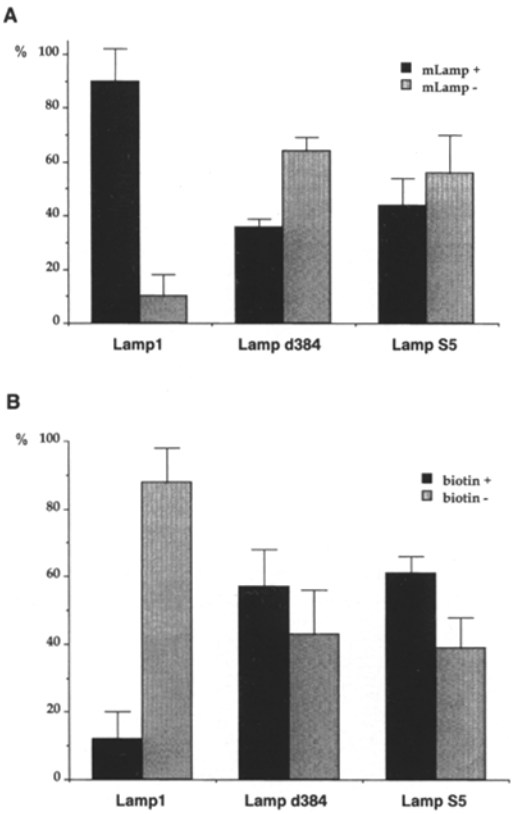

Figure 8. Quantitative analysis of the immunoelectron microscopy data. Bar graphs showing the relative distribution of the human lamp1 constructs with respect to (*A*) endogenous murine lamp1 and (*B*) surface-biotinylated proteins after 7.5 min at 37°C. In *A*, the wild-type human lamp1 expressed in the transfected L cells shows 90% colocalization with endogenous lamp1 (*filled bars*). The striated bars show that 10% did not overlap with endogenous lamp. In contrast, only 44% of internal Lamp S5 colocalized with the endogenous lamp1 whereas 56% was observed in murine lamp1-negative vesicles. This trend was also observed with Lamp d384 where 64% of the internal label is in murine lamp1-negative vesicles. In *B*, the internal wild-type human lamp1 showed limited colocalization with internalized, biotinylated plasma membrane constituents (12%) whereas Lamp d384 and Lamp S5 exhibited 57% and 61% overlap, respectively.

ment and delivery to late endosomal structures is felt to be signal mediated (Mayor et al., 1993).

Our finding that ~40% of Lamp d384 and Lamp S5 is present at the cell surface at steady-state even though these molecules are being internalized at relatively rapid rates indicates that these mutants are in a recycling pathway. The immunoelectron microscopy studies show that both Lamp d384 and Lamp S5 are greatly enriched in early endosomal structures compared to the wild-type lamp1. These data are consistent with wild-type lamp1 after the direct routing pathway shown in Fig. 9 *A* (steps 1, 2, and 3) and the mutant lamps following one or both of the recycling pathways shown in Fig. 9 *B* (steps 4, 5, 6, and 7 or steps 1, 6, 7, and 5).

An examination of Fig. 9 shows that three, and possibly four, of the trafficking steps require sorting signals. These are (*a*) sorting into vesicles at the TGN for delivery to endosomes (step 1), (*b*) sorting into clathrin-coated pits at the plasma membrane for rapid internalization (step 5), (*c*) sorting in early/sorting endosomes for delivery to late endosomes (step 2), and possibly (*d*) sorting in late endosomes for efficient transfer to lysosomes (step 3). Previous studies of lysosomal membrane proteins have identified short amino acid sequences in the cytoplasmic tails of these molecules that mediate one or more of these sorting events (for reviews see Fukuda, 1991; Sandoval and Bakke, 1994). Tyrosine-based sorting motifs have been identified in lamp1 and lgp120 (Williams and Fukuda, 1990; Harter and Mellman, 1992; Guarnieri et al., 1993; Höning and Hunziker, 1995), the precursor of lysosomal acid phosphatase (Peters et al., 1990; Eberle et al., 1991), CD63 (Metzelaar et al., 1991) and HLA-DM (Marks et al., 1995) whereas an LZ (Z denotes a hydrophobic amino acid) based motif has been shown to regulate the sorting of Limp II (Ogata and Fukuda, 1994; Sandoval et al., 1994). When substitutions of the tyrosine or the LZ sequence are made the mutant proteins accumulate at the cell surface, and in those cases where it has been examined, the proteins are internalized very slowly, in contrast to the findings with Lamp d384 and Lamp S5. The tyrosine and LZ mutations probably prevent sorting in the TGN (step 1, Fig. 9) resulting in delivery of the protein to the cell surface where it accumulates due to a block in step 5. A less likely possibility is that these proteins are transported from the TGN to the early/sorting endosomes, and then cycle to the cell surface where they are trapped due to blocks in steps 2 and 5.

