The Pathway and Targeting Signal for Delivery of the Integral Membrane Glycoprotein LEP100 to Lysosomes

Paul M. Mathews, John B. Martinie, and Douglas M. Fambrough

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

Abstract. A complete set of chimeras was made between the lysosomal membrane glycoprotein LEP100 and the plasma membrane-directed vesicular stomatitis virus G protein, combining a glycosylated lumenal or ectodomain, a single transmembrane domain, and a cytosolic carboxyl-terminal domain. These chimeras, the parent molecules, and a truncated form of LEP100 lacking the transmembrane and cytosolic domains were expressed in mouse L cells. Only LEP100 and chimeras that included the cytosolic 11 amino acid carboxyl terminus of LEP100 were targeted to lysosomes. The other chimeras accumulated in the plasma membrane, and truncated LEP100 was secreted. Chimeras that included the extracellular domain of vesicular stomatitis G protein and the carboxyl terminus of LEP100 were targeted to lysosomes and very rapidly degraded. Therefore, in chimera-expressing cells, virtually all the chimeric molecules were newly synthesized and still in the biosynthesis and lysosomal targeting pathways. The behavior of one of these chimeras was studied in detail. After its processing in the Golgi apparatus, the chimera entered the plasma membrane/endosome compartment and rapidly cycled between the plasma membrane and endosomes before going to lysosomes. In pulse-expression experiments, a large population of chimeric molecules was observed to appear transiently in the plasma membrane by immunofluorescence microscopy. Soon after protein synthesis was inhibited, this surface population disappeared. When lysosomal proteolysis was inhibited, chimeric molecules accumulated in lysosomes. These data suggest that the plasma membrane/early endosome compartment is on the pathway to the lysosomal membrane. This explains why mutations that block endocytosis result in the accumulation of lysosomal membrane proteins in the plasma membrane.

THE limiting membrane of the lysosome is greatly enriched in a class of heavily glycosylated, extensively sialylated integral membrane proteins (reviewed in Kornfeld and Mellman, 1989). Although the function of these glycoproteins is not known, they may protect the lysosomal membrane from hydrolysis (Kornfeld and Mellman, 1989; Granger et al., 1990). These glycoproteins are also found in lower abundance in endocytic vesicles and in the plasma membrane (Lippincott-Schwartz and Fambrough, 1987; Griffiths et al., 1988; Mane et al., 1989). The avian lysosomal membrane glycoprotein LEP100 and its encoding DNA have been characterized (Lippincott-Schwartz and Fambrough, 1986, 1987; Fambrough et al., 1988; Zot and Fambrough, 1990). LEP100 has a cleaved amino-terminal signal peptide, a lumenal domain of 361 amino acids with 17 N-linked oligosaccharides, a single transmembrane domain of 24 amino acids, and a cytoplasmic carboxyl terminus of 11 amino acids. Homologues of LEP100 occur in mammals, including human hLAMP-1 (Viitala et al., 1988; Fukuda et al., 1988), rat lgp120 (Howe et al. 1988), and mouse mLAMP-1 (Chen et al., 1988). Amino acid sequence similarity among these four lysosomal membrane glycoproteins increases from the amino terminus to the carboxyl terminus, with the cytoplasmic domain showing complete sequence identity.

The intracellular trafficking leading to the lysosomal delivery of many soluble lysosomal enzymes involves the mannose 6-phosphate (M6P)[1] signal/receptor system. Newly synthesized lysosomal enzymes are modified by the addition of phosphate to the 6-hydroxy position on mannose residues (von Figura and Hasilik, 1986). This M6P signal is recognized by the M6P receptor in the *trans*-Golgi network (TGN) (Griffiths and Simons, 1986), and M6P-tagged enzymes are transported by vesicular traffic to late endosomes (Lemansky et al., 1987; Marquardt et al., 1987; Griffiths et al., 1988). Here the enzymes dissociate from their receptor and are transported on to lysosomes while the receptor is recycled to the TGN (Brown et al., 1986; Duncan and Kornfeld, 1988; Goda and Pfeffer, 1988). The lysosomal membrane glycoproteins, however, do not contain the M6P modification and must be targeted to lysosomes by a different mechanism (D'Souza and August, 1986; Lippincott-Schwartz and

1. *Abbreviations used in this paper*: CHX, cycloheximide; LAP, lysosomal acid phosphatase; M6P, mannose 6-phosphate; TGN, *trans*-Golgi network; VSV-G, vesicular stomatitis virus coat glycoprotein.

Fambrough, 1986; Barriocanal et al., 1986; Granger et al., 1990).

Previous reports have documented the lysosomal targeting of chimeric proteins consisting of the ectodomain of a plasma membrane protein linked to the transmembrane and cytoplasmic domains of LEP100 or the cytoplasmic domain of hLAMP-1 (Mathews, P. M., and D. M. Fambrough, 1988. *J. Gen. Phys.* 92:18*a*; Williams and Fukuda, 1990). We report here on a complete battery of chimeras between LEP100 and the plasma membrane protein vesicular stomatitis virus coat glycoprotein (VSV-G) in which each of the three domains of LEP100 was replaced by the corresponding domain of VSV-G and vice versa. The behavior of these chimeras and a truncated form of LEP100 that was secreted suggests that only the 11-amino acid cytoplasmic domain of LEP100 is involved in lysosomal targeting.

The mechanism whereby the cytoplasmic tail of LEP100 directs the lysosomal targeting of newly synthesized molecules remains unknown. The single tyrosine in the cytoplasmic tail has been shown to be necessary for the lysosomal targeting of hLAMP-1 and lgp120. Mutating this tyrosine resulted in proteins that accumulate in the plasma membrane, as did eliminating most of the cytoplasmic tail of hLAMP-1 (Williams and Fukuda, 1990; Hunziker et al., 1991). This mistargeting has been interpreted as resulting from a defect in sorting in the TGN: newly synthesized molecules fail to target to lysosomes at the TGN and are therefore transported to the plasma membrane along the constitutive secretory pathway. This interpretation was based predominantly on the work of Green et al. (1987), in which metabolically labeled lgp120 was found to appear rapidly in a lysosomal subcellular fraction, and very little lgp120 was found in the plasma membrane and early endosomes. Recently Harter and Mellman (1992) revised this interpretation, proposing a branched pathway of targeting of lgp120 partly via the TGN to late endosomes and lysosomes and partly via the plasma membrane.

Contrary to these interpretations, recent experiments indicate that most or all molecules of a very closely related lysosomal membrane glycoprotein (LAMP-2) pass through the basolateral plasma membrane of polarized MDCK cells before lysosomal delivery (Nabi et al., 1991). LAMP-2 molecules, which share some sequence identity with LEP100, have been cloned from a number of mammalian species (Fukuda et al., 1988; Cha et al., 1990; Granger et al., 1990). Another lysosomal protein, human lysosomal acid phosphatase (LAP), travels to the plasma membrane as an integral membrane protein prior to endocytosis, lysosomal delivery, and cleavage to a soluble form (Waheed et al., 1988; Braun et al., 1989). In our study we made use of a LEP100 deletion mutant and chimeras between LEP100 and VSV-G protein to study the pathway by which the lysosomal targeting signal in LEP100 directs molecules to the lysosomes. These three cases (LAMP-2, LAP, and LEP100) suggest that the delivery of integral membrane proteins to the lysosome via the cell surface is a major pathway in animal cells.

Materials and Methods

Mutagenesis and Construction of Chimeric cDNAs

The cDNA encoding VSV-G was the generous gift of Dr. J. K. Rose (Yale University School of Medicine, New Haven, CT) (Rose and Shafferman, 1981; Rose and Bergmann, 1982). Mutations creating unique restriction endonuclease sites were introduced by the method of Kunkel et al. (1987). Briefly, dUTP-containing single stranded cDNA was prepared, mutagenic synthetic oligonucleotides were used to prime second strand synthesis, and mutated DNA was recovered after transforming dUTP-intolerant bacteria. A PvuII restriction site was introduced into the VSV-G cDNA just 5′ to the region encoding the transmembrane domain; this site is in-frame with a unique PvuII site located just 5′ to the region encoding the transmembrane domain in the LEP100 cDNA. HpaI sites were introduced into both cDNAs near the 3′ end of the region encoding the transmembrane domain. Chimeric cDNAs were constructed using these restriction sites (see Fig. 1).

The chimera in which the cytoplasmic domain of LEP100 replaced that of VSV-G was constructed using the polymerase chain reaction (PCR) (Saiki et al., 1988). A hybrid primer with the sequence 5′-GGA CTA TTC TTG GTT CTC AGA AAG AGG AGC CAC-3′ was used to generate a 448-bp fragment consisting of a 18-bp region of identity to the 3′-most transmembrane encoding region of VSV-G, the entire cytoplasmic domain and 3′ untranslated regions of the LEP100 cDNA, and an additional 68 bp extending beyond the polylinker in pSVDF-4 (see below). This fragment was isolated and used as primers with VSV-G cDNA in the Bluescript vector (Stratagene, La Jolla, CA) for six PCR cycles. Then a large fragment encoding most of the lumenal and all of the transmembrane regions of VSV-G and the cytoplasmic domain of LEP100 was generated by adding appropriate primers and carrying out 30 additional PCR cycles. This fragment was cut with NcoI and EcoRI and cloned into Bluescript containing the 5′-609 bp of VSV-G (up to the unique NcoI site).

To generate the DNA encoding a secretory form of LEP100, a PCR fragment representing just the lumenal encoding domain of LEP100 was generated, using as 3′ end primer the oligonucleotide 5′-AGG GAA TTC TTA CTA CAT GTT GTT TTC ATC CAG-3′ that introduced two tandem stop codons followed by an EcoRI site after the last codon preceding the transmembrane encoding region. This fragment was digested with BglII and EcoRI and cloned into the Bluescript vector containing the 5′-790-bp EcoRI-BglII fragment of the LEP100 cDNA.

The nucleotide sequences of all the splice regions of the chimeric DNAs and all the PCR-generated coding regions were confirmed by DNA sequencing (Sanger et al., 1977). The structures of the normal, mutant, and chimeric proteins are summarized in Fig. 1 along with the nomenclature used for them in this report.

