Accumulation of Membrane Glycoproteins in Lysosomes Requires a Tyrosine Residue at a Particular Position in The Cytoplasmic Tail

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Abstract. Human lysosome membrane glycoprotein h-lamp-1 is a highly *N*-glycosylated protein found predominantly in lysosomes, with low levels present at the cell surface. The signal required for delivery of h-lamp-1 to lysosomes was investigated by analyzing the intracellular distribution of h-lamp-1 with altered amino acid sequences expressed from mutated cDNA clones. A cytoplasmic tail tyrosine residue found conserved in chicken, rodent, and human deduced amino acid sequences was discovered to be necessary for efficient lysosomal transport of h-lamp-1 in COS-1 cells. In addition, the position of the tyrosine residue relative to the membrane and carboxyl terminus also determined lysosomal expression. Supplanting the wild-type h-lamp-1 cytoplasmic tail onto a cell surface reporter glycoprotein was sufficient to cause redistribution of the chimera to lysosomes. A similar chimeric protein replacing the cytoplasmic tyrosine residue with an alanine was not expressed in lysosomes. Altered proteins that were not transported to lysosomes were found to accumulate at the cell surface, and unlike wild-type lysosomal membrane glycoproteins, were unable to undergo endocytosis. These data indicate that lysosomal membrane glycoproteins are sorted to lysosomes by a cytoplasmic signal containing tyrosine in a specific position, and the sorting signal may be recognized both in the *trans*-Golgi network and at the cell surface.

A CLASS of integral membrane glycoproteins specific to lysosomes has been identified from rat, mouse chicken, and human cells (for review, see Kornfeld and Mellman, 1989), and the human form is designated lysosome-associated membrane glycoprotein (lamp).[1] At least four subclasses of lamps have been identified based on apparent molecular weight, ranging from $M_r \approx 35{,}000$ to 130,000 (Barriocanal et al., 1986), and the best studied of these subclasses are those of $M_r \approx 90{,}000$–120,000, identified in humans as lamps 1 and 2 (h-lamp-1 and h-lamp-2). The corresponding glycoproteins identified from different species have similar mature molecular weights, containing polypeptide portions with $M_r \approx 40{,}000$–50,000, with the high mature molecular weight due to the 16–18 N-linked carbohydrates attached to the proteins. The role of lysosomal membrane glycoproteins in the cell has not been determined, but several possibilities exist. As integral membrane proteins, lamps could play a role in maintaining lysosomal structure, and their presence in the endocytic pathway implies a possible function in the control of the internalization and recycling process.

Experiments designed to examine the intracellular distribution of lamps have had conflicting results, varying with the species origin of the cells studied. Kinetic and morphological study of the chicken lamp-1 analogue LEP100 suggested a rapid cycling of lamp between lysosomes and the cell surface, with a steady-state distribution of ≈90% lysosome, 2–3% plasma membrane, and 5–8% in endosomes (Lippincott-Schwartz and Fambrough, 1986; Lippincott-Schwartz and Fambrough, 1987), and surface LEP100 molecules were associated with clathrin-coated pits. In contrast, immunoelectronmicroscopy and cell fractionation experiments did not detect the rat analogue lgp120 at the cell surface, only in intracellular structures (Geuze et al., 1988; Green et al., 1987). This glycoprotein, termed lgp107 in other studies (Himeno et al., 1989*b*; Noguchi et al., 1989), was later found to circulate between the cell surface and lysosomes via the endocytic pathway in primary cultured rat hepatocytes (Furuno et al., 1989*a*; Furuno et al., 1989*b*). The human lamp-1 glycoprotein has also been studied in several cell lines (Carlsson et al., 1988; Mane et al., 1989), and it was detected at the cell surface in low concentrations with decreasing cell surface expression in more differentiated cells (Mane et al., 1989).

Soluble lysosomal enzymes contain phosphate additions

1. *Abbreviations used in this paper*: CI-MPR, cationic independent mannose 6-phosphate receptor; endo H, endo-β-acetylglucosaminidase H; HA, hemagglutinin; hCG-α, human gonadotropin alpha chain; hCG-α^M, membrane anchored form of hCG-α; lamp, lysosome-associated membrane glycoprotein; h-lamp-1, human lamp 1; h-lamp-2, human lamp 2; TRITC-WGA, rhodamine-labeled wheat germ agglutinin; TR-O, Texas red–conjugated ovalbumin; VSV-G, vesicular stomatitus virus glycoprotein.

to Asn-linked carbohydrate mannose residues that are recognized by specific mannose 6-phosphate receptors, and the enzyme-receptor complex is directed into the lysosome biogenesis pathway (for review, see Kornfeld and Mellman, 1989). Lamps do not contain mannose 6-phosphate modifications and do not require carbohydrate for delivery to lysosomes (Barriocanal et al., 1986). It has been suggested that the *trans*-Golgi network pathway for delivery of newly synthesized components to lysosomes merges with the endocytic pathway, and sorting of lysosome components occurs in an "intermediate compartment" (Griffiths et al., 1988; Geuze et al., 1988). It is likely that lamps contain a positive signal in their peptide sequence that operates in this compartment to direct lamp sorting to lysosomes. To determine if a signal exists in the h-lamp-1 peptide that indicates lysosomal expression, mutations were made in an h-lamp-1 cDNA clone (Fukuda et al., 1988*b*), and altered cDNA was expressed in monkey cells. Data presented here indicate that a tyrosine residue in a specific position in the cytoplasmic domain is necessary for lysosomal expression of h-lamp-1 and for efficient lysosomal expression of a reporter molecule containing the h-lamp-1 cytoplasmic tail.

Materials and Methods

Cell Culture and Transfection

COS-1 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in DME supplemented with 10% FCS. Confluent cells were passaged the day before transfections, and subconfluent cells were transfected with CsCl-purified plasmid DNA using DEAE-dextran (Fisher Scientific Co., Pittsburgh, PA) in TBS (Guan and Rose, 1984). Generally, a 3.5-cm tissue culture dish containing a glass cover slip with attached cells was washed with TBS, and then the cells were incubated at 37°C for 45 min in 1 ml buffer containing 10 μg DNA and 0.5 mg/ml DEAE-dextran. The cells were then washed, replaced in medium, and incubated at 37°C for 40 h without glycerol, DMSO, or chloroquine treatment (Williams and Lamb, 1986). Cells to be metabolically labeled were grown and transfected in 3.5-cm tissue culture dishes, and then washed in medium lacking methionine. The cells were pulse labeled with 100 μCi Tran^{35}S label (ICN K&K Laboratories Inc., Plainview, NY) in methionine-deficient medium for 15 min, and then the label was chased for increasing times using serum supplemented complete medium.