In the case of lgp120, the glycine preceding the YQTI motif is required to prevent the protein from appearing at the cell surface before its delivery to lysosomes but internalization occurs at a normal rate followed by efficient delivery to lysosomes (Harter and Mellman, 1992; Höning and Hunziker, 1995). Thus, the substitution of an alanine for the glycine produces a block that is specific for step 1. This result shows that the same YQTI signal is deciphered differently in the TGN and at the plasma membrane depending on whether or not the glycine is present. Our data indicate that the YXXI motif also regulates sorting at the level of the early/sorting endosome (step 2). The fact that the mutant lamp 382-85A/SK is transported to dense lysosomes even though all of the amino acids in the cytoplasmic tail have been altered, except for the membrane anchor KRK and the YXXI motif, excludes the involvement of other amino acids at this sorting step. Since changing the spacing of the YXXI motif relative to the membrane by deleting just one amino acid (Lamp d384) or adding five alanines (Lamp S5) abolished lysosomal targeting, we conclude that spacing of the signal relative to the transmembrane domain is crucial for it to be effective at step 2. However, this parameter is much less important for internalization at the plasma membrane (step 5). Our data do not allow us to decide whether sorting in the TGN (step 1) is also impaired. An indication that this might not be the case comes from the pulse chase experiment which showed that the peak of cell surface appearance of the Lamp d384 and Lamp S5 mutants is delayed compared to the wild-type lamp1 (3 h vs 1 h). This could potentially come about if the mutant lamps were routed to endosomes before traveling to the cell surface (steps 1, 6, and 7 in Fig. 9 *B*), whereas the small percentage of lamp1, Lamp S1, and