Expression of DNAs in Ltk⁻ Cells

Mouse Ltk⁻ cells (Kit et al., 1963) were maintained in DME, 10% FBS, and 50 μg/ml gentamicin (Gibco Laboratories, Grand Island, NY) at 37°C in a 5% CO_2 atmosphere.

The cDNAs encoding LEP100, VSV-G, and the chimeras were cloned into the mammalian expression vector pSVDF-4 (Takeyasu et al., 1987), permitting butyrate-inducible expression driven by the SV-40 early promoter (Gorman et al., 1983). For stable lines, mouse Ltk⁻ cells were cotransfected using either a calcium phosphate precipitate (Small and Scangos, 1984) or lipofectin (Gibco-BRL, Rockville, MD) used according to manufacturers protocol, with ~ 20 μg of expression plasmid DNA and 2 μg of pHSVtk5′ Δ 0.2 DNA (McKnight, 1980) per 100-mm tissue culture dish of sub-confluent Ltk⁻ cells. After 24 h the medium was replaced by HAT medium (Boehringer Mannheim Biochemicals, Indianapolis, IN). HAT-resistant clones (Littlefield, 1964) were isolated, expanded, and assayed for expression of LEP100 or VSV-G epitopes by immunofluorescence microscopy.

The cDNA encoding truncated LEP100 was cloned into an EcoRI site introduced just 3′ of the human cytomegalovirus immediate early promoter in the expression vector pCB6 (Brewer and Roth, 1991). L cell lines expressing truncated LEP100 were established by lipofectin-mediated transfection and selection in 400 μg/ml G418 (Sigma Chemical Co., St. Louis, MO) in DME 10% FBS. Approximately 150 colonies from a single 100-mm tissue culture dish were pooled for use in immunofluorescence and metabolic labeling experiments. Expression required butyrate induction (10 mM for 24 to 48 h).

Transient expression was employed in some immunofluorescence experiments. L cells were plated on glass coverslips in 35-mm tissue culture dishes, 4 μg DNA was added as lipofectin/DNA complex in 1 ml DME, 1 ml DME with 10% FBS was added 4 h later, and at 24 h the growth medium was replaced with DME 10% FBS containing 10 mM butyrate. After an additional 36 to 48 h these cultures were used for immunofluorescence microscopy. The recombinant vaccinia virus/T7 RNA polymerase expression system (Fuerst et al., 1986; Moss, 1991) was used for short-term

expression of high levels of protein. Chimeric cDNAs were cloned into the Bluescript vector downstream from the T7 promoter. Newly confluent Ltk⁻ cells growing on 25-mm-diam glass coverslips were infected with the recombinant vaccinia virus at a multiplicity of 10 pfu/cell in 0.6 ml DME. 30 min later, transfection was begun by adding 4 to 5 μg DNA, 10 μl lipofectin in 1 ml DME. After 4 h, 1 ml DME 10% FBS was added. In "pulse expression" experiments (see Results), 75 μg/ml cycloheximide (Sigma Chemical Co.) was added at this time. When leupeptin was used to prevent lysosomal degradation of the ectodomain of VSV-G, 20 μM leupeptin (Sigma Chemical Co.) was added 3 to 4 h before infection and maintained throughout the experiment.

Immunofluorescence Microscopy

Anti-LEP100 monoclonal IgG (LEP100-mAb) (Lippincott-Schwartz and Fambrough, 1986) recognizes a lumenal epitope on LEP100 and does not cross-react with murine Ltk⁻ cells. mAb II (VSV-G-mAb), the hybridoma a gift of Dr. D. S. Lyles (Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC), binds to an extracellular epitope of VSV-G (LeFrancois and Lyles, 1982). Antibodies were purified from ascites fluid as previously described by Lippincott-Schwartz and Fambrough (1986). FITC or TRITC (Molecular Probes, Eugene, OR) was conjugated to purified antibody as described by Anderson and Fambrough (1983). FITC and TRITC-conjugated goat anti-mouse-IgG sera were purchased from Cappel Laboratories (Malvern, PA). For location of endogenous m-LAMP1 in mouse L cells, rat monoclonal anti-m-LAMP1 (Chen et al., 1985) and FITC-conjugated goat anti-rat-IgG serum (Cappel Laboratories) were used.

Cells grown on 25-mm coverslips were fixed in 1% fresh formaldehyde, 100 mM sucrose, 20 mM Na phosphate (pH 7.2) at room temperature for 10 min, rinsed, labeled for 30–60 min with 5 μg/ml mAb in Hepes-buffered (20 mM pH 7.2) MEM containing 10% horse serum, and washed repeatedly with Hepes/MEM containing 10% horse serum. Cells were permeabilized by the addition of 0.125% saponin (wt/vol) (Sigma Chemical Co.) to the antibody containing and the wash solutions. For indirect immunofluorescence labeling, coverslips were washed after primary antibody binding, and secondary antibody was then bound followed by 30 min of washes. Cells were mounted in 90% glycerol, 10% PBS, 1 mg/ml *p*-phenylene diamine and examined with a (Carl Zeiss, Oberkochen, Germany) epifluorescence microscope.

Metabolic Labeling and Immunoprecipitation

Approximately 5×10^5 cells were plated in 35-mm tissue culture dishes and after 16 h were induced with 10 mM butyrate for 36 to 48 h. Cultures were incubated in methionine-free MEM containing 10 mM butyrate for 30 min before a 15-min incubation with 100–200 μCi/ml [^{35}S]methionine and [^{35}S]cysteine (TRAN^{35}S-LABEL) (ICN Biomedicals, Inc., Costa Mesa, CA). After four rinses with DME 10% FBS at 4°C, cells were grown in DME, 10% FBS for the chase times specified in the text. For some experiments, cells were treated with 20 μM leupeptin beginning 4–5 h before methionine starvation and continuing through the labeling and chase periods.

^{35}S-labeled cells were rinsed once with PBS, extracted with 1 ml buffer A (150 mM NaCl, 50 mM Tris, 10 mM EDTA, 1% Triton X-100, pH 7.5) containing protease inhibitors (0.3 mg/ml benzamidine, 0.6 mg/ml *N*-ethylmaleimide, 1.0 mg/ml bacitracin), and the extracts were frozen. Extracts were thawed and centrifuged at 12,000 *g* 10 min. Each supernatant was mixed with 50 μg mAb covalently coupled to Sepharose 4B beads (Fambrough and Bayne, 1983) and incubated at 4°C overnight. Immunobeads were washed eight times with buffer A, twice with PBS, and eluted with SDS sample buffer (10% [vol/vol] glycerol, 30 mg/ml SDS, 5% [vol/vol] 2-mercaptoethanol, 62 mM Tris, pH 6.8). Immune precipitates were analyzed by SDS-PAGE and fluorography. Radioactivity in the bands was quantified with a Molecular Dynamics PhosphorImager and the ImageQuant software package.

^{125}I-mAb Binding

Sodium [^{125}]iodide was purchased from Amersham Corp. (Arlington Heights, IL), Iodogen from Pierce Chemical Company (Rockford, IL). Purified monoclonal IgGs were iodinated by the Iodogen method (Salicinski et al., 1981). Specific activities of iodinated antibodies were determined from knowledge of the antibody concentration (input antibody amount determined spectrophotometrically) and the radioactivity measured in aliquots of the antibody solution after iodination. These values were typically on the order of 1 cpm/10^6 antibody molecules. Cells grown in 35-mm tissue culture dishes were fixed at room temperature for 10 min (as for immunofluorescence microscopy) and incubated for 1–2 h with ^{125}I-mAb (5 μg/ml) in MEM, 10% horse serum, 20 mM Hepes (pH 7.2). After unbound antibody was removed by three 10 min washes in 1–2 liters of medium at 4°C, bound ^{125}I-mAb was eluted with 2 ml of 1 N NaOH and counted in a gamma counter. To permeabilize cells, saponin (0.125% wt/vol) was added to the binding medium. Non-specific binding was determined using a 50- or 100-fold excess unlabeled mAb in the binding solution. Data points represent the average of two or three measurements of non-specific binding subtracted from the average of three to five measurements of total binding.

Results

Only LEP100/VSV-G Chimeras Containing the Cytoplasmic Domain of LEP100 Are Targeted to Lysosomes

VSV-G was chosen for the construction of chimeric molecules because of the overall similarity in topography to LEP100, and because, unlike LEP100, VSV-G accumulates in the plasma membrane of transfected cells (Rose and Bergmann, 1982). Chimeric cDNAs were constructed in which regions of the DNA encoding the three domains of LEP100 (lumenal, transmembrane, and cytoplasmic) were replaced singly and in combinations by DNA encoding the corresponding domains of VSV-G (Fig. 1). The resulting chimeras were expressed in mouse L cells, and their cellular locations were determined by immunofluorescence microscopy with monoclonal antibodies specific for either the lumenal domain of LEP100 or the extracellular domain of VSV-G. For all chimeras containing the ectodomain of VSV-G, the protease inhibitor leupeptin was added to cells 16 h before fixation to prevent proteolytic destruction of this domain in lysosomes (see below). When expressed in L cells (Fig. 2) VSV-G showed the expected cell-surface localization, while LEP100 showed a lysosomal distribution. (In numerous experiments co-localization of LEP100 and endogenous mouse LAMP-1 was found; occasionally one or two internal structures showed selective immunolabeling with LEP100 or LAMP-1.)

Three of the chimeras (L/V/V, L/L/V, and V/L/V) accumulated in the plasma membrane (Fig. 2). These chimeras have in common the cytoplasmic domain of VSV-G. The apparent cell surface distribution of VSV-G, L/V/V, and L/L/V was quantified by ^{125}I-VSV-G mAb binding to intact and permeabilized cells. After 48 h of butyrate induction and 16 h of leupeptin treatment, ≥75% of molecules were in the plasma membrane (Table I).

The three chimeras incorporating the cytoplasmic domain of LEP100 (V/L/L, V/V/L, and L/V/L) accumulated in lysosomes (Fig. 2), co-localizing with mLAMP1. Although LEP100 and V/L/L were predominantly intracellular, ^{125}I-antibody binding experiments detected some molecules at the cell surface (Table I). The presence of these molecules in the plasma membrane is similar to the situation for LEP100 in chicken embryo fibroblasts, where ~2–3% of molecules were found on the surface (Lippincott-Schwartz and Fambrough, 1986, 1987).