Plasmid Construction and Mutagenesis

The expression vector used was a kind gift of Dr. J. K. Rose (Yale University School of Medicine, New Haven, CT), and is a derivative of pJC119 (Sprague et al., 1983) termed phCG-α^M (Guan et al., 1988), used previously to express human chorionic gonadotropin alpha chain (hCG-α) and membrane-bound hCG-α chimera in COS-1 cells (Guan et al., 1988). This vector contains SV-40 sequences required for replication in COS-1 cells, and contains the hCG-α^M chimera coding region inserted under the control of the SV-40 late region promoter (Guan et al., 1988). A cDNA clone containing the full-length coding sequence of h-lamp-1 and a truncated 3′ untranslated region was constructed in the plasmid Bluescript (Stratagene, La Jolla, CA) as follows. λ clones 15b (Fukuda et al., 1988*b*) and i202 (Viitala et al., 1988) encoding h-lamp-1 were digested with Eco RI, and the released fragments were cloned in separate Bluescript vectors with the 5′ ends near the vector Xho I site and 3′ ends near the vector Bam HI site. cDNA encoding the carboxy terminal two-thirds of h-lamp-1 was excised by digestion with Sac I and Bam HI from the plasmid containing the i202 clone, which has a truncated 3′ untranslated region and 5′ coding region. This fragment was subcloned in place of the Sac I–Bam HI fragment of 15b, a full-length clone. The resulting plasmid, phL1-15/202, contains a full-length h-lamp-1 coding region flanked by an Xho I site at the 5′ end and a Bam HI site following an 86-bp 3′ untranslated region. These restriction sites were used to excise the insert and to subclone it into the SV-40 expression vector remaining after Xho I and Bam HI digestion of phCG-α^M, forming pSVhL1. The Sac I–Bam HI fragment of the i202 clone was also inserted into M13mp19 for use in oligonucleotide-directed, site-specific mutagenesis. Site-specific amino acid replacement mutagenesis was done by the two-oligonucleotide method (Zoller and Smith, 1984) as described previously (Williams and Lamb, 1986), and the wild-type and identified mutant DNAs were excised from M13 RFI DNA, cloned in place of the wild-type 3′ two-thirds of h-lamp-1 cDNA in pSVhL1, and designated by the name and position of the amino acid change. To substitute the sequences, encoding the h-lamp-1 cytoplasmic tail in place of the corresponding vesicular stomatitus virus–glycoprotein (VSV-G) domain, a similar strategy was used. An Xho I–Pst I fragment of phCG-α containing almost all the hCG-α coding region was cloned in Bluescript, while a Pst I–Bam HI fragment containing the remainder of hCG-α sequences and the VSV-G sequences was subcloned in M13mp18. A 63-mer oligonucleotide was used to insert DNA encoding the entire h-lamp-1 cytoplasmic domain and stop codon exactly between the sequences encoding the VSV-G transmembrane anchor and cytoplasmic tail. In addition, this oligonucleotide encoded a Pst I site just 3′ of the h-lamp-1 tail stop codon. Pst I digestion released a DNA fragment encoding the VSV-G anchor and h-lamp-1 tail that was subcloned downstream of the hCG-α DNA in Bluescript, forming the hCG-α/G/L^Y insert. The M13 clone with inserted h-lamp-1 sequences was also used for another round of mutagenesis to alter the cytoplasmic tyrosine codon in order to specify Ala at that position. This mutant was also subcloned into the Bluescript hCG-α clone to make the hCG-α/G/L^A insert. Both chimeric DNA fragments were then inserted into SV-40 expression vectors using the flanking Xho I and Bam HI sites to make the plasmids pSVhCG-α/G/L^Y and pSVhCG-α/G/L^A.

Immunoprecipitation and Endoglycosidase Treatment

Labeled cells were lysed as described previously (Carlsson et al., 1988) and h-lamp-1 was immunoprecipitated as described previously (Carlsson et al., 1988; Williams and Lamb, 1986) using monoclonal antibody BB6, which specifically immunoprecipitates h-lamp-1 (Carlsson and Fukuda, 1989), rabbit anti–mouse IgG, and protein A agarose beads (Bio-Rad Laboratories, Cambridge, MA). Purified h-lamp-1 was removed from agarose beads and treated with 10 mU endo-β-actylglucosaminidase H (endo H; Genzyme Corp., Boston, MA) according to the manufacturer's instructions, and the treated proteins were analyzed by SDS-PAGE and fluorography.

Indirect Immunofluorescence

COS-1 cells growing on glass coverslips were transfected with plasmid DNA, and after 40 h were washed in PBS and fixed in freshly made 0.5% formaldehyde. Fixed cells were treated with 4 mg/ml BSA and 0.2% saponin for 10 min at room temperature, and then incubated 45 min at room temperature with primary antibody diluted in the same buffer. Rabbit anti-hCG antiserum was used to detect hCG-α chimerae, and a monoclonal antibody used to recognize h-lamp-1 (H5G11, a kind gift of Dr. J. T. August, Johns Hopkins Medical School). HSG11 does not cross react with the endogenous monkey lamp-1 (Fig. 1). Cells were washed in PBS containing 0.2% saponin, and then incubated 45 min with FITC-conjugated goat anti–mouse IgG for anti-(h-lamp-1), or goat anti–rabbit IgG for anti–hCG antibody, washed, and mounted on slides in 90% glycerol and 0.05% P-phenylenediamine in 10 mM Tris HCl, pH 9.0. To label the Golgi body preferentially, fixed permeabilized cells were incubated with TRITC-conjugated wheat germ agglutinin (TRITC-WGA) in addition to FITC-conjugated secondary antibody. To prelabel lysosomes, transfected cells were incubated in medium containing 20 μM leupeptin and 100 μg/ml Texas red–conjugated ovalbumin (TR-O) at 37°C for 2 h before fixation. Media containing TR-O had been clarified by ultracentrifugation before addition to cells. For antibody internalization assays, duplicate coverslips of transfected cells were incubated on ice for 30 min with primary antibody diluted in PBS containing Ca^{++} and Mg^{++}. Coverslips were washed in ice-cold buffer, and one slip of each pair was immediately fixed, while the other was returned to medium and incubated at 37°C for 1 h before fixation. Fixed cells were washed, treated with BSA and saponin, and the primary antibody was detected with FITC-conjugated secondary antibody as described above. Anti-hCG antiserum and FITC-labeled secondary antibodies were preabsorbed to fixed COS-1 cells and clarified by ultracentrifugation before use. Rabbit anti-hCG antiserum and all secondary antibodies were from Cappel Laboratories (Malvern, PA); P-formaldehyde, saponin, P-phenylenediamine, TRITC-WGA, and leupeptin were from Sigma Chemical Co. (St. Louis, MO). TR-O was from Molecular Probes, Inc. (Eugene, OR). All slides were visualized with a Zeiss Axioplan microscope and photographed using Kodak TMAX film.