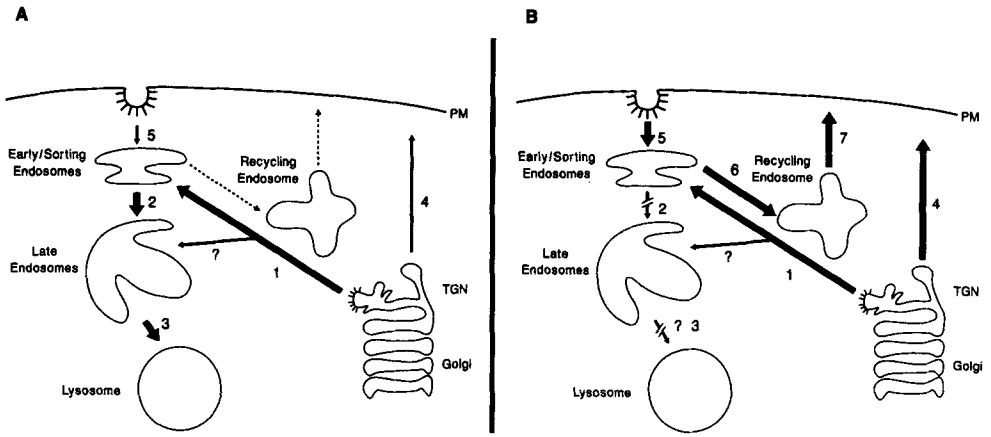

Figure 9. Models for the trafficking of normal and mutant lamp1. (*A*) Wild-type lamp1 is synthesized in the rough endoplasmic reticulum and transported to the Golgi where it is sorted in the TGN for delivery to early/sorting endosomes (step 1). It is then transported to late endosomes (step 2) and lysosomes (step 3). In addition, some of the lamp1 molecules may be delivered to later endosomal structures. A small percentage of the Lamp1 escapes to the plasma membrane, either by recycling from early/sorting endosomes via the recycling endosomes or by direct transport from the TGN (step 4). The lamp1 molecules at the cell surface are rapidly internalized (step 5) and targeted to late endosomes and lysosomes. The thick arrows illustrate the major flow of wild-type lamp1 as well as Lamp S1 and Lamp S3. (*B*) The lamp1 spacing mutants traffic from the TGN to the plasma membrane either directly (step 4) or via the recycling pathway (steps 1, 6, and 7). Once at the cell surface, the mutant lamps are internalized and enter early/sorting endosomes (step 5). Since their movement to late endosomes is blocked (step 2), they follow the recycling pathway to return to the plasma membrane. Those molecules that bypass the block at step 2 appear to be unable to enter dense lysosomes, consistent with an additional block at step 3. The thick arrows illustrate the major flow of the Lamp d384 and Lamp S5.

Lamp S3 molecules that are missorted in the TGN reach the cell surface directly (step 4 in Fig. 9).

The immunoelectron microscopy studies revealed that a portion of Lamp d384 and Lamp S5 overlaps with the endogenous murine lamp1, suggesting that some of these molecules either leak through the block in the early/sorting endosome and enter later endocytic compartments, or are delivered directly to these compartments from the TGN (Fig. 9, step 1). This is consistent with the finding that ~40% of the intracellular Lamp d384 and Lamp S5 (~20% of the total molecules) are in compartments that are not labeled after a 7.5-min uptake of surface-biotinylated proteins. If these mutant lamps are truly in late endosomes, it is striking that they do not enter dense lysosomes. One possibility is that this step requires a fourth sorting signal (Fig. 9, step 3) which is nonfunctional in the mutant lamps due to the change in spacing. Green et al. (1994) have presented evidence for a lysosomal-sorting signal on P-selectin, but this signal could be acting at the level of the early/sorting endosome (Fig. 9, step 2). In the case of the CD-MPR, we have shown previously that there is a default pathway from late endosomes to lysosomes (Rohrer et al., 1995). However, routing via this pathway is relatively slow compared to the signal-mediated pathways, and combined with the fact that the majority of the mutant lamps are already depleted from late endosomes due to the block in the early/sorting endosomes, the delivery of the mutant forms from late endosomes to lysosomes by default may be too inefficient to allow accumulation of significant amounts of these proteins.

The finding that a tyrosine-based motif on lamp1 is required for sorting in endosomes is consistent with results obtained in polarized cells where it has been shown that common tyrosine-based signals regulate polarized sorting in the TGN and in endosomes (Matter et al., 1993; Aroeti and Mostov, 1994). Further, a similar tyrosine-based motif has been demonstrated to target TGN38 to the TGN (Bos et al., 1993; Humphrey et al., 1993; Wong et al., 1993). Considering the similarity of the tyrosine-based motifs, it has been difficult to understand how the sorting machinery distinguishes between these signals. Our findings with lamp1 suggest that the spacing of the signals relative to the membrane may serve as a determinant for the differential sorting of these molecules. In this regard it is notable that the tyrosine-based signals of the various lamps are closer to the membrane than the equivalent signals found on the other proteins (Trowbridge et al., 1993). It will be of considerable interest to determine whether subtle alterations in this spacing influence the trafficking of other classes of molecules, as occurs with lamp1.

In summary, our findings show that the same signal (YXXI) on lamp1 can be read at three, and possibly four, different sorting sites. Small changes in the presentation of the signal affect sorting at some of these sites, but not others. The spacing of sorting signals relative to the membrane may prove to be an important determinant of the trafficking of membrane proteins.

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