The Lumenal Domain of LEP100 Is Secreted When Expressed as a Truncated Protein

The lumenal domain of LEP100 appears to lack lysosomal targeting information, since L/V/V and L/L/V chimeras do not accumulate in lysosomes. However, it is possible that tar-

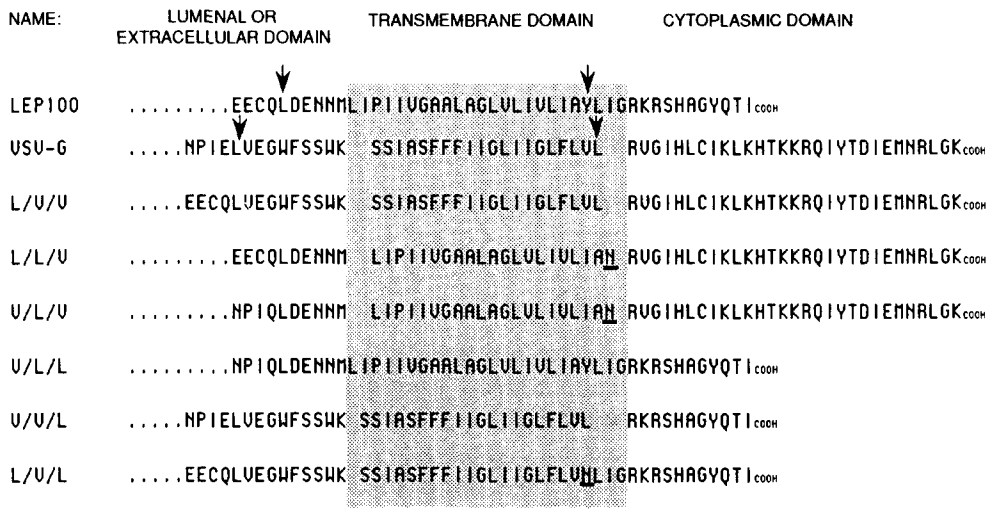

Figure 1. Nomenclature and amino acid sequences of LEP-100/VSV-G chimeric proteins. The amino acid sequences for LEP100, VSV-G, and the chimeras are shown beginning a few amino acid amino terminal to the transmembrane domain (shaded). Constructions of chimeras employed PvuII sites in the DNA encoding the lumenal domain of LEP100 and the extracellular domain of VSV-G and Hpa I sites in the DNA encoding the transmembrane domains (arrows indicate corresponding amino acid locations). Chimeras are named according to the scheme lumenal-extracellular domain/transmembrane domain/cytoplasmic domain with "L" for LEP100 and "V" for VSV-G; e.g., L/V/L consists of the lumenal domain of LEP100/transmembrane domain of VSV-G/cytoplasmic domain of LEP100. Truncated LEP100 consists of the entire lumenal domain without the transmembrane of cytoplasmic domains. The asparagine residue (*underlined*) in the transmembrane domain of L/L/V, V/L/V and L/V/L is an amino acid change resulting from the generation of the HpaI site.

geting information in the lumenal domain of LEP100 might be overridden by information in the cytoplasmic domain of VSV-G. If the lumenal domain of LEP100 contains intrinsic lysosomal targeting information and reaches the lysosome by a route analogous to that taken by M6P-tagged lysosomal enzymes, then the lumenal domain expressed alone should accumulate in lysosomes. Otherwise, this domain, expressed as a truncated protein, should be secreted. To test these possibilities, we constructed a truncated LEP100 consisting of the entire lumenal domain without the transmembrane and cytoplasmic domains (Fig. 1). When truncated LEP100 was expressed in mouse L cells, immunofluorescence labeling revealed a reticular pattern and nuclear ring, indicative of predominantly ER localization. No lysosomal pattern of labeling was detected even when cells were cultured in the presence of leupeptin for 16 h before immunofluorescence microscopy; and when cycloheximide was also included in the medium for the final few hours, immunoreactivity disappeared, consistent with secretion of truncated LEP100.

The fate of the truncated LEP100 protein was verified by pulse-chase metabolic labeling experiments. After a 30-min [^{35}S] methionine, [^{35}S]cysteine pulse but no chase, truncated LEP100 was recovered from the cell lysate. The truncated LEP100 isolated from the cells was the high mannose intermediate form, migrating in SDS-PAGE with an M_r of 85 kD, 6 kD less than that of the high mannose form of full-length LEP100 expressed in L cells. During the chase period the amount of truncated LEP100 associated with the cells decreased rapidly (Fig. 3, *A* and *B*). The complex carbohydrate form of truncated LEP100 was not apparent in immunoprecipitates from cell lysates even with overexposure of x-ray film. However, this form with M_r of 100–110 kD appeared in the medium during the 1–2-h chase and remained relatively stable to 10 h (Fig. 3, *A* and *B*). Thus, there appears to be no pathway for transport of the lumenal domain of LEP100 from the Golgi apparatus to lysosomes.

The VSV-G Ectodomain of V/L/L Is Rapidly Degraded in Lysosomes

The chimera V/L/L did not accumulate sufficiently in lysosomes for detection by immunofluorescence microscopy except when the protease inhibitor leupeptin was present in the medium (see Fig. 2). Presumably, the VSV-G ectodomain was degraded rapidly upon delivery to the lysosome. To study this, L cells from a cell line expressing V/L/L were treated for increasing times with leupeptin and total V/L/L molecules quantified by ^{125}I-VSV-G-mAb binding (Fig. 4). The number of V/L/L molecules tripled by 12 h, beginning a few hours after addition of leupeptin, and doubled again by 16 h (see Table II). The lag in accumulation was presumably due to slow onset of leupeptin inhibition of proteolysis. (For this reason, treatment with leupeptin was begun 4 h before metabolic labeling or transient expression with the recombinant vaccinia virus/T7 system) (see below).

If V/L/L molecules are targeted to lysosomes and rapidly degraded there, the population of V/L/L in cells not treated with leupeptin should be comprised of V/L/L molecules in the biosynthetic pathway and in the lysosomal targeting pathway. Treatment of expressing cells with the protein synthesis inhibitor cycloheximide (CHX) should result in the disappearance of V/L/L molecules as they transit these pathways and arrive in lysosomes. The kinetics of disappearance of V/L/L in CHX-treated cells is shown in Fig. 5. Approximately 80% of the molecules were lost within 90 min. The kinetics of loss of V/L/L molecules would be expected to be complex, reflecting the transport of newly synthesized V/L/L molecules through the biosynthetic pathway and their delivery to the lysosome. Transport events preceding delivery to the lysosome are likely to be rate limiting and the actual degradation of the VSV-G ectodomain in the lysosome may be a very rapid event. This interpretation is supported by pulse-chase metabolic labeling experiments described below.