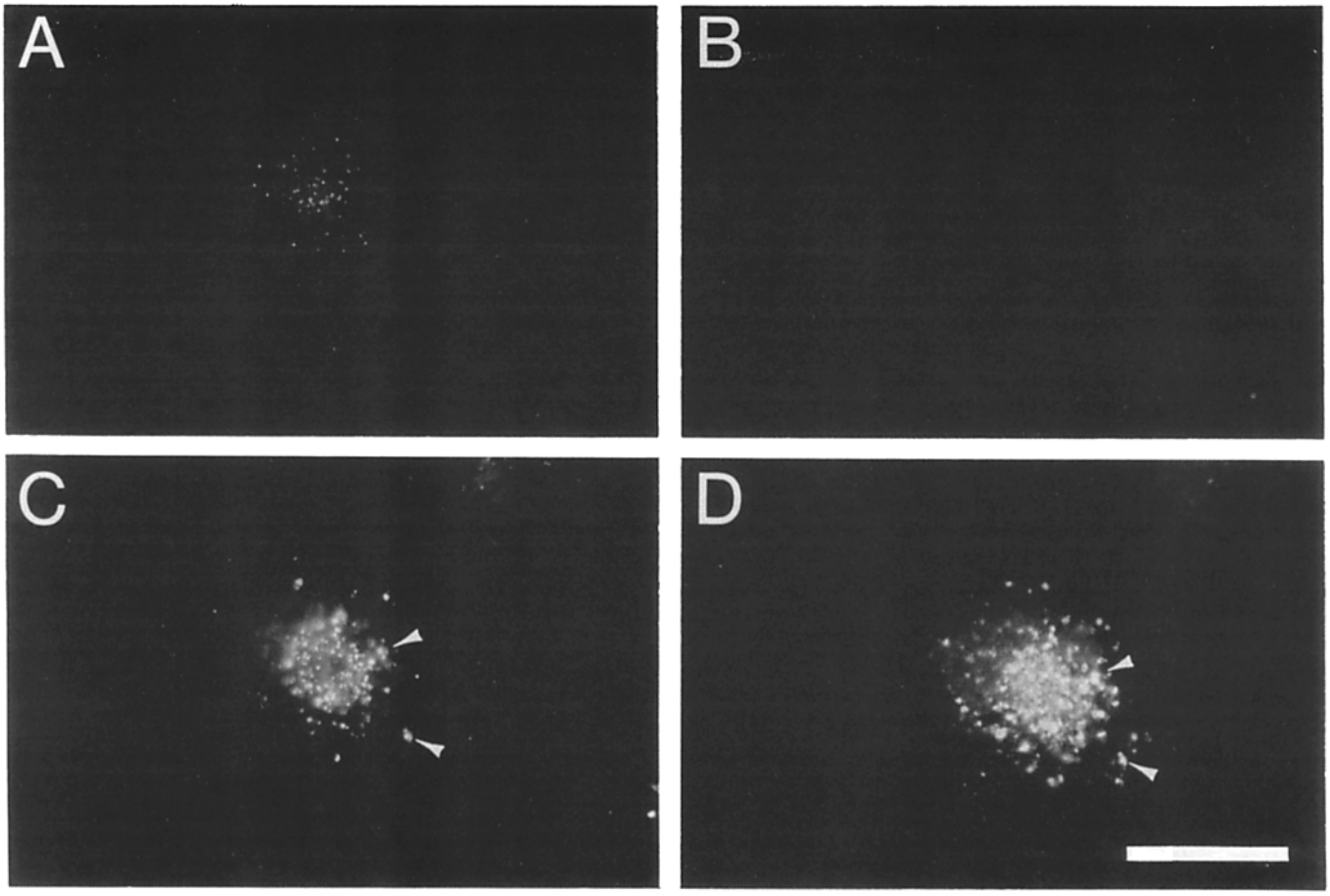


Figure 1. Indirect immunofluorescence detects h-lamp-1 in COS-1 cell lysosomes. Subconfluent COS-1 cells were mock transfected (*A* and *B*) or transfected with the pSVhL1 expression vector containing wild-type h-lamp-1 cDNA (*C* and *D*). 30 h after transfection, lysosomes were labeled by incubating cells in medium containing 20 μM leupeptin and 100 μg/ml TR-O for 2 h. Cells then were washed, fixed in formaldehyde, and permeabilized with saponin. The intracellular distribution of h-lamp-1 was detected with monoclonal anti-(h-lamp-1) antibody H5G11 and FITC-conjugated goat anti-mouse IgG. The distribution of TR-O and h-lamp-1 were compared in the same cells, using rhodamine filters for TR-O (*A* and *C*) and fluorescein filters for FITC-IgG (*B* and *D*). The exposure time for the photograph shown in *B* was manually adjusted to equal that of *D*. (*A*) Mock transfected, TR-O; (*B*) the same cell as *A*, anti-(h-lamp-1); (*C*) pSVhL1 transfected, TR-O; (*D*) the same cell as *C*, anti-(h-lamp-1). Arrowheads denote specific examples of co-localized staining. Bar, 20 μm.

Results

Lysosomal Accumulation of h-lamp-1 in COS-1 Cells Requires a Cytoplasmic Tyrosine Residue

The nucleotide sequence of cDNA clones corresponding to two human lysosomal glycoproteins, h-lamp-1 and h-lamp-2, have been determined (Fukuda et al., 1988*b*). The deduced amino acid sequences of h-lamp-1 and h-lamp-2 have 37% overall shared sequences, with some domains in common (Fukuda et al., 1988*b*). It is likely that a signal calling for the localization of h-lamp-1 and h-lamp-2 to lysosomes would be found in a domain conserved between the two glycoproteins. The peptide portions of h-lamp-1 and h-lamp-2 are decorated very heavily with carbohydrate in their lumenal domains, with ~60% of the mature molecular weight of the lumenal domain due to carbohydrate residues (Fukuda et al., 1988*b*; Carlsson et al., 1988), which could limit accessibility of recognition for lumenal peptide signals. h-lamp-1 and h-lamp-2 share an identical 4 amino acid sequence in the cytoplasmic tail. Inspection of the deduced mouse lamp-1 (Chen et al., 1988) and analogous chicken (Fambrough et al., 1988) and rat (Howe et al., 1988) lamp amino acid sequences showed that this domain, His-Ala-Gly-Tyr (HAGY), is conserved in other lamps. In addition, human lysosome acid phosphatase is translocated to lysosomes as a transmembrane protein independent of mannose 6-phosphate receptors (Waheed et al., 1988), and the cytoplasmic tail contains a tyrosine in a relative cytoplasmic position similar to h-lamp-1 (Fukuda et al., 1988*b*; Pohlmann et al., 1988). It seemed likely that the lamp cytoplasmic domain could contain a lysosomal localization signal, and to examine the effects of alterations of h-lamp-1 on intracellular protein targeting, h-lamp-1 was introduced into a heterologous cell line, COS-1.

Plasmid DNA encoding wild-type h-lamp-1 was cloned in an SV-40 expression vector (pSVhL1), and delivered by DEAE-dextran mediated transfection (Lopata et al., 1984) into COS-1 cells, a cell line allowing efficient production of mRNA from the SV-40 late promoter of transfected DNA (Gluzman, 1981). These transfected cells efficiently produced h-lamp-1, which was identified intracellularly by indirect immunofluorescence (Fig. 1 *D*). The anti-(h-lamp-1) monoclonal antibody used is specific to the human lamp-1

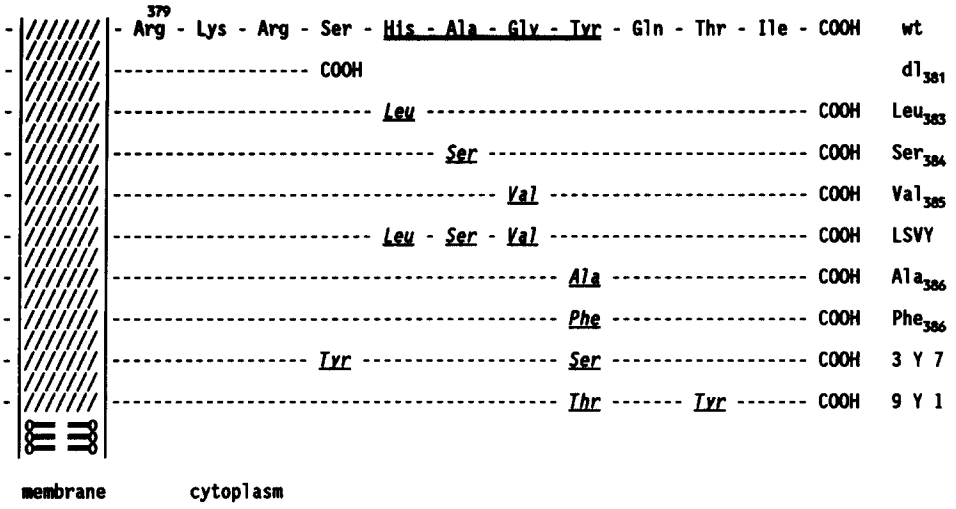


Figure 2. Mutations in the cytoplasmic tail of h-lamp-1. The amino acid sequence of the wild-type (*wt*) h-lamp-1 cytoplasmic tail (residues 379–389) is shown schematically. The H-A-G-Y sequence conserved between h-lamp-1 and h-lamp-2 is doubly underlined, and mutations described in the text are shown below the wild-type sequence. The mutants are designated as in the text, and only the changed amino acids are shown. The deletion mutant dl_{381} was truncated at the position shown, and the amino acid residues changed in the point mutations are underlined and italicized in each case.

molecule, as no cross-reaction with endogenous monkey lamp-1 was detected (Fig. 1 *B*). To insure that h-lamp-1 expressed in COS-1 cells accumulated in lysosomes, the intracellular distribution of h-lamp-1 was compared to that of a lysosomal marker by indirect immunofluorescence colocalization. Lysosomes of mock-transfected and pSVhL1-transfected COS-1 cells were prelabeled by incubation in media containing Texas red–conjugated ovalbumin, a marker that accumulates in lysosomes through endocytosis (Lippincott-Schwartz and Fambrough, 1987; Steinberg et al., 1988). The intracellular location of h-lamp-1 in the TR-O–labeled cells was visualized by indirect immunofluorescence, and Fig. 1 shows that h-lamp-1 is concentrated in the same intracellular structures as TR-O (compare Figs. 1 *C* and 1 *D*), suggesting that these structures are lysosomes.

To investigate whether amino acids present in the cytoplasmic carboxy-terminal tail of h-lamp-1 are necessary for efficient lysosomal expression, mutations were made in the h-lamp-1 COOH-terminal region by using a fragment of the h-lamp-1 cDNA corresponding to the COOH terminal two-thirds of the protein as a template for oligonucleotide-directed, site-specific mutagenesis, as described in Materials and Methods. Mutants produced to investigate the role of the cytoplasmic domain in lysosomal localization are shown in Fig. 2. These mutant cDNAs were cloned in the SV40 expression vector in place of wild-type h-lamp-1 cDNA, and were expressed in COS-1 cells (Fig. 3). First, a deletion mutant was made that eliminated the cytoplasmic tail at amino acid 409, by changing the codon AGU (Ser) to UAG (STOP), such that the final eight residues were eliminated, while three basic amino acids were left as a "stop transfer" sequence to help maintain membrane anchorage. The glycoprotein h-lamp-1_{dl381} produced from this mutant cDNA was analyzed by immunofluorescence of transfected cells, and was not found in lysosomes. Instead, Fig. 3 *B* shows that the protein was detected intracellularly in a perinuclear distribution.

