J. Biochem. 120, 1088-1094 (1996)

Biosynthetic Transport of a Major Lysosome-Associated Membrane Glycoprotein 2, Lamp-2: A Significant Fraction of Newly Synthesized Lamp-2 Is Delivered to Lysosomes by Way of Early Endosomes

Kenji Akasaki,[1] Akihiro Michihara, Yoshiteru Fujiwara, Kouichi Mibuka, and Hiroshi Tsuji

Faculty of Pharmacy and Pharmaceutical Sciences, Gakuencho, Fukuyama University, Fukuyama, Hiroshima 729-02

Received for publication, May 29, 1996

Lysosomal membranes contain two highly glycosylated proteins, designated as lamp-1 and lamp-2, as major components. Lamp-1 and lamp-2 are similar to each other in the protein structure. Here, we investigated the biosynthetic transport of lamp-2 through the endocytic vacuoles in cultured rat hepatocytes in comparison with that of lamp-1, which has previously been studied [Akasaki *et al.* (1995) *Exp. Cell Res.* 220, 464-473]. Newly synthesized lamp-2 (NS-lamp-2) was transported to the *trans*-Golgi from rough endoplasmic reticulum with a half time ($t_{1/2}$) of 32 min, more slowly than NS-lamp-1 ($t_{1/2}$=13 min). After leaving the *trans*-Golgi, NS-lamp-2 is transferred to at least three compartments; the cell surface ($t_{1/2}$=47 min), cell peripheral early endosomes ($t_{1/2}$=38 min) and perinuclear late endosomes ($t_{1/2}$=48 min). NS-lamp-2 transported to any compartment is delivered finally to lysosomes ($t_{1/2}$=90 min). A significant fraction of NS-lamp-2 (45% of the total) was transported from the *trans*-Golgi to early endosomes, and then delivered to dense lysosomes *via* perinuclear late endosomes, whereas a major portion of NS-lamp-1 follows an intracellular route to late endosomes without passing through the cell periphery. NS-lamp-2 leaves the cell peripheral region more rapidly than NS-lamp-1. The kinetic and quantitative data for biosynthetic transport of NS-lamp-2 to early endosomes and the cell surface indicate that NS-lamp-2 may be transported first to early endosomes, from which a small portion of it (~3.5% of the total) moves to the plasma membrane *via* a recycling system. In contrast, a small fraction of NS-lamp-1 is transported to the plasma membrane directly from the *trans*-Golgi, since NS-lamp-1 is delivered to the plasma membrane and early endosomes with almost the same half times.

Key words: biosynthesis, endocytosis, endosome, lysosome, membrane glycoprotein.

Cells actively sequester macromolecules from the extracellular space by endocytosis. Receptor-mediated endocytosis is the best-documented process by which extracellular proteins such as low density lipoproteins and asialoglycoproteins are efficiently internalized from the cell surface, and delivered to lysosomes through two compartments, designated as early endosomes and late endosomes. The internalized proteins appear initially in cell peripheral early endosomes that exhibit tubulovesicular structures. Incubation at low temperature (16-20°C) causes accumulation of the internalized ligands in early endosomes. The ligands are subsequently delivered to late endosomes, which are primarily localized in the perinuclear region. Late endosomes are often larger and exhibit a more complex organization of internal membranes than early endosomes. It has been shown that cation-independent mannose 6-phosphate receptors that serve for targeting of lysosomal enzymes are concentrated in late endosomes. Lysosomes are single membrane-bounded organelles operating in the final stage of the endocytic process. They contain a vast number of soluble acid hydrolases which contribute to degradation of the endocytosed macromolecules [reviewed by Holtzman (*1*), Hubbard (*2*), Kornfeld and Mellman (*3*), and Gruenberg and Howell (*4*)].

The lysosomal membranes contain several highly *N*-glycosylated proteins among which the best described are lamp-1 (lgp A) and lamp-2 (lgp B). These two glycoproteins are structurally similar and evolutionarily related. They contain a large luminal domain which is heavily *N*-glycosylated, a single membrane-spanning domain, and a short cytoplasmic domain. A tyrosine-based motif present in the cytoplasmic domains of lamp-1 and lamp-2 is involved in targeting these proteins to lysosomes [reviewed by Fukuda (*5*), Peters and von Figura (*6*), and Hunziker and Geuze (*7*)]. Additionally, lamp-1 and lamp-2 cycle between lysosomes and the cell surface along the endocytic pathway in certain cell types (*8-14*).

There has been an increasing number of studies concerning biosynthetic routes of lysosomal membrane proteins, including lamp-1 and lamp-2 (*15-22*), although it is still debated whether newly synthesized lysosomal membrane proteins are transported from the *trans*-Golgi first to the

[1] To whom correspondence should be addressed.