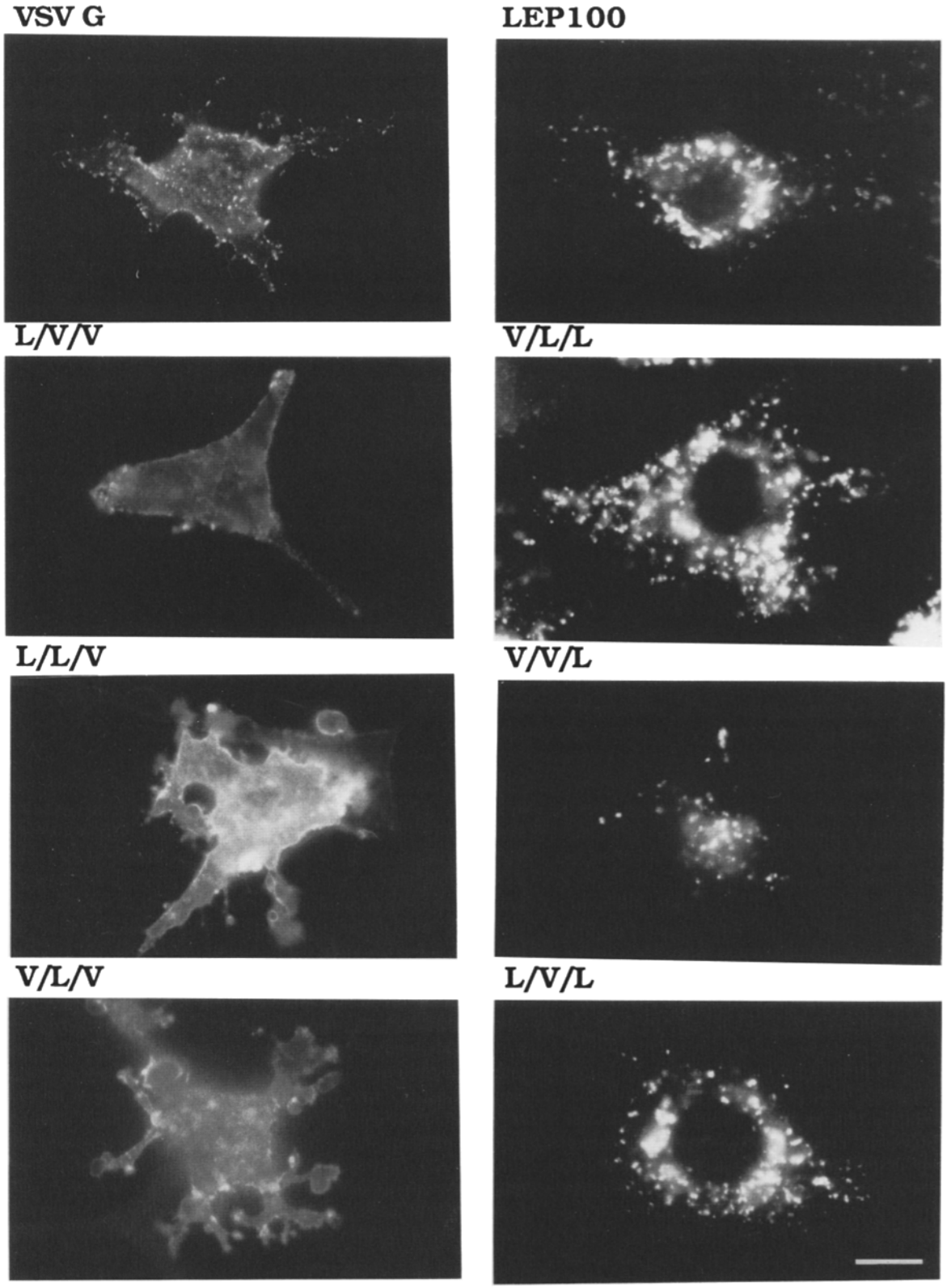

Figure 2. Immunofluorescence microscopy of cells expressing chimeric proteins. L cells expressing VSV-G, L/V/V, L/L/V, V/L/V (left panel top to bottom), LEP100, V/L/L, V/V/L, and L/V/L (right panel top to bottom) were labeled with either FITC conjugated VSV-G-mAb or LEP100-mAb. All cells were permeabilized by saponin with the exception of those expressing VSV-G. In order to see lysosomal accumulation of chimeras containing the ectodomain of VSV-G (V/L/V, V/L/L, and V/V/L), it was necessary to add leupeptin to the cells during the latter part of the expression period (generally 16–20 h of leupeptin treatment was chosen). VSV-G, L/V/V, L/L/V, LEP100, and V/L/L were expressed in stably transfected L cell lines. V/L/V and L/V/L were expressed in transient expression assays employing lipofectin. The chimera V/V/L was expressed using the vaccinia virus/T7 expression system. For this photomicrograph, V/V/L expression was allowed to proceed for 4 h, then cycloheximide was added for an additional 4 h, followed by fixation and fluorescent antibody labeling. Protein synthesis was blocked with cycloheximide to allow newly synthesized V/V/L molecules to move out of the biosynthetic pathway and into lysosomes, simplifying the immunofluoresence pattern. Bar, 10 μm.

Table I. Cell Surface Expression of LEP100, VSV-G, and Chimeras in Stably Expressing L Cell Lines

Cell line expressing	Percentage of total binding at cell surface
VSV G	73.4 ± 7.9%*
L/V/V	86.8 ± 2.6%*
L/L/V	91.7 ± 1.7%*
LEP100	3.7 ± 2.4% (n = 2)‡
V/L/L	0.8 ± 0.1% (n = 3)‡

Sets of five dishes of cells (3 measurements of total binding, two of non-specific binding) were incubated with ^{125}I-labeled mAb either with or without permeabilization with saponin, and the percentage of specific binding sites on the plasma membrane was determined. All cell lines were treated with 20 μM leupeptin for the final 16 h of a 48 h butyrate induction.
* (Average surface binding)/(average total binding) ± (range of surface binding)/(average of total binding), all expressed as a percentage.
‡ Mean ± standard error.
n = number of independent experiments.

In the CHX treatment experiments a small fraction of V/L/L molecules was relatively long lived. Pulse-chase metabolic labeling experiments did not reveal a subset of V/L/L molecules which were long-lived without leupeptin treatment (see Fig. 6). Among possible explanations for this discrepancy is that CHX treatment might have resulted in depletion of lysosomal hydrolases necessary for the rapid degradation of the VSV-G ectodomain, thus extending the stability of V/L/L in the lysosome.

Fig. 6 shows SDS-PAGE analysis of V/L/L from L cells pulse-labeled for 15 min with [^{35}S]methionine and [^{35}S]cysteine followed by various chase times. Without the addition of leupeptin, ^{35}S-labeled V/L/L was no longer detected after a 2-h chase. This is consistent with the disappearance of the majority of VSV-G-mAb binding sites within 90 min after the addition of CHX (Fig. 5). Fully glycosylated V/L/L first appeared after 20 min of chase, indicating passage into

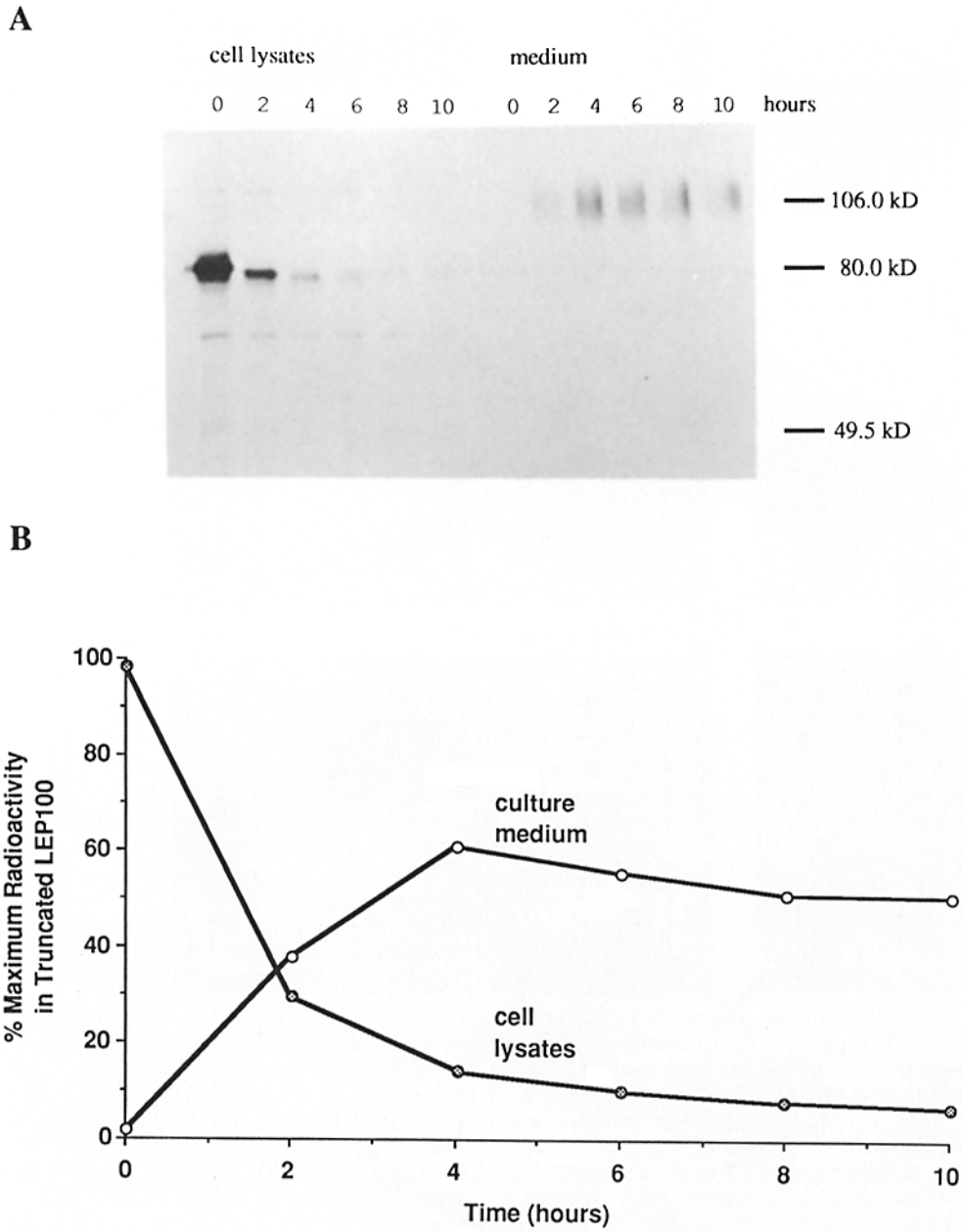

Figure 3. Secretion of a truncated LEP100. Truncated LEP-100 was expressed in a pooled population of cell lines. (*A*) Expressing cells, grown in 60-mm-diam tissue culture dishes and butyrate induced for 40 h, were metabolically labeled for 30 min with TRAN^{35}S-LABEL. The chase medium and the detergent lysate of the cells at each chase time were incubated overnight at 4°C with LEP100-mAb immunobeads. Immunobeads were washed extensively and bound material eluted directly in SDS sample buffer and analyzed by SDS-PAGE and fluorography. (*B*) Radioactivity in electrophoretic bands was quantified by PhosphorImager analysis and expressed as percentages of ^{35}S originally incorporated into LEP100 during the pulse.

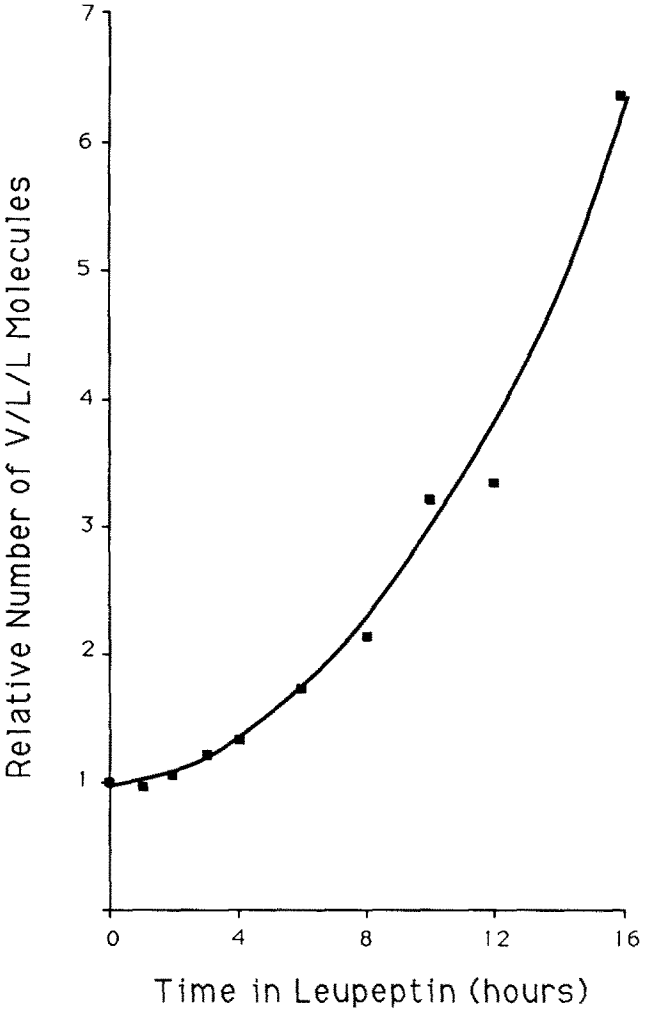

Figure 4. Accumulation of V/L/L following leupeptin treatment. An L cell line expressing the chimera V/L/L was grown in 35-mm-diam tissue culture dishes, induced for 48 h with butyrate, and treated with 20 μM leupeptin for the final portion of this time period, as indicated. Each point represents the average of six measurements of ^{125}I-labeled VSV-G-mAb binding minus the average of two measurements of non-specific binding. Specific binding for each period of time in medium containing leupeptin was normalized to the binding in cultures not treated with leupeptin, yielding relative numbers of V/L/L molecules in the cultured cells.