The indirect immunofluorescence shown in Fig. 3 was done using fixed transfected cells permeabilized with saponin, a treatment that allows detection of intracellular structures and visualization of antigens at the cell surface (Willingham and Pastan, 1985) by (h-lamp-1)–specific monoclonal antibody H5G11, and the photographs of cells shown were exposed for an equal length of time. Comparison of Fig. 3 (*A* and *B*) suggests that a larger amount of h-lamp-1_{dl381} is expressed at the cell surface than found in wild-type h-lamp-1. The contribution of each of the four conserved residues in the carboxy-terminal HAGY sequence to lysosomal targeting was investigated using site-specific replacement mutation. As a first test, proteins changed at only one of the four residues (Fig. 2) were analyzed by transfection and immunofluorescence for intracellular distribution. Fig. 3 (*C*, *D*, and *E*) shows that altering positions 383, 384, and 385 from His, Ala, or Gly to Leu, Ser, and Val does not affect the intracellular distribution of the altered h-lamp-1 molecules. In contrast, when the Tyr residue at position 386 was substituted by an Ala (Fig. 3 *F*), the distribution changed, such that h-lamp-1_{Ala386} was detected in a perinuclear structure. Introduction of His at position 386 gave the same result (data not shown). Fig. 3 *F* also shows that h-lamp-1_{Ala386} is detected at the cell surface at increased levels. These data suggest the Tyr residue found in lamp cytoplasmic tails is necessary for efficient lysosomal accumulation.

To determine whether the other three conserved amino acid residues could have a cooperative function with the required Tyr_{386}, a mutant was produced in which residues 383–385 were all changed (to Leu-Ser-Val). Fig. 3 *G* shows the intracellular distribution of the LSVY mutant, with this protein detected in lysosomes, and perhaps a slight increase in the amount detected at the cell surface compared to wild type (Fig. 3 *A*). These data indicate that the other three conserved amino acid residues are not absolutely required for Tyr_{386} signal function. In support of this conclusion, recent sequence data indicates that only the Gly and Tyr residues are conserved in this cytoplasmic region of lgp96, a rat lamp analogous to h-lamp-2 (Noguchi et al., 1989). To investigate whether the presence of Tyr_{386} is an absolute requirement for lysosomal accumulation, another aromatic amino acid residue, Phe, was substituted in its place. Fig. 3 *H* shows that h-lamp-1_{Phe386} is transported to lysosomes but most of this glycoprotein is detected at the cell surface. This suggests that an aromatic residue in the cytoplasmic tail of h-lamp-1 is necessary for lysosomal expression, but the most efficient

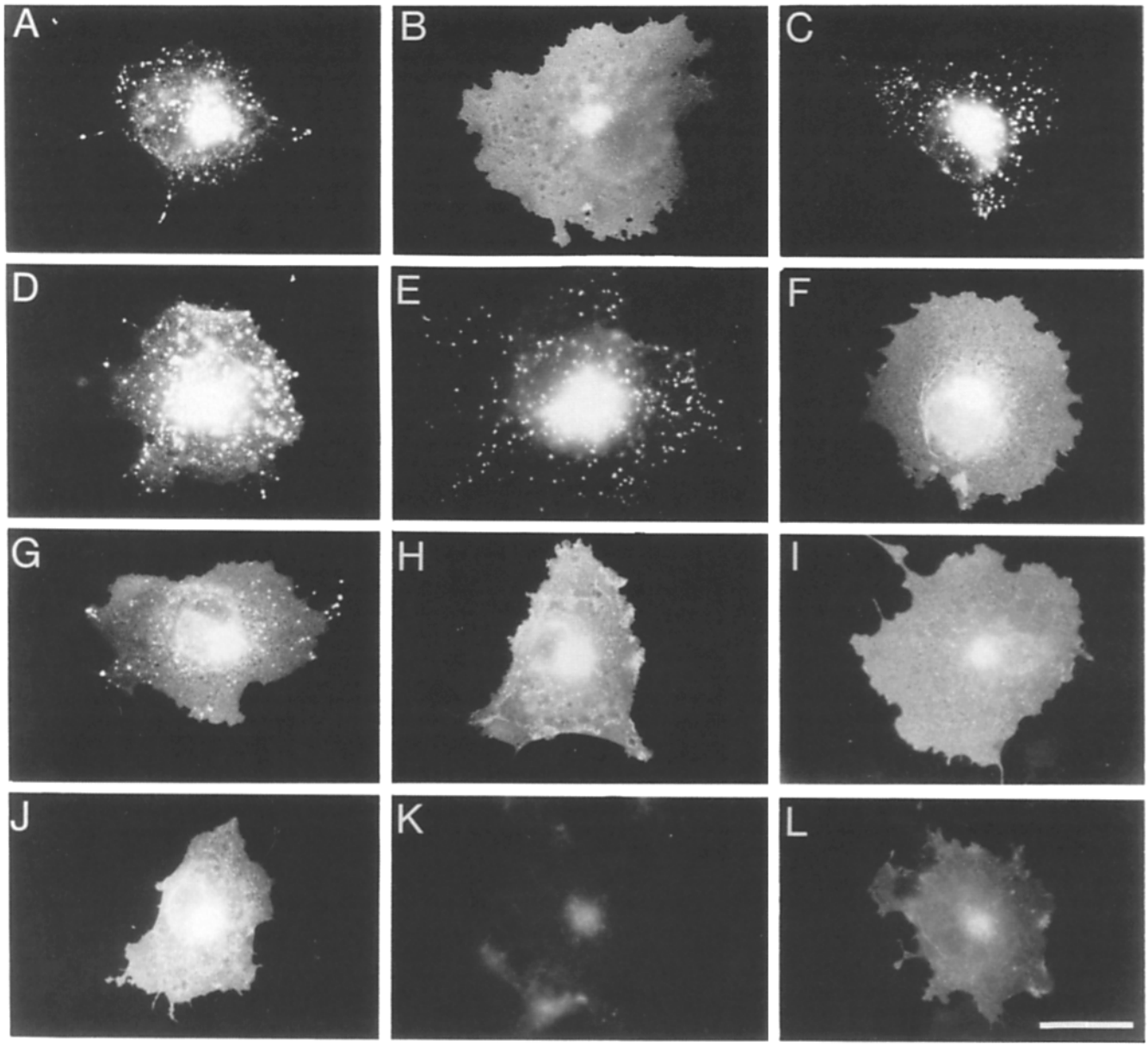
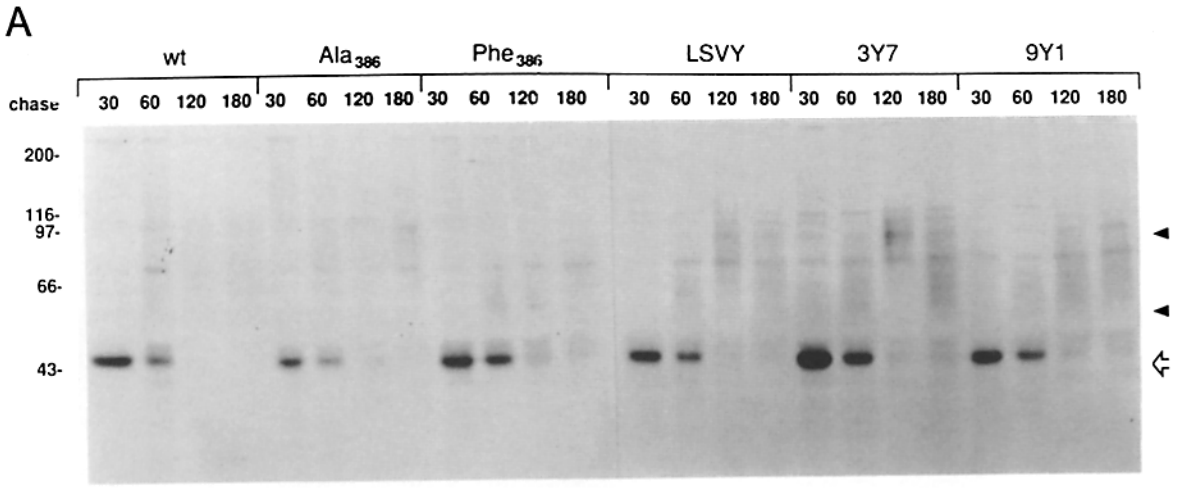


Figure 3. Immunofluorescence of COS-1 cells transfected with pSVhL1 wild-type and mutant DNA identifies a cytoplasmic tyrosine necessary for lysosomal expression. Subconfluent COS-1 cells were transfected with the pSVhL1 expression vector containing wild-type h-lamp-1 or cytoplasmic tail mutant cDNAs. Fixed cells were permeabilized with saponin and the intracellular distribution of h-lamp-1 was detected with a monoclonal anti-(h-lamp-1) antibody and FITC-conjugated goat anti-mouse IgG (*A–K*). Cells shown in *K* were incubated with TRITC-WGA after secondary antibody to distinguish the Golgi body, and the same cell is shown in *K* (FITC-IgG) and *L* (TRITC-WGA). The photographs of cells shown in *A–J* were exposed for an equal length of time. (*A*) h-lamp-1 wild type; (*B*) dl_{381}; (*C*) Leu_{383}; (*D*) Ser_{384}; (*E*) Val_{385}; (*F*) Ala_{386}; (*G*) LSVY; (*H*) Phe_{386}; (*I*) 3-Y-7; (*J*) 9-Y-1; (*K*) Ala_{386}; (*L*) Ala_{386}. Bar, 20 μm.

expression in lysosomes is dependent on the presence of a cytoplasmic Tyr.