Abbreviations: lamp-1 and 2, lysosome-associated membrane glycoproteins 1 and 2, respectively; NS-lamp-1 and 2, newly synthesized lamp-1 and 2, respectively; PNS, post-nuclear supernatant.

cell periphery and subsequently delivered to lysosomes along the endocytic pathway. We have recently reported that newly synthesized lamp-1 (NS-lamp-1) leaving the *trans*-Golgi enters the endocytic pathway *via* three distinct points; the cell surface, early endosomes and late endosomes (*22*). Green *et al.* (*15*) reported that NS-lamp-1 and NS-lamp-2 are transported to lysosomes at a similar rate without passing through the cell surface in mouse macrophages and normal rat kidney cells. On the other hand, Nabi *et al.* (*18*) have later shown that NS-lamp-1 and NS-lamp-2 appear in the cell peripheral region and then are transported to lysosomes with similar kinetics. More information on the comparative biosynthetic transport of lamp-1 and lamp-2 through the endocytic compartments is required to understand better the biogenesis of lysosomal membranes. Here, we studied the biosynthetic transport of lamp-2 through the endocytic organelles using a combination of pulse-chase labeling, cell surface immunoprecipitation, and subcellular fractionation of monolayer cultures of rat hepatocytes under the same conditions as in the previous study on lamp-1. The data obtained here were compared with those for lamp-1.

MATERIALS AND METHODS

Materials—Male Wistar rats weighing 200 g were obtained from Shimizu Experimental Animal (Kyoto). [^{35}S]-L-Methionine was purchased from HAS (Hungary). Percoll, Protein A-Sepharose CL-4B and Sepharose 4B were obtained from Pharmacia LKB (Uppsala, Sweden). All other chemicals were of reagent grade, and purchased from various commercial sources.

Preparation of Anti-Lamp-2 Antibody—Lamp-2 was purified from lysosomes of rat liver as described (*23*). Polyclonal antisera against lamp-2 were produced in white rabbits by giving them multiple muscular and subcutaneous injections of an emulsified mixture of purified lamp-2 and Freund's complete adjuvant. Three injections were given, at the beginning, middle, and end of a 2-week period. Specific polyclonal anti-lamp-2 IgG was purified from the antiserum preparation by immunoaffinity chromatography on immobilized lamp-2 resin.

Cultured Hepatocytes—Hepatocytes were obtained from rats by collagenase perfusion as described by Seglen (*24*). Hepatocytes (3×10^6) were diluted with Eagle's essential medium containing 10% fetal calf serum, washed with Hanks' solution, then incubated in humidified air containing 5% CO_2 at 37°C for 24 h.

Cell Fractionation—Cells incubated on 60 mm tissue culture dishes were washed several times in cold Hanks' buffer, then in a cold isotonic sucrose solution (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl buffer, pH 7.3), and removed from the dish using a rubber policeman. About 2×10^6 cells in 2 ml of the sucrose solution were homogenized with 5 strokes in a Teflon homogenizer, then centrifuged at $650\times g$ for 2 min. The post-nuclear supernatant (PNS) was diluted with Percoll to a final concentration of 30% and centrifuged at 25,000 rpm for 40 min in a Beckman 70.1 Ti rotor. Following centrifugation, the gradients were divided into 18×0.5 ml fractions by downward displacement. The densities of the gradient fractions were obtained from the refractive indices.

Assay of Enzymes—β-Glucuronidase were assayed as described by Robins *et al.* (*25*).

Protein Determination—Proteins were determined by the method of Lowry *et al.* (*26*) using bovine serum albumin as the standard.

Gel Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% slab gels according to Laemmli (*27*).

Immunoblot Procedures—Proteins in SDS-slab gel were transferred to a nylon membrane by electrophoresis, using a modified version of the procedure of Towbin *et al.* (*28*). The positive bands were visualized by means of ECL Western blotting detection kits (Amersham, Bucks, UK), that contain a sensitive chemiluminescent substrate for horseradish peroxidase.

Pulse-Labeling of Hepatocytes—The cell monolayers were washed twice in Hanks' solution supplemented with vitamins, 5% fetal calf serum and amino acids except for methionine, then incubated for 20 min in this medium containing 100 μCi of [^{35}S]-L-methionine. The cell monolayers were washed once in Eagle's minimal essential medium containing 5% fetal calf serum and 2 mM methionine, then incubated in the same medium until solubilization, unless otherwise stated.

Immunoprecipitation of Labeled Lamp-2—Labeled cells were washed with ice-cold Hanks' buffer and lysed in 0.2 ml of 1% SDS/0.5% Triton X-100/0.15 M NaCl/2 mM EDTA/10 mM Tris-HCl (pH 7.0). After centrifugation at $10,000\times g$ for 60 min, the supernatant was diluted 10 times with 0.5% Triton X-100/0.15 M NaCl/2 mM EDTA/10 mM Tris-HCl (pH 7.0)/protease inhibitors (leupeptin, pepstatin A, chymostatin, and antipain, 10 μg/ml each). The lysates were incubated with nonimmune IgG-Sepharose 4B for 8 h at 4°C followed by centrifugation. The supernatants were incubated with anti-lamp-2 IgG for 3 h at 4°C followed by incubation with protein A-Sepharose CL-4B beads for 12 h at 4°C. The sedimented beads were washed five times with 1% Triton X-100/0.5% deoxycholate/0.15 M NaCl/2 mM EDTA/0.1% BSA/10 mM Tris-HCl (pH 7.0) (buffer A), five times with buffer A containing 2 M KCl and then twice with 0.1% SDS/0.5% Triton X-100/0.15 M NaCl/10 mM Tris-HCl (pH 8.6). The immunocomplex beads were suspended in 100 μl of sample buffer for electrophoresis containing 5% β-mercaptoethanol. The mixtures were boiled in a hot bath for 3 min. The supernatants collected by centrifugation were resolved by SDS-PAGE. Radioactive bands were detected by fluorography using ENHANCE (NEN, Boston, USA) on Kodak XAR-5 film. Positive protein bands were quantified using a Shimadzu chromatoscanner (model CS-910) in the transmission mode.