Table II. Distribution of V/L/L in Cells with and without Leupeptin Treatment

Treatment	^{125}I-VSV-G-mAb bound to permeabilized cells	^{125}I-VSV-G-mAb bound to non-permeabilized cells
	(*cpm*)	(*cpm*)
20 μM leupeptin	956,000 ± 60,000	6,500 ± 90
no leupeptin	155,000 ± 4,100	5,000 ± 550

Cultures of V/L/L-expressing cells were induced for 48 h with butyrate and some cells were treated for the last 16 h with 20 μM leupeptin. The numbers of specific total ^{125}I-VSV-G-mAb binding sites and cell surface binding sites were determined in saponin permeabilized and non-permeabilized cells as described in Materials and Methods. Leupeptin treatment results in some cell loss from tissue culture dishes (<20%) as ascertained by counting cell numbers on multiple duplicate dishes with and without leupeptin treatment. The data for leupeptin treatment are adjusted to reflect this.

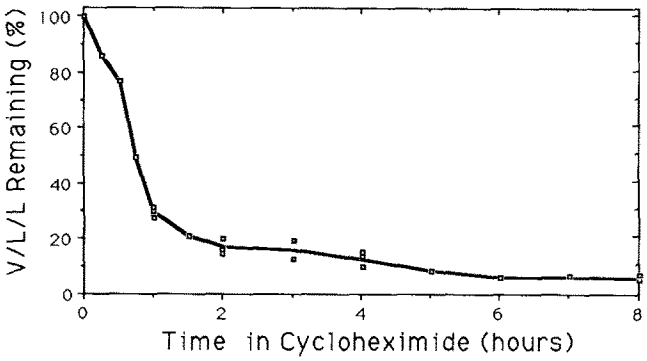

Figure 5. Stability of V/L/L following cycloheximide block of protein synthesis. An L cell line expressing the chimera V/L/L was grown in 35-mm-diam tissue culture dishes, induced with 10 mM butyrate for 36 h, and then treated with 75 μg/ml cycloheximide for the indicated times. After fixation, specific binding of ^{125}I-VSV-G-mAb to saponin-permeabilized cells was measured. The data are combined from four experiments and each data point represents the average of triplicate measurements of total binding minus the average of two measurements of non-specific binding.

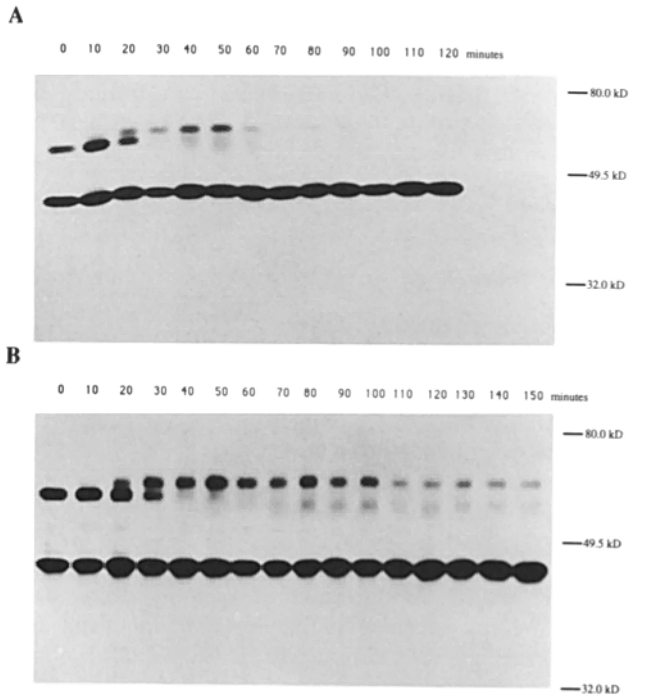

Figure 6. Biosynthesis and degradation of V/L/L in L cells with and without leupeptin treatment. An L cell line expressing V/L/L was grown in 35 mm-diameter tissue culture dishes and induced with 10 mM butyrate for 2 d before a 15 min metabolic labeling. Chase times are indicated. V/L/L was immunoaffinity purified from detergent lysates of cells with VSV-G-mAB immunobeads and subjected to SDS-PAGE and fluorography. (*A*) The material immunoprecipitated from cells without leupeptin treatment. (*B*) 20 μM leupeptin was added to cell cultures 4 h before the metabolic labeling and then maintained throughout metabolic labeling and chase. The high mannose intermediate form of V/L/L appears immediately following metabolic labeling while the complex carbohydrate form appears during the chase. Additionally, a clipped form with an apparent molecular weight of ~60 kD is seen following longer chase periods in leupeptin treated cells. The 44-kD band on both gels is a contaminant mouse protein always seen when VSV-G-mAb immunobeads were used for V/L/L isolation.

the Golgi apparatus by this time. Degradation products, that appear in SDS-PAGE as a smeared band migrating ahead of the mature form of V/L/L, were apparent after 40 to 50 min of chase. In cells treated with leupeptin, ^{35}S-labeled V/L/L remained throughout a 2.5-h chase period (Fig. 6 *B*) and was still recovered after a 10-h chase.

A decrease in apparent molecular weight of ^{35}S-labeled V/L/L occurred during the chase with leupeptin present (Fig. 6 B). This indicates that V/L/L was partially degraded to a "clipped" form (M_r, 60 kD) that retained the epitope recognized by VSV-G-mAb. The clipped protein was ~7 kD smaller than fully glycosylated V/L/L and probably resulted from the removal of the amino terminal region of the protein, since a polyclonal anti-serum raised against a peptide corresponding to the cytoplasmic tail of LEP100 (Nabi et al., 1991) bound to the clipped form of V/L/L in immunoblots. Removal of the two N-linked oligosaccharides would also result in about a 7 kD decrease in molecular weight, a possibility which was not ruled out. Clipped V/L/L first appeared after 60 to 70 min of chase and became the only form of the molecule present by the end of a 10-h chase period. If the clipping occurs in the lysosomes, the delivery of V/L/L to lysosomes must begin less than 75 to 85 min after synthesis.

Taken together, these experiments support the hypothesis that V/L/L molecules are targeted to lysosomes through a biosynthesis/transport process of 60–80-min duration and that, unless leupeptin is present, V/L/L molecules are degraded within minutes of reaching the lysosomes.

Some Newly Synthesized V/L/L Molecules Are Found at the Cell Surface

Since V/L/L molecules appear to be degraded quickly upon arrival in lysosomes, the detection of V/L/L molecules at the cell surface of cells not treated with leupeptin would strongly suggest that newly synthesized V/L/L molecules can pass through the plasma membrane before lysosomal delivery. We determined the relative number of V/L/L molecules in the plasma membrane of cells expressing V/L/L with or without leupeptin treatment by measuring the binding of ^{125}I-VSV-G-mAb to non-permeabilized as well as permeabilized cells (Table II). 0.7% of the total VSV-G-mAb binding sites were found at the cell surface in leupeptin treated cells while 3.3% of the total VSV-G-mAb binding sites were found at the cell surface without leupeptin treatment. As would be expected, the total number of VSV-G-mAb binding sites increased substantially (sixfold in 16 h of leupeptin treatment). The absolute number of V/L/L molecules at the cell surface, however, did not change dramatically (only 1.3-fold). Thus, the steady state level of V/L/L in the plasma membrane is relatively independent of the accumulation of V/L/L molecules in lysosomal membranes.

A Wave of Recently Synthesized V/L/L and LEP100 Molecules Passes through the Cell Surface following a Synchronized Pulse of Expression

The transient expression of recently synthesized V/L/L or LEP100 molecules in the plasma membrane was visualized by immunofluorescence microscopy following synchronized expression with the recombinant vaccinia virus/T7 system. L cells pre-treated for 4 h with leupeptin were infected with recombinant vaccinia virus and 30 min later transfected with V/L/L-DNA or LEP100-DNA cloned just 3′ to the T7 promoter in pBluescript. In these experiments the T7 RNA polymerase–driven synthesis of V/L/L or LEP100 was allowed to proceed for a maximum of 4 h, terminated by addition of CHX. At 4 h the predominant intracellular immunofluorescence was in the ER (identified by the presence of a circum-nuclear ring) and intense perinuclear labeling taken to be Golgi apparatus (Fig. 7 *A*). Lysosomal labeling was not apparent. Labeling of the plasma membrane was strong at this time, however (Fig. 7, *B* and *D*). Cells still expressed V/L/L at the surface 1 h after the addition of CHX (Fig. 7 *E*). After 2 h in CHX almost no surface labeling could be detected (Fig. 7 *F*) and the characteristic lysosomal pattern of immunofluorescence was seen (see Fig. 9 *C*). Comparable results were obtained for LEP100 expression. The expression of V/L/L at the cell surface was independent of the lysosomal accumulation of V/L/L. Without leupeptin treatment V/L/L was readily detected both on the cell surface and in the ER and Golgi regions, but it did not accumulate in lysosomes and it disappeared completely within 2 h of CHX treatment.

Cell Surface V/L/L Rapidly Cycles Between the Plasma Membrane and Endosomes and Is Transported more Slowly to Lysosomes

In chicken fibroblasts a few percent of LEP100 molecules are found in the plasma membrane, and these undergo continuous, rapid exchange with a larger population of molecules in early endosomes (Lippincott-Schwartz and Fambrough, 1986, 1987). Cell-surface V/L/L molecules were also found to exchange rapidly with intracellular molecules (Fig. 8 *A*). When cell-surface V/L/L molecules were saturated with VSV-G-mAb at 4°C and the cells subsequently warmed to 37°C, a new population of binding sites for VSV-G-mAb appeared at the surface with a half-time of two to three minutes. In this experiment a small fraction of the newly appearing molecules should be attributable to newly synthesized molecules arriving from the Golgi apparatus. The kinetics of such a component should be linear (whereas recycling kinetics are exponential) and its magnitude might be ~20% of the molecules detected in the warm-up period, an effect too small to be seen clearly in experiments such as shown in Fig. 8 *A*. The rate of internalization of ^{125}I-VSV-G-mAb bound to V/L/L molecules from the cell surface at 37°C was also very rapid (Fig. 8 *B*).