Lysosomal Expression Is Dependent upon h-lamp-1 Cytoplasmic Tyrosine Position

Experiments designed to determine the residue responsible for endocytosis of cell surface glycoproteins showed that introduction of a Tyr residue at a specific site in the short cytoplasmic tail of influenza A virus hemagglutinin (HA), a protein that normally avoids coated pits, causes the mutant HA to enter coated pits and subsequently be subject to endocytosis (Lazarovitz and Roth, 1988). Introduction of Tyr residues in other cytoplasmic positions, NH_2 terminal and COOH terminal of the targeting position, was not enough to cause efficient HA endocytosis (Lazarovitz and Roth, 1988). To determine whether there is a similar position effect for the h-lamp-1 cytoplasmic Tyr found to be necessary for lysosomal localization, the relative position of the Tyr was altered in two mutants. Fig. 2 shows that the wild-type position of the cytoplasmic Tyr is 8 residues COOH terminal of the membrane, and 4 from the COOH terminus (7-Y-3). The first



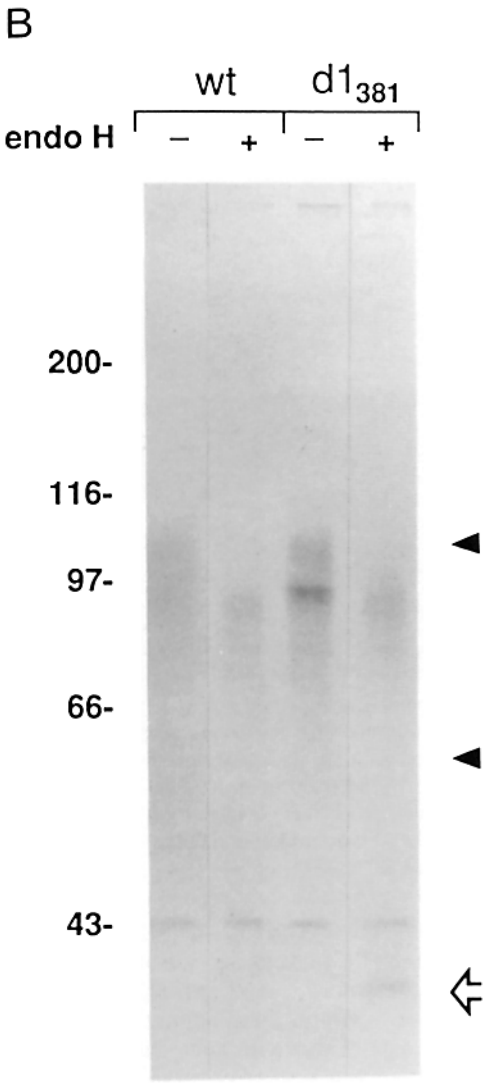


Figure 4. Endoglycosidase H analysis of h-lamp-1 Golgi transport kinetics. (*A*) COS-1 cells were transfected with pSVhL1 wild-type and cytoplasmic tail mutant DNAs, and, 40 h after transfection, the cells were pulse labeled with 100 μCi Tran^{35}S label for 15 min. The cells were then replaced in media and the label chased for 30, 60, 120, or 180 min. Cells were lysed, the labeled glycoproteins were immunoprecipitated and then treated with 10 mU endo H for 16 h at 37°C, and the results visualized by SDS-PAGE and fluorography. Sizes of marker proteins (in kilodaltons) are shown to the left of the figure. The open arrow denotes the position of the h-lamp-1 protein resulting from removal of high mannose carbohydrates, and the closed arrowheads show the range of molecular masses of the heterogeneous molecular mass, endo H resistant, mature h-lamp-1. (*B*) COS-1 cells expressing h-lamp-1 wild-type and mutant dl_{381} were pulse labeled, and the label was chased 120 min. Labeled wild-type and mutant h-lamp-1 molecules were immunoprecipitated and were treated (+ lanes) or mock treated (– lanes) with endo H, and the reaction products were visualized by SDS-PAGE and fluorography.

mutant produced replaced Ser_{382} with Tyr, and position 386 was changed to Ser (3-Y-7), while the second mutant has a penultimate Tyr residue and Thr_{386} (9-Y-1). These mutants were examined for intracellular accumulation in transfected cells, as shown in Fig. 3 (*I* and *J*), and were found in an intracellular perinuclear structure and at the cell surface. A small amount of mutant 9-Y-1 protein may be seen in punctate intracellular structures, suggesting that a small amount of this protein is delivered to lysosomes. These data indicate that the presence of a Tyr in the h-lamp-1 cytoplasmic tail is not

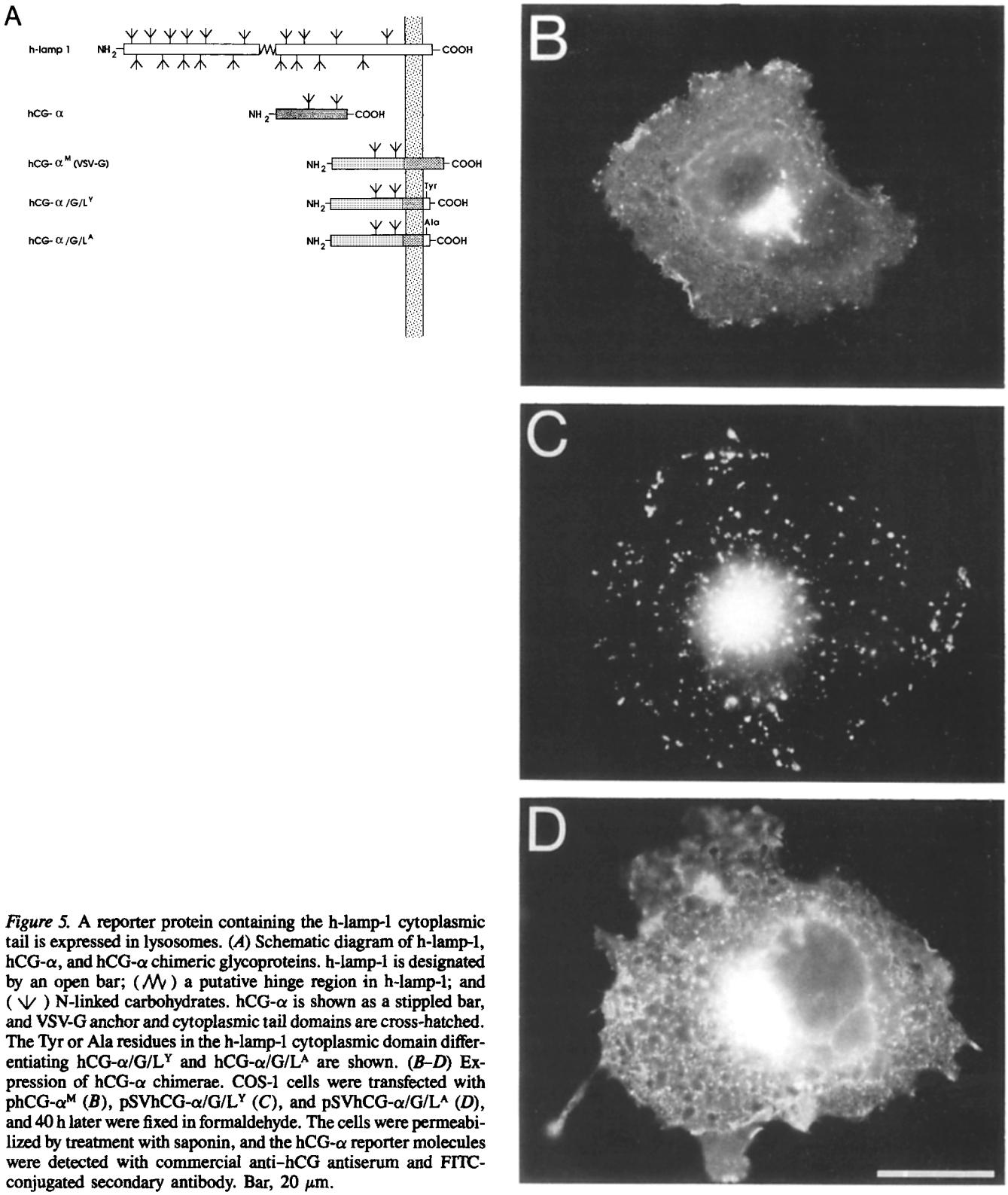
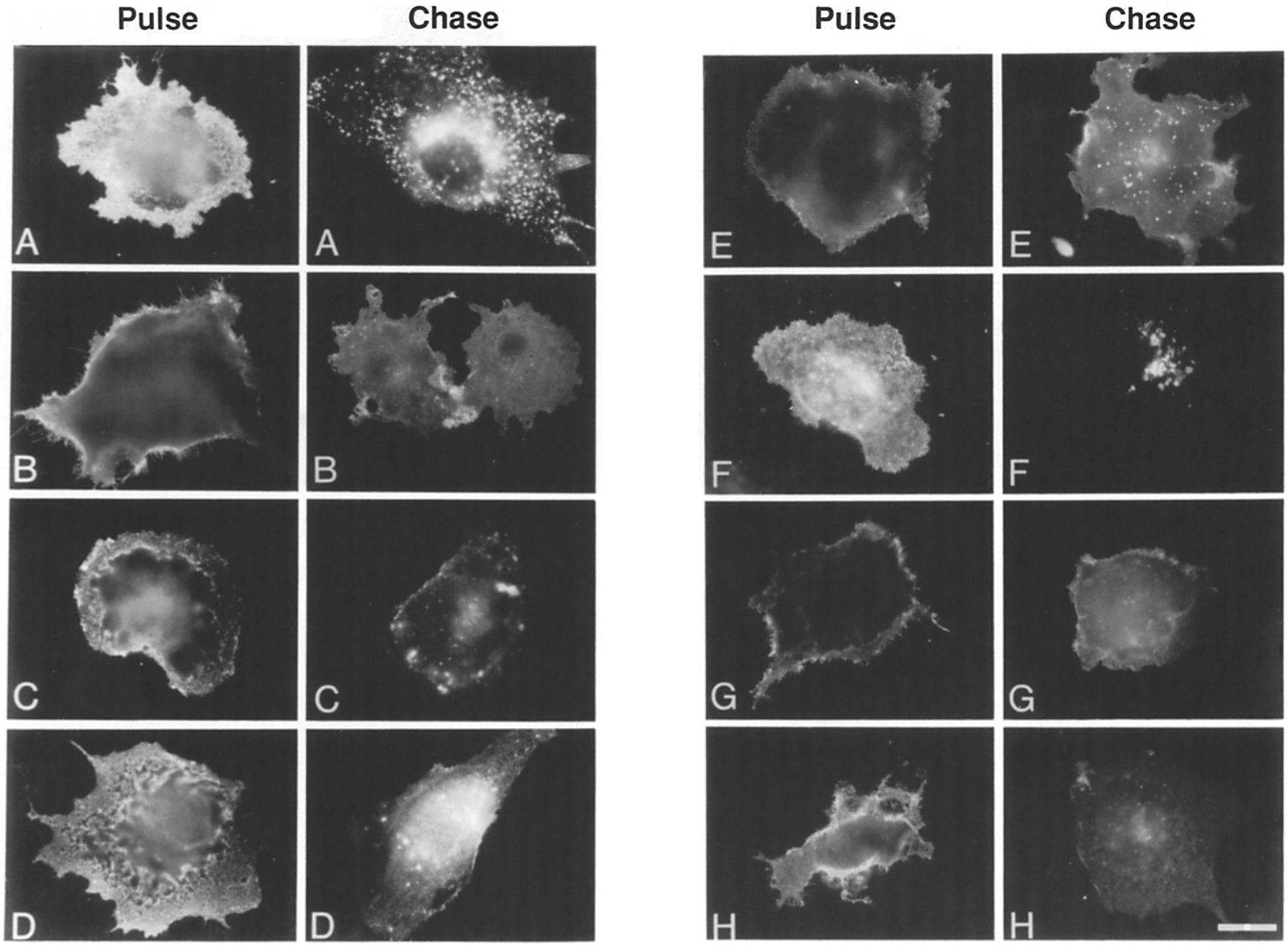


Figure 5. A reporter protein containing the h-lamp-1 cytoplasmic tail is expressed in lysosomes. (*A*) Schematic diagram of h-lamp-1, hCG-α, and hCG-α chimeric glycoproteins. h-lamp-1 is designated by an open bar; (⋀⋁) a putative hinge region in h-lamp-1; and (⋁) N-linked carbohydrates. hCG-α is shown as a stippled bar, and VSV-G anchor and cytoplasmic tail domains are cross-hatched. The Tyr or Ala residues in the h-lamp-1 cytoplasmic domain differentiating hCG-α/G/L^Y and hCG-α/G/L^A are shown. (*B–D*) Expression of hCG-α chimerae. COS-1 cells were transfected with phCG-α^M (*B*), pSVhCG-α/G/L^Y (*C*), and pSVhCG-α/G/L^A (*D*), and 40 h later were fixed in formaldehyde. The cells were permeabilized by treatment with saponin, and the hCG-α reporter molecules were detected with commercial anti-hCG antiserum and FITC-conjugated secondary antibody. Bar, 20 μm.

solely sufficient for steady-state accumulation in lysosomes, but rather that the position of the Tyr relative to the membrane and/or the COOH terminus also determines the accumulation signal.

Elimination of the Lysosome Address Label Leads to Accumulation of h-lamp-1 at the Cell Surface

The h-lamp-1 mutants dl_{381} and Ala_{386} have similar pheno-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types, different from wild-type h-lamp-1, as indirect immunofluorescence demonstrated cell surface expression of these proteins and a concentration of these proteins in a perinuclear region (Fig. 3, *B* and *F*). Permeabilized transfected cells were incubated with rhodamine-labeled wheat germ agglutinin (TRITC-WGA), a lectin specific for carbohydrate structures found mainly in the Golgi complex (Virtanen et al., 1980), before antibody detection of mutant h-lamp-1 protein. TRITC-WGA bound to the same intracellular perinuclear structure (Fig. 3 *K*) containing high concentrations of h-lamp-1_{Ala386} (Fig. 3 *L*) and h-lamp-1_{dl381} (data not shown). These data support the hypothesis that these mutants do not accumulate in lysosomes, since all the intracellular h-lamp-1 mutant protein was found associated with the Golgi. These data also suggest that the mutant h-lamp-1 detected intracellularly represents protein in transit through the Golgi, en route to the cell surface.

A possibility exists that slight modifications in the h-lamp-1 cytoplasmic tail could cause a slowing of transport through the Golgi, leading to altered carbohydrate modification or misrouting of the protein. To investigate the effects of altered cytoplasmic tails on the rate of h-lamp-1 Golgi transport, pulse-chase experiments were performed to determine the time required for h-lamp-1 mutants to acquire resistance to endo H. Human lamp-1 and lamp-2 are first synthesized as $M_r \approx 85$ kD, glycosylated precursors containing all high-mannose carbohydrates, and h-lamp-1 is reduced to $M_r \approx 39.5$ kD upon endo H digestion of the precursor (Carlsson et al., 1988). This precursor is modified to a higher, heterogeneous molecular weight mature form ($t_{1/2} \approx 45$ min) with most of its 18 N-linked carbohydrate chains becoming resistant to endo H treatment (Carlsson et al., 1988), indicating passage through the Golgi body (Tarentino and Maley, 1974; Hubbard and Ivatt, 1981). Cells transfected with wild-type h-lamp-1 or cytoplasmic tail mutants were analyzed for the time required to become endo H resistant, as shown in Fig. 4. Carbohydrate moieties of h-lamp-1 were processed to complex type and became mostly resistant to endo H with $t_{1/2} \approx 45$ min (Fig. 4 *A*). Mutants Ala_{386}, Phe_{386}, LSVY, and Tyr position mutants 3-Y-7 and 9-Y-1 were analyzed similarly and found to have similar rates of Golgi transport (Fig. 4 *A*). The mutant dl_{381} acquired endo H resistance at a slower rate, and a fraction remained entirely endo H sensitive after 120 min of chase (Fig. 4 *B*).