In chicken embryo fibroblasts, LEP100 is internalized from the plasma membrane and transported to lysosomes. When cells were exposed to LEP100-mAb, the antibody was carried to lysosomes and accumulated there if cells were treated with leupeptin (Lippincott-Schwartz and Fambrough, 1987) (Fig. 9 *C*). The same sort of process occurred in cells expressing the V/L/L chimera: grown in medium containing VSV-G-mAb and leupeptin, the cells accumulated VSV-G-mAb and showed the punctate, lysosomal pattern of fluorescence when subsequently permeabilized and labeled with fluorescent anti-mouse-IgG second antibody (Fig. 9, *A* and *B*).

Cell Surface Appearance of Newly Synthesized V/L/L Is Not an Artifact of Overexpression

In the recombinant vaccinia expression system there is massive synthesis of V/L/L (making it easy to demonstrate the cell surface appearance of newly synthesized V/L/L by im-

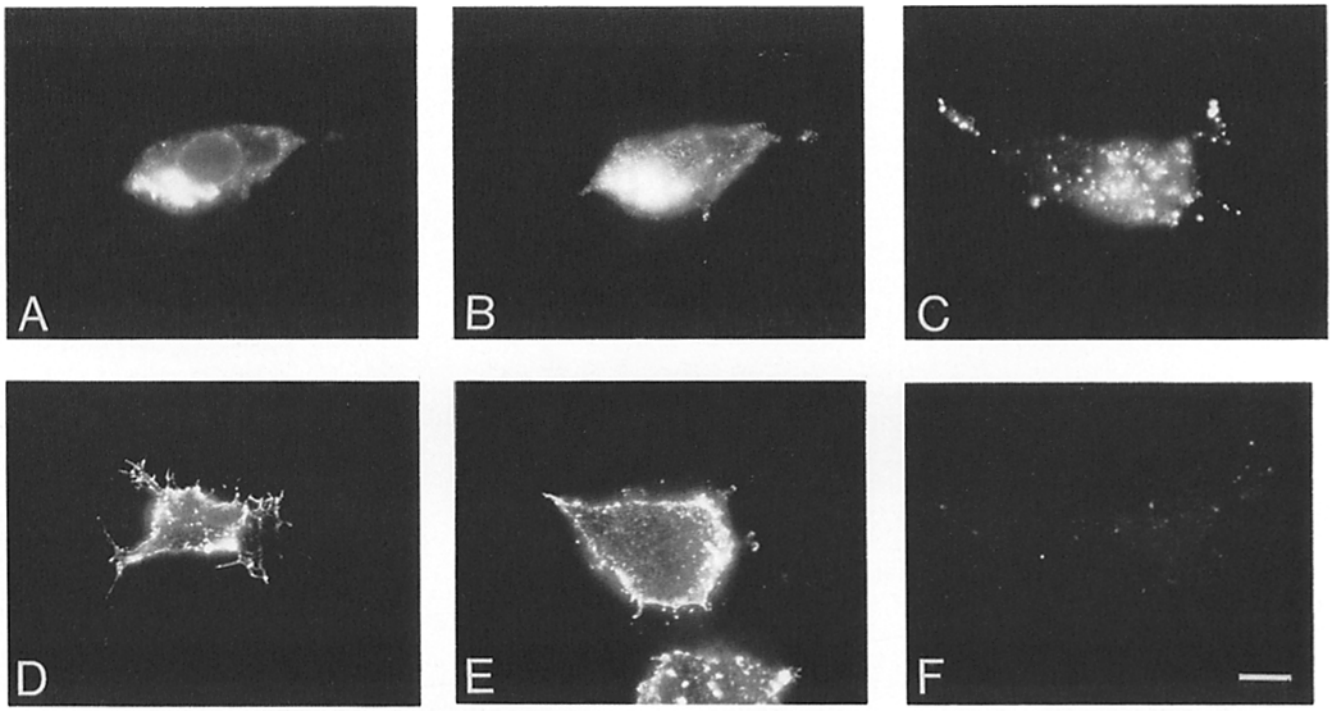

Figure 7. A wave of V/L/L passes through the plasma membrane following synchronized expression using the vaccinia virus/T7 system. 30 min after Ltk⁻ cells were infected with vaccinia virus/T7, DNA encoding V/L/L was introduced as a DNA/lipofectin complex. This complex was prepared as a stock solution and an equal volume added to each coverslip. 4 h later the medium was replaced with medium containing 75 μg/ml cycloheximide. After additional incubation, cells were fixed and labeled with VSV-G-mAb and then FITC-conjugated goat anti-mouse antibody. Immunofluorescence micrographs of representative cells are shown at each time point following cycloheximide addition. For total cell staining, 20 μM leupeptin was added to dishes to be transfected with V/L/L four hours prior to vaccinia virus infection: this concentration of leupeptin was maintained throughout the experiment. (*A*) Total cell labeling with 0 h CHX treatment. (*B*) The same cell as in A with the plane of focus adjusted to show cell surface labeling. (*C*) Total cell labeling following 2 h CHX treatment. (*D*) Cell surface labeling (without saponin) at 0 h CHX treatment. (*E*) Cell surface labeling at 1 h CHX treatment. (*F*) Cell surface labeling at 2 h CHX treatment. Bar, 10 mm.

munofluorescence microscopy) (Fig. 7). It is conceivable that this level of expression might swamp an intracellular sorting mechanism and lead to misdirection of V/L/L molecules to the cell surface. However, with the exception of experiments documented in Fig. 7, the experiments describing the behavior of V/L/L molecules were conducted with stable L cell lines expressing V/L/L in response to induction by butyrate. To assess the effect of expression level on appearance of V/L/L at the cell surface in these cells, we conducted a series of experiments at different levels of expression. In these experiments we measured the average number of V/L/L molecules per cell (Table III). Expression levels appeared to be less than or comparable to the expected endogenous level of expression of LEP100 type molecules (see Discussion).

Then we performed experiments to assess the relative number of V/L/L molecules coming to the plasma membrane en route to lysosomes at lower and higher levels of expression in these same cultures. The population of V/L/L molecules in these cultures consists almost entirely of molecules in the biosynthesis and targeting pathway. If cultures could be exposed to an infinite concentration of ^{125}I-VSV-G-mAb, every molecule of V/L/L coming to the plasma membrane would become labeled. If lysosomal degradation could be inhibited quantitatively and instantly by leupeptin, this antibody would accumulate in lysosomes in amounts identical to the number of V/L/L molecules that had passed through the plasma membrane compartment. If this experiment were done by adding leupeptin, cycloheximide, and the ^{125}I-VSV-G-mAb to the culture medium simultaneously, by 2 h the V/L/L-mediated uptake of antibody would cease. If all the V/L/L passed through the plasma membrane compartment, an amount of antibody equal to the starting amount of V/L/L would have accumulated in the lysosomes. In practice, the inhibition of lysosomal degradation by leupeptin develops slowly (Fig. 4) even at high concentrations of leupeptin, and the binding of antibody to antigen proceeds with a half-time of a few minutes at practical antibody concentrations. Nevertheless, this experimental design can be employed to assess the relative amount of V/L/L passing through the cell surface en route to lysosomes at the various expression levels. The results of such experiments are tabulated in Table IV and the experimental details are described in the legend. The V/L/L-dependent uptake of ^{125}I-VSV-G-mAb did plateau by 100 min, as expected (data not shown), and at 2 h the cells contained an amount of ^{125}I-VSV-G-mAb equal to the amount of V/L/L present in parallel cultures treated for 2 h with leupeptin and cycloheximide. These results were independent of the level of V/L/L expression.

Discussion

The Cytoplasmic Domain of LEP100 Is Necessary and Sufficient for Lysosomal Targeting