These data suggest that the amino acid substitutions in the cytoplasmic tail of h-lamp-1 studied do not alter the rate of Golgi transport or carbohydrate processing, and that the altered intracellular accumulation found for these mutants is due to a change in glycoprotein sorting at a late or post-Golgi pathway branch point. Truncation of the cytoplasmic tail alters the carbohydrate processing rate more drastically, but the shortened protein eventually does leave the Golgi complex, as may be seen by the cell surface expression of h-lamp-1 dl_{381} (Fig. 3 *B*).

The Lysosome Localization Signal Can Be Transferred to a Heterologous Protein

To examine whether the lysosomal sorting signal identified in the cytoplasmic tail of h-lamp-1 could enable a protein normally not found in lysosomes to be delivered there, chimeric reporter proteins bearing only the h-lamp-1 cytoplasmic tail were expressed in COS-1 cells. The h-lamp-1 cytoplasmic tail was transferred to the same relative position in a reporter glycoprotein, hCG-α^M, by oligonucleotide replacement loop mutagenesis. hCG-α^M is a form of hCG-α bound to membrane through the VSV-G transmembrane anchor and cytoplasmic tail domains (Guan et al., 1988). hCG-α^M was found to be efficiently expressed at transfected cell surfaces (Guan et al., 1988), indicating that it does not contain "retention sequences" for internal cellular structures. The N-linked carbohydrate chains of hCG-α were found to be modified to contain polylactosaminoglycan when the protein was anchored in membranes by VSV-G sequences (Fukuda et al., 1988*a*), which could help stabilize the hCG-α reporter domain in the proteolytic lysosomal environment. These data suggested that hCG-α could serve as a good reporter molecule for accumulation in lysosomes. The final amino acid residue of soluble hCG-α (Ser) (Fiddes and Goodman, 1979) corresponds to the presumed ultimate lumenal residue of VSV-G (Rose and Gallione, 1981), and the presumed first cytoplasmic residue (Arg) is the same in VSV-G and h-lamp-1 (Rose and Gallione, 1981; Fukuda et al., 1988*b*), and so the chimeric glycoproteins shown schematically in Fig. 5 *A* were designed to reflect exactly the original context of each domain with no loss or addition of other amino acid residues. Two chimera were produced from hCG-α^M. hCG-α/G/L^Y was made by appending nucleotide sequences encoding the wild-type h-lamp-1 cytoplasmic tail immediately after the VSV-G transmembrane anchor domain. A second chimera was made in the same manner, but recreated the Ala_{386} mutation in the cytoplasmic tail to form hCG-α/G/L^A. Plasmid DNA encoding these two chimera and authentic hCG-α^M were used to transfect cells and the intracellular distribution of the glycoproteins produced were analyzed. All three glycoproteins were recognized by anti-hCG antiserum, and were modified to much larger, heterogeneous molecular weight forms consistent with polylactosaminoglycan formation with similar rates (data not shown). Indirect immunofluorescence of transfected cells permeabilized with saponin shows that hCG-α^M accumulated at the cell surface (Fig. 5 *B*), as found previously (Guan et al., 1988). hCG-α/G/L^Y was seen to accumulate in circular cytoplasmic vesicles with morphology identical to lysosomes (Fig. 5 *C*), and had very little surface expression. hCG-

Figure 6. Elimination of the cytoplasmic tyrosine blocks h-lamp-1 internalization from the cell surface. COS-1 cells were transfected with pSVhL1 wild-type or cytoplasmic tail mutant, phCG-α^M, pSVhCG-α/G/L^Y, or pSVhCG-α/G/L^A DNA. 40 h after transfection, living cells were incubated on ice with appropriate antibody for 30 min to bind cell surface molecules, and then either fixed immediately (pulse panels), or replaced in media at 37°C for 1 h before fixation (chase panels). Fixed cells were treated with saponin, and antibodies bound to proteins remaining at the cell surface or internalized proteins were detected with FITC-conjugated secondary antibody. Photographs shown were exposed for varying times to visualize signal effectively. (*A*) h-lamp-1 wild type; (*B*) Ala_{386}; (*C*) 3-Y-7; (*D*) 9-Y-1; (*E*) Phe_{386}; (*F*) hCG-α/G/L^Y; (*G*) hCG-α^M; (*H*) hCG-α/G/L^A. Bar, 20 μm.

α/G/L^{Y} colocalizes with the lysosomal marker TR-O, suggesting that these structures are lysosomes (data not shown). The chimera hCG-α/G/L^{A} (Fig. 5 *D*) did not accumulate in lysosomes, but rather showed an intracellular distribution similar to h-lamp-1$_{Ala386}$ and hCG-α^{M}, with high concentrations at the transfected cell surface and in the Golgi body. These data suggest that the h-lamp-1 cytoplasmic tail contains a signal sufficient to redirect a glycoprotein to lysosomes rather than the cell surface. Corresponding with intact h-lamp-1, alteration of this cytoplasmic tail to substitute another amino acid in place of the tyrosine destroys the lysosome address function, allowing transport of hCG-α/G/L^{A} to the cell surface.

Elimination of Cytoplasmic Tyrosine Blocks h-lamp-1 Internalization

The chicken lamp-1 analogue LEP100 has been found to cycle between lysosomes and the plasma membrane via endocytic vesicles (Lippincott-Schwartz and Fambrough, 1987; Lippincott-Schwartz and Fambrough, 1988), and h-lamp-1 has been localized at cell surfaces, albeit in concentrations varying with cell type (Mane et al., 1989). To investigate whether h-lamp-1 molecules undergo endocytosis when expressed in COS-1 cells, movement of h-lamp-1 was studied by antibody capture experiments. Duplicate cultures of live transfected cells were incubated on ice with antibody to h-lamp-1 or hCG-α for 30 min to bind cell surface molecules, and then one culture was immediately fixed while the other was replaced in media at 37°C for 1 h before fixation. Fixed cells were then permeabilized by treatment with saponin and the location of antigen-antibody complexes detected with FITC-labeled secondary antibody. The intracellular distribution of primary antibody represents the movement over the 1-h chase period of glycoprotein labeled at the cell surface, and these cells were compared to cells that were fixed without chase. Fig. 6 (both *A* panels) shows two cells labeled in this way, and these data suggest that some wild-type h-lamp-1 is available for antibody binding at the surface (Fig. 6 *A*, *pulse*), and that no antibody penetrates the cell during the labeling, since no intracellular structures are detected by FITC-secondary antibody. At the end of the chase period, a large proportion of anti-(h-lamp-1) antibody labeled h-lamp-1 was transported into the cell, and may be seen to accumulate in lysosomes (Fig. 6 *A*, *chase*). These data indicate that h-lamp-1 is transported from the surface of transfected COS-1 cells, and is delivered to lysosomes. Mutants of h-lamp-1 in which the cytoplasmic tail was altered were used for similar experiments, and Fig. 6 *B*, *C*, and *D* show that Ala$_{386}$, 3-Y-7, 9-Y-1 are not internalized efficiently from the cell surface. Mutant Ala$_{386}$ protein seems not to be internalized at all, but interestingly, the mutants in which the cytoplasmic Tyr residue is present, but its position is altered, seem to have differing phenotypes. Mutant 3-Y-7 contains a Tyr closer to the membrane than wild type, and seems incompetent for internalization, similar to Ala$_{386}$, but the mutant protein-antibody complexes seem to accumulate in large, irregular regions of the cell surface. Mutant 9-Y-1, with a more COOH terminal Tyr, may be seen to move into the cell at much lower but detectable levels compared to wild type. Phe$_{386}$ also was found to be internalized at lower efficiency (Fig. 6 *E*) compared to wild type. These data support the conclusion that the cytoplasmic tyrosine of h-lamp-1 is necessary for proper cycling of the glycoprotein, and suggest that the internalization signal can be Phe$_{386}$ or Tyr$_{388}$, but that these mutations lead to lowered internalization efficiencies. This pattern of transport is controlled only by the cytoplasmic tail, since hCG-α/G/L^{Y}, which contains only that portion of h-lamp-1 attached to the hCG-α/VSV-G reporter molecule, is seen to be subject to similar removal from the surface and efficient transport to lysosomes (Fig. 6 *F*). The movement from the cell surface is probably not due to antibody cross-linking and indiscriminate clearance from the cell surface, since hCG-α^{M} was found to remain mainly at the cell surface in the 60-min chase period (Fig. 6 *G*). The chimera hCG-α/G/L^{A} differs from hCG-α/G/L^{Y} only by having Ala substituted for the cytoplasmic tyrosine, and Fig. 6 *H* shows that hCG-α/G/L^{A} is not internalized, analogous to h-lamp-1 mutant Ala$_{386}$. Previous studies have suggested that the VSV-G transmembrane and cytoplasmic domains, when appended to some glycoproteins, enable the endocytosis (Roth et al., 1986) and delivery to lysosomes (Rizzolo, 1989) of the chimeric glycoproteins. This could explain the small amount of punctate intracellular staining visible in the chase panel of Fig. 6 *G*, but comparing the chase panels of Fig. 6 (*G* and *F*) demonstrates that the h-lamp-1 cytoplasmic tail is much more effective in mediating endocytosis than the corresponding VSV-G domain. Together, these data indicate that the cytoplasmic tyrosine of h-lamp-1 is in a context that signals the majority of the protein to be delivered to lysosomes, and allows the small amount of h-lamp-1 that reaches the surface of transfected COS-1 cells to be removed and delivered to lysosomes.

Discussion

Lysosome membrane glycoproteins are abundant molecules present in all species and cell types tested, but the function of these glycoproteins remains unknown. Lamp molecules previously have been detected in low concentrations at the cell surface, and have been found to recycle between the cell surface, Golgi body, and lysosomes (Lippincott-Schwartz and Fambrough, 1986; Lippincott-Schwartz and Fambrough, 1987; Mane et al., 1989; Furuno et al., 1989*a*; Furuno et al., 1989*b*). Data presented here indicate that, dissimilar to lysosomal enzymes, the signal that causes h-lamp-1 to be efficiently concentrated in lysosomes is a tyrosine residue at a specific site in the short h-lamp-1 cytoplasmic tail, and that signal may be transferred to other integral membrane proteins resulting in the hybrids' accumulation in lysosomes.

Cytoplasmic domains have been found to be required for efficient endocytosis for a number of cell surface receptors. The low density lipoprotein (Lehrman et al., 1985), transferrin (Rothenberger et al., 1987), epidermal growth factor (Prywes et al., 1986), polymeric immunoglobulin (Mostov et al., 1986), constant region (Miettinen et al., 1989) insulin-like growth factor-II/cation independent mannose 6-phosphate (CI-MPR; Lobel et al., 1989) receptors, and an HLA class I molecule (Vega and Strominger, 1989) were shown to require cytoplasmic domains to be efficiently internalized from the cell surface, and a specific Tyr residue was found to be required for low density lipoprotein receptor (Davis et al., 1986, 1987) and CI-MPR (Lobel et al., 1989) endocyto-

sis. Influenza A virus HA normally does not enter coated pits and avoids endocytosis, but a mutant HA containing a tyrosine residue in one position in the cytoplasmic tail was found to enter cell surface coated pits, to be rapidly internalized, and eventually to reach lysosomes (Lazarovitz and Roth, 1988). Data presented here indicate h-lamp-1 molecules lacking a cytoplasmic tyrosine residue are not localized in lysosomes, but were found to accumulate at the cell surface. Unlike wild-type h-lamp-1, these mutants were unable to enter the endocytic pathway efficiently, suggesting that the movement of lamp from cell surface coated pits to lysosomes described previously (Lippincott-Schwartz and Fambrough, 1986) takes place via a mechanism similar to that used for receptor endocytosis. This mechanism has been proposed to include a class of coated pit accessory proteins termed adaptins (Robinson, 1987; Pearse and Robinson, 1984; Pearse, 1988), proteins purified from cell surface coated pits found to bind specifically to peptides containing tyrosine residues corresponding to receptor and HA(Tyr) cytoplasmic tails, but not to similar peptides lacking tyrosine (Pearse, 1988).

Does the intracellular route taken by de novo synthesized h-lamp-1 necessarily include the cell surface before arrival in lysosomes? Lysosomal acid phosphatase was recently discovered to pass through the plasma membrane during transport to lysosomes (Braun et al., 1989). cDNA sequence analysis of the human (Pohlmann et al., 1988) and rat (Himeno et al., 1989*b*) forms of this lysosomal enzyme showed that it contains a cytoplasmic tyrosine similar to lamp, but kinetic studies indicate that the enzyme requires much longer time to reach lysosomes from the endosome–cell surface cycle than lamp (Braun et al., 1989), suggesting that its sorting is slightly different than lamp. Direct evidence describing lamp movement out of the *trans*-Golgi network is lacking, and studies of lamp biosynthesis kinetics have not yielded clear-cut results. Lysosome membrane glycoproteins have been described to cycle between lysosomes and the cell surface in some cell types, but were not detected at the cell surface in others. The differences reported for lamp cell surface expression may reflect an inability to detect small amounts of rapidly transported glycoprotein by electron microscopy, or are perhaps due to differences in lamp transport kinetics in cells at varying stages of differentiation in different species. Data presented here suggest that if h-lamp-1 does not first go to the cell surface before lysosomes, and sorting of lysosome membrane proteins occurs in the *trans*-Golgi network or a later, intracellular compartment, then the same signal, a tyrosine-containing cytoplasmic tail, is used for both intracellular sorting and for efficient endocytosis.

The 11-residue length of the lamp cytoplasmic tail may function to limit interactions with cytoplasmic proteins by limiting overlapping protein binding. Separate domains for lysosomal enzyme sorting and endocytosis were identified in the CI-MPR cytoplasmic tail (Lobel et al., 1989). CI-MPR is internalized from the cell surface and also segregates mannose 6-phosphate containing enzymes from the *trans*-Golgi network into the lysosome biogenesis pathway (Morgan et al., 1987), but does not progress towards lysosomes beyond prelysosomal/endosomal vesicles (Griffiths et al., 1988; Geuze et al., 1988) and is recycled to the *trans*-Golgi network (Duncan and Kornfeld, 1988). The lamp tail is likely too short to contain such binding sites, but the finding that slight alterations in the relative Tyr position cause much less lysosomal accumulation and less effective internalization suggests that h-lamp-1 could interact very specifically with cytoplasmic factors. Further experiments are required to determine if the h-lamp-1 cytoplasmic tail interacts with specific proteins at the cell surface or in intracellular vesicles, and the identity and function of these proteins in lysosomal membrane glycoprotein sorting.

Sorting of lamp from cell surface resident proteins could begin in the *trans*-Golgi complex, as previously suggested (Green et al., 1987), when lamp cytoplasmic tails could associate with a specific sorting protein that would direct them into the lysosome biogenesis pathway perhaps through recognition of the lamp tyrosine-containing cytoplasmic domain. Overproduction of lamps could saturate the sorting pathway, analogous to the epidermal growth factor receptor (Wiley, 1988), explaining differences described in the amount of lamp at the cell surface. h-lamp-1 molecules would concentrate first in prelysosomal vesicles that are most likely contiguous with, or the same as, endosomal vesicles (Griffiths et al., 1988; Geuze et al., 1988), and mixing in this branch-point/sorting vesicle would allow a small, constant amount of lamp to reach the cell surface via the receptor recycling pathway, and also allow internalized cell surface lamp to be sorted to lysosomes. Lamp molecules in lysosomes could reenter the sorting mechanism through the mechanism described for the exchange of lysosomal contents via membrane-bound vesicles (Deng and Storrie, 1988; Draye et al., 1988). Disruption of lysosome pH and morphology with chloroquine was found to cause the redistribution of chicken lamp LEP100 to the cell surface and endocytic vesicles (Lippincott-Schwartz and Fambrough, 1987). This redistribution may occur by blocking lysosome formation but not recycling, causing an increase in the number of sorting vesicles and limiting lamp to cycling between the cell surface and the prelysosome block point. This model differs somewhat from another recently proposed model for lamp intracellular transport (Kornfeld and Mellman, 1989), but it accounts for lysosome accumulation and recycling of lamp molecules, and it suggests several avenues for testing its validity.

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