This study began as an exploration of the location of

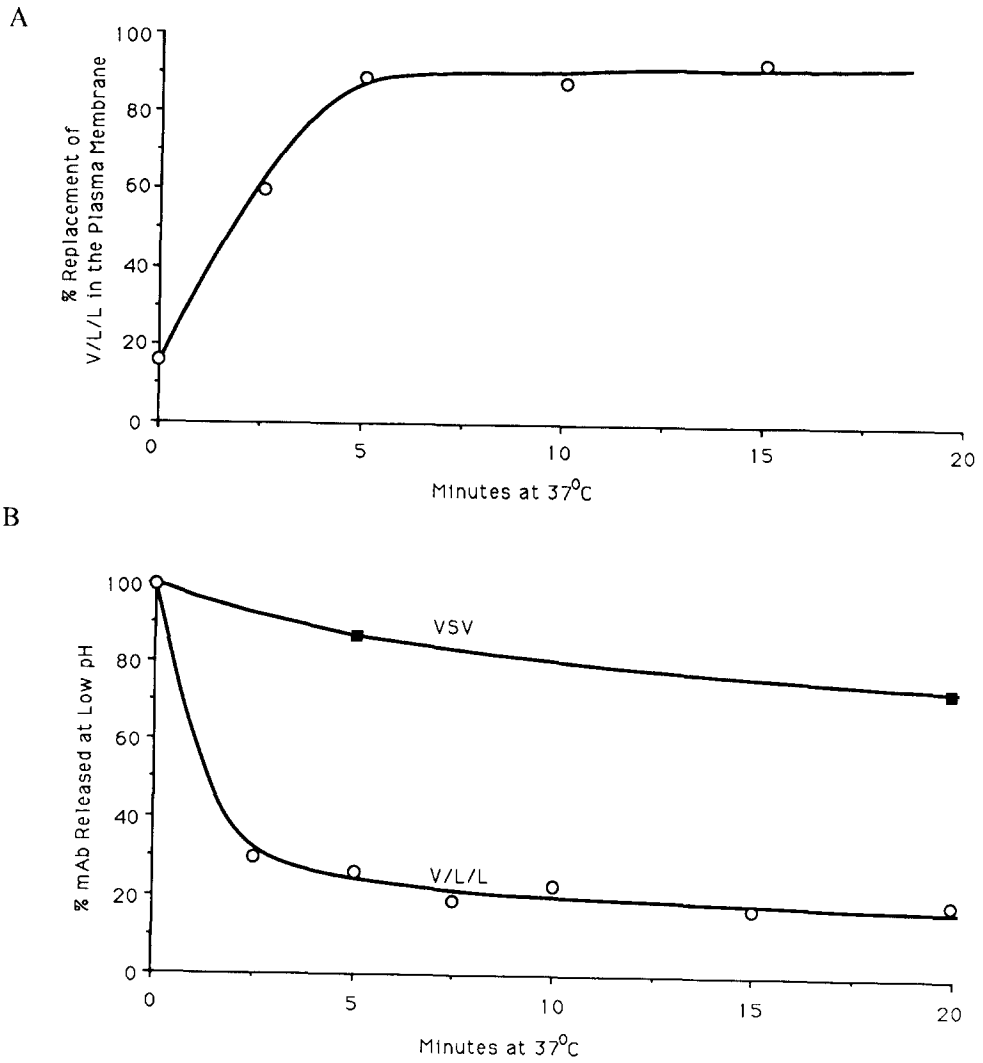

Figure 8. Cycling of V/L/L at the plasma membrane. (*A*) Cell-surface VSV-G-mAb binding sites were blocked by incubating cell lines of V/L/L-expressing cells with excess antibody (50 μg/ml) at 4°C. Following warm-up at 37°C, cells were fixed at the indicated times and ^{125}I-VSV-G-mAb binding sites measured. (*B*) ^{125}I-VSV-G-mAb was bound for 6 h at 4°C to the surface of living L cells expressing V/L/L or VSV-G grown in 35-mm-diam tissue culture dishes. After warming to 37°C for the times specified, surface-associated antibody was eluted by treating for 5 min with ice-cold 0.1 M acetic acid, 1.5 M NaCl. ^{125}I-VSV-G-mAb eluted by this low pH, high-salt treatment is plotted as a percentage of radiation eluted without 37°C warm up. For both experiments, each data point represents the average measurement for a set of five dishes of cells.

lysosomal targeting information in the LEP100 molecule. The parent molecules (LEP100 and VSV-G) and all six possible topologically equivalent chimeras involving their ectodomain, transmembrane domain, and cytoplasmic domain (L/L/V, L/V/V, V/L/V, V/L/L, and L/V/L, and V/V/L) were expressed in mouse L-cells. All chimeras containing the cytoplasmic domain of LEP100 accumulated in lysosomes; those that did not, accumulated in the plasma membrane. These results confirm and extend similar work by Williams and Fukuda (1990) that explored the targeting information in the cytoplasmic domain of hLAMP-1, which has a cytoplasmic domain identical to that of LEP100. In their work, mutations of the single Tyr in this domain to Ala, Ser, or Thr resulted in accumulation of the mutant hLAMP-1 molecules in the plasma membrane. The normal cytoplasmic domain was shown to be sufficient for lysosomal targeting of a chi-

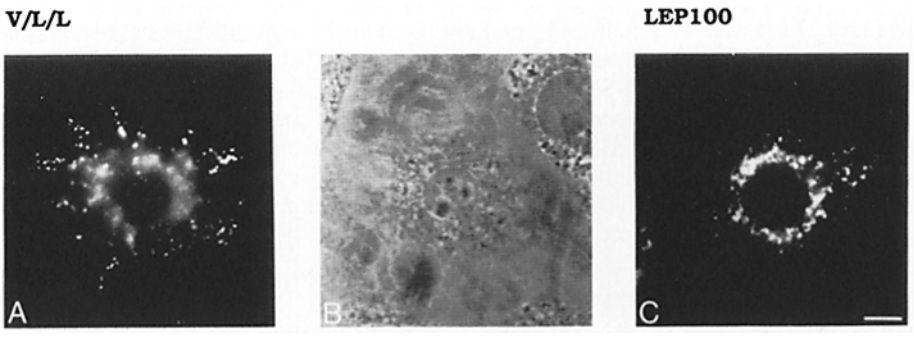

Figure 9. Antibody uptake by cells expressing V/L/L and LEP100. Stably transfected L cell lines were plated onto glass coverslips. After 30 h of butyrate induction, 20 μM leupeptin, and 5 μg/ml VSV-G-mAb (V/L/L expressing cells) or LEP100-mAb (LEP100 expressing cells) were added to the butyrate-containing medium. Incubations were continued for an additional 12 h. Cells were then washed extensively with PBS, fixed, and incubated with FITC-conjugated goat anti-mouse antibody in the presence of saponin. (*A*) Immunofluorescence micrograph of a single cell expressing V/L/L. (*B*) Phase contrast photomicrograph of the same cell, showing surrounding cells not expressing V/L/L. (*C*) Immunofluorescence micrograph of a cell expressing LEP100. Bar, 10 μm.

Table III. Expression Levels of V/L/L in Butyrate-induced Mouse Cell Lines

Butyrate treatment	Cells ($\times 10^6$) per dish	V/L/L molecules per culture	V/L/L molecules per cell
(*h*)			
0	3.8	1.1×10^8	29
22	3.7	7.6×10^8	210
24	3.8	1.9×10^9	520
25	3.2	1.8×10^9	550
48	3.2	7.1×10^9	2,210
50	3.5*	1.6×10^{10}	4,570

Confluent cultures of mouse cells stably transfected with DNA encoding V/L/L were induced in 10 mM butyrate containing medium for the specified times. Cell numbers were calculated from direct counts on phase micrographs of representative areas of the cultures at the time of analysis and varied between 3.2 and 3.8 $\times 10^6$ per dish.
Numbers of V/L/L molecules were determined as the numbers of specific ^{125}I-VSV-G-mAb binding sites in saponin permeabilized cells, using ^{125}I-VSV-G-mAb of known specific activity (see Materials and Methods).
* Indicates experiment in which cell numbers in the confluent cultures were not counted but only estimated as the average of the values found in the other experiments.

meric protein that consisted of a secretory protein, human gonadotropin α-chain, linked to the transmembrane domain of VSV-G, and the cytoplasmic domain of hLAMP-1. Whether the lumenal or transmembrane domains of hLAMP-1 might also contain lysosomal targeting information was not explored.

The Lumenal Domain and Transmembrane Domain of LEP100 Are Neither Necessary Nor Sufficient for Lysosomal Targeting of Chimeras, and the Lumenal Domain of LEP100 Expressed Alone Is a Secretory Protein

Chimeras between LEP100 and VSV-G that lacked the cytoplasmic domain of LEP100 were not targeted to lysosomes even if they contained the lumenal domain or the transmembrane domain of LEP100 or both. Since it was conceivable that a strong plasma membrane targeting signal in the cytoplasmic domains of chimeras and mutants might override a lysosomal targeting signal in the lumenal domain of LEP100, a truncated form lacking the transmembrane and cytoplasmic domains of LEP100 was expressed. It was secreted efficiently by the cells and only unprocessed precursor could be detected in cell lysates. The prevailing idea has been that lysosomal membrane proteins are sorted for targeting to the lysosomes at the *trans*-Golgi network (TGN) (see Green et al., 1987; Kornfeld and Mellman, 1989; Williams and Fukuda, 1990; Hunziker et al., 1991). Soluble lysosomal proteins are sorted at this site and sent to lysosomes by a process mediated in part by M6P receptors (reviewed in Kornfeld and Mellman, 1989). If this were the case for the lysosomal membrane proteins, any bias towards lysosomal sorting should have sent some of the truncated LEP100 to lysosomes.

Table IV. Effect of Expression Level on V/L/L-mediated ^{125}I-VSV-G-mAb Uptake

Butyrate treatment	V/L/L molecules expressed per cell	V/L/L per cell after 2 h in CHX+ leupeptin	VSV-G-mAb uptake per cell after 2 h in CHX+ leupeptin
(*h*)			
22	210	ND	60
24	520	110	300
25	550	180	180
48	2,210	510	420
50	4,570	1,920	1,960

Confluent cultures of mouse cells stably transfected with DNA encoding V/L/L were induced in 10 mM butyrate containing medium for the specified times. Numbers of V/L/L molecules per cell (column 2) are taken from Table III. Sets of cultures from the same batches were exposed to leupeptin (80 or 100 μg/ml) and CHX for 2 h, fix, permeabilized, and specific ^{125}I-VSV-G-mAb binding sites determined. In parallel, other cultures were maintained in medium with leupeptin and CHX for 2 h at 37°C in the presence of 1 μg/ml ^{125}I-VSV-G-mAb with (three or five dishes) or without (two or three dishes) a 50-fold excess unlabeled antibody. After the 2-h incubations, cultures wre washed at 4°C and radioactivity measured in a gamma counter. Number in the third column were calculated by multiplying the number of V/L/L molecules per cell (second column) by the ratio of specific ^{125}I-VSV-G-mAb binding to CHX + leupeptin treated cells vs. binding to untreated cells. To obtain the numbers in the fourth column, specific uptake was calculated by subtracting uptake in the presence of 50-fold unlabeled antibody from total ^{125}I-mAb uptake. Specific uptake was converted to molecules of antibody taken up per cell by using the known specific activity of the antibody (Materials and Methods) and the number of cells per culture dish (Table III).

The Lumenal Domain of LEP100 Stabilizes LEP100 in the Lysosome but Fails to Protect the Lumenal Domain of Nearby V/L/L Molecules

To observe the lysosomal delivery of chimeric proteins lacking the lumenal domain of LEP100 it was necessary to block lysosomal proteases with leupeptin. Even with leupeptin treatment, the VSV-G domain was clipped rapidly upon delivery of the chimeras to lysosomes. Immunofluorescence microscopy showed clearly that newly synthesized molecules are delivered to most or all of the lysosomes in the cell and therefore newly arriving molecules must exist in a surround of endogenous lysosomal membrane glycoprotein molecules. The half-life in lysosomes of chimeras containing the VSV-G domain is no more than a few minutes, whereas that of LEP100 is about two days (Lippincott-Schwartz and Fambrough, 1986, 1987).

Most or All Chimeric Molecules Directed to the Lysosome by the LEP100 Cytoplasmic Domain Are First Incorporated into the Plasma Membrane

The V/L/L chimera was most useful in determining the pathway taken by newly synthesized molecules to the lysosome because the VSV-G ectodomain was rapidly degraded upon delivery to lysosomes. Pulse/chase experiments (Fig. 6) showed that many of the V/L/L molecules in expressing cells had not been processed by Golgi enzymes and that Golgi-processed molecules were degraded within minutes. Rapid loss of the population of V/L/L molecules when protein synthesis was inhibited is consistent with this interpretation of the pulse/chase experiments. Leupeptin treatment of the cells stabilized the V/L/L molecules, allowing them to accumulate in lysosomes (Figs. 2 and 4). The movement of newly synthesized V/V/L and V/L/L through the plasma membrane was shown most graphically in the pulse-expression experiments (Fig. 7). Although the time resolution was limited in those experiments, the detection of V/L/L molecules in the plasma membrane for up to 1 h after CHX treatment, but not after 2 h, is consistent with the kinetics of V/L/L transport through the biosynthetic pathway (includ-

ing the plasma membrane) found in the pulse/chase labeling experiments.

The rapid degradation of the VSV-G ectodomain in lysosomes predicts that compartments upstream from the lysosome should contain newly synthesized V/L/L molecules regardless of leupeptin treatment whereas the lysosome and compartments downstream from the lysosome should contain V/L/L molecules only following leupeptin treatment. Therefore, the relative insensitivity of the number of cell surface V/L/L molecules to leupeptin treatment is strong evidence that the plasma membrane is upstream from the lysosome (Table II). The slightly greater number of V/L/L molecules found in the plasma membrane following 16 h of leupeptin treatment may reflect the presence of older molecules at the cell surface which, due to leupeptin treatment, have accumulated in lysosomes and have cycled out of lysosomes back to the plasma membrane, as described for LEP100 molecules in chick fibroblasts (Lippincott-Schwartz and Fambrough, 1986, 1987).

In cells expressing V/L/L, the steady-state expression of V/L/L in the plasma membrane was ~4% of the V/L/L molecules. Since about half of the V/L/L molecules were in the high-mannose state characteristic of location in the ER (Fig. 6 *A*) and another fraction of V/L/L molecules must be in the Golgi apparatus, the post-Golgi compartments are expected to contain roughly 25% of the V/L/L molecules. The 4% of molecules in the plasma membrane are in rapid exchange with an endosomal pool of V/L/L molecules, and the size of this pool can be estimated from the curves in Fig. 8 (for discussion see Lippincott-Schwartz and Fambrough, 1987) to be about four to five times the size of the plasma membrane population, or around 20% of total V/L/L molecules. Thus the size of the combined populations of V/L/L molecules rapidly exchanging between the plasma membrane and endosomes is about the size of the entire post-Golgi population. While this study does not completely rule out the possibility that some molecules traveled more directly from the TGN to lysosomes, direct transport of a significant fraction of molecules is unlikely.

In our experiments the levels of V/L/L in the biosynthesis/targeting pathway ranged up to ~4,500 molecules per cell (Table III). While we do not have exact data for the quantity of the endogenous homolog of LEP100 (m-lamp-1) in these cells, this value must be similar to that reported for chick fibroblasts (Lippincott-Schwartz and Fambrough, 1986): nearly 400,000 molecules per cell. These endogenous molecules have a metabolic half-life of about 40 h, and therefore to sustain an endogenous level of even 200,000 molecules per cell, a biosynthetic rate of about 5,000 molecules per hour would be required. Moreover, there are probably several different lysosomal membrane proteins that employ the same sorting machinery, and therefore the sorting machinery normally processes roughly 10,000 molecules/h. Thus, it seems unlikely that the exogenously expressed V/L/L disturbs the sorting/targeting behavior of the cells, even if expression levels varied considerably from cell to cell in the population. This is supported by the data in Table IV that show no effect of expression levels on V/L/L-mediated antibody uptake. The antibody uptake data do not in themselves distinguish between appearance of antigen at the plasma membrane or the flow of medium through the endocytic pathway by fluid phase uptake and then exocytosis (Besterman et al., 1981; Adams et al., 1982; Swanson et al., 1985; Swanson, 1989; Bomsel et al., 1989) with retention in the endosomes being antigen dependent. However, the experiments that directly demonstrate cycling (Fig. 8) suggest that large volume fluid flow through the endocytic pathway need not be invoked to account for antibody uptake. In either mechanism newly synthesized LEP100-type molecules must be in the plasma membrane/early endosome compartment to mediate the saturable uptake of antibody.

Recently Harter and Mellman (1992) demonstrated that high levels of over-expression of lgp120 (a homolog of LEP100) in CHO cells resulted in higher steady state levels on the plasma membrane. Harter and Mellman (1992) also reported immunofluorescence evidence for enhanced surface expression of another endogenous lysosomal membrane protein in the over-expressing cells and interpreted this as missorting resulting from competition with exogenously expressed lgp120 for sorting receptors in the TGN. In these experiments the expression levels were up to 15 times the endogenous; at these levels sorting might be significantly perturbed. Harter and Mellman (1992) also reported that in nontransfected pulse-labeled NRK cells ^{35}S-labeled lgp120 was detected at the plasma membrane (1.8% of total) by biotinylation of cells at 0°C, a level not greatly different from the 4% V/L/L found at the surface of fixed cells in our experiments. The small size of this fraction is not directly an indicator of the significance of the cell surface in the transport pathway to lysosomes. This size depends upon the balance between distribution in surface and early endosome compartments as well as the rate of exit from early endosomes in the pathway to lysosomes.

Most of the results reported here involve the behavior of V/L/L molecules. We have made the assumption that the ectodomain of VSV-G protein is behaving passively in the sorting/targeting behavior of the chimera. We cannot rule out the possibility that this domain somehow masks the targeting signal for direct delivery of LEP100 from the TGN to lysosomes. Clearly it does not prevent efficient delivery to lysosomes via the cell surface.

The Major Pathway of Targeting LEP100 to the Lysosomes Includes the Plasma Membrane

Our data fit a model in which newly synthesized LEP100 is transported to the cell surface early endosome compartment, is shuttled rapidly between the plasma membrane and endosomes by endocytosis and exocytosis, and is then delivered to lysosomes. Because of this rapid shuttling, it would be very difficult to distinguish direct transport of newly synthesized LEP100 molecules from the Golgi apparatus to the plasma membrane from indirect transport via early endosomes. Our data are not consistent with the TGN being the site of sorting of LEP100-type lysosomal membrane glycoproteins for delivery to late endosomes or lysosomes.

If the plasma membrane is in the pathway for lysosomal targeting of a lysosomal membrane protein, then that protein must have a structure suitable for efficient endocytosis. A number of integral membrane proteins undergo endocytosis as a normal part of their function (Ktistakis et al., 1990). This process generally depends upon the structure of their cytoplasmic domain, e.g., the LDL receptor (Lerman et al., 1985; Davis et al., 1987), the EGF receptor (Prywes

et al., 1986), the polymeric IgA/IgM receptor (Mostov et al., 1986), the transferrin receptor (Rothenberg et al., 1987), and the Fc receptor (Miettinen et al., 1989). At least one tyrosine occurs in the cytoplasmic domain of each of these receptors, and, where it has been examined, changing a critical tyrosine either eliminates or greatly reduces endocytosis (e.g., Davis et al., 1987; Lobel et al., 1989; Jing et al., 1990). This is also true for the transmembrane form of LAP (Peters et al., 1990). Mutations of the tyrosine in the cytoplasmic domain of hLAMP-1 and lgp120 result in proteins that accumulate in the plasma membrane (Williams and Fukuda, 1990; Hunziker et al., 1991). In light of our data showing the passage of newly synthesized LEP100, V/V/L, and V/L/L through the plasma membrane and the secretion of the truncated LEP100, it appears that accumulation of these mutants in the plasma membrane results from a defect in endocytosis and not from missorting in the TGN.

Although the cytoplasmic domains of the lysosomal membrane proteins share features of a tyrosine-containing endocytosis motif with the plasma membrane receptors discussed above (Ktistakis et al., 1990), plasma membrane receptors generally avoid delivery to the lysosome. It is an interesting possibility that there may be a graded set of internalization signals: from those involved mainly in endocytosis to those involved in endocytosis and efficient lysosomal delivery. In fact, the properties of these signals may regulate not only subcellular distribution of membrane proteins but also their lifetimes, by determining the probability that they will be transported from endosome to lysosome.

Recent experiments in MDCK cells indicate that most molecules of LAMP-2 (at least 70%) are targeting basolaterally before lysosomal delivery (Nabi et al., 1991). LAMP-2 (Fukuda et al., 1988; Cha et al., 1990; Granger et al., 1990) is a type of lysosomal membrane glycoprotein closely related to the LEP100/LAMP-1 type. Human LAP, a lysosomal enzyme synthesized as a transmembrane protein, has also been shown to travel to the plasma membrane before endocytosis, delivery to lysosomes, and cleavage to the soluble form (Waheed et al., 1988; Braun et al., 1989). Our studies of LEP100 together with the data on the other lysosomal glycoproteins, LAMP-2 and LAP, suggest that the delivery of transmembrane proteins to the lysosome via the cell surface is a major pathway in animal cells.

The question of a branched pathway (as proposed by Harter and Mellman, 1992) or quantitative delivery of newly synthesized lamp-type molecules to the cell surface/early endosome cycle, as argued here, cannot easily be resolved with present technologies. Since it is virtually impossible to prove a negative, the existence of a minor direct pathway from TGN to lysosomes cannot be disproved. The apparent quantitative differences between cell types might reflect different balances of direct and indirect pathways or simply different kinetics of individual transport steps in the cell surface route to lysosomes. The biosynthetic rate of the LEP100-type lysosomal membrane proteins is very modest, for they have long lifetimes in the cells. It would not require very robust expression of sorting machinery to deliver newly synthesized molecules efficiently from the TGN to lysosomes if that were the intended biological route. There must be an important biological reason for sending LEP100 to the surface before retrieval and targeting to lysosomes. One exciting possibility is that this targeting route plays a normal role in cell–cell or cell–matrix interactions.

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