Cell Surface Immunoprecipitation—The hepatocytes monolayers were pulse-labeled with 100 μCi of [^{35}S]-L-methionine for 20 min and chased for various periods as described above. The cells were incubated with 50 μg of anti-lamp-2 IgG in Hanks' solution containing 5% fetal calf serum on ice for 30 min, then washed seven times with PBS containing 1% BSA followed by detergent lysis. After clearing the lysate with nonimmune IgG-Sepharose 4B, lamp-2 that appeared on the cell surface and bound to antibody was precipitated from the lysate using protein A-Sepharose CL-4B. The immunocomplex-bound protein A-Sepharose CL-4B beads were extensively washed and resolved by SDS-PAGE as described above.

RESULTS

Occurrence of Lamp-2 in Early Endosomes, Late Endosomes, and Lysosomes in a Steady State—We have established a procedure to separate the endocytic organelles by using Percoll density centrifugation of rat hepatocyte PNS (Fig. 1) (*22*). Early endosomes are located on the lightest buoyant fraction (fraction 1) since the endocytic tracers are accumulated in this fraction after incubation with the tracers at 20°C. When the cells are incubated at 37°C, the tracers are transferred to the dense lysosomal fraction (fractions 17 and 18) *via* the intermediate fraction (fractions 13 and 14), indicating that late endosomes are recovered around fraction 13. We examined the distribution of lamp-2 on the Percoll gradient in order to determine whether lamp-2 occurs in early and late endosomes of rat hepatocyte in the steady state. As shown in Fig. 1B, lamp-2 was detected to a significant degree in the early endosomal fraction (fraction 1), late endosomal fraction (fractions 13 and 14), and lysosomal fraction (fractions 17 and 18), as observed for lamp-1. Therefore, we isolated early endosomal, late endosomal, and lysosomal lamp-2 from fraction 1, fractions 13 and 14, and fractions 17 and 18, respectively, in the following experiments. Lamp-2 on the plasma membrane was isolated by cell surface immunoprecipitation as described under "MATERIALS AND METHODS," since the plasma membrane fractionated by Percoll density centrifugation was contaminated with microsomal and Golgi elements under these conditions.

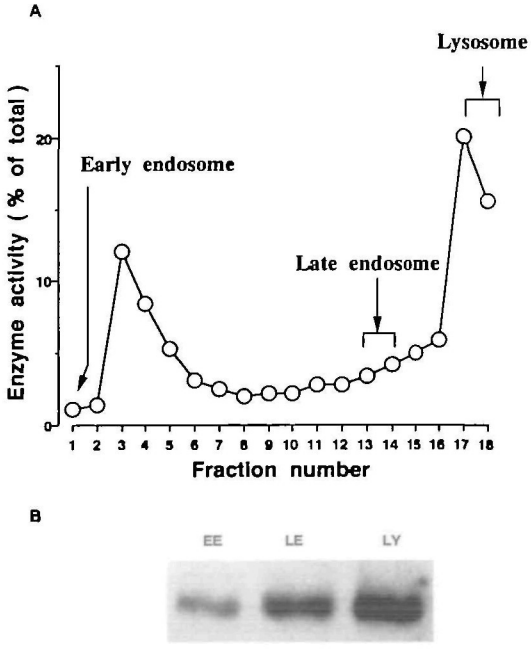

Fig. 1. **Immunoblot detection of lamp-2 in early endosomal, late endosomal, and lysosomal fractions.** Primary monolayer cultures of hepatocytes were homogenized and centrifuged at 650×*g* for 2 min. The resultant supernatant (PNS) were centrifuged on a Percoll gradient as described under "MATERIALS AND METHODS." The gradient was divided into 18 fractions in a downward direction. (A) The distribution of β-glucuronidase (○) as a marker of lysosomes and endoplasmic reticulum is shown. Early endosomes, late endosomes, and lysosomes are recovered mainly in fraction 1, fractions 13 and 14, and fractions 17 and 18, respectively, as indicated by the previous tracer experiments (see Fig. 1 in Ref. *22*). (B) After centrifugation at 150,000×*g* for 30 min to remove Percoll particles, an equal volume (50 μl) of each fraction was subjected to SDS-PAGE followed by immunoblotting with anti-lamp-2 antibody. EE, early endosomes; LE, late endosomes; LY, lysosomes.

Carbohydrate Processing of NS-Lamp-2—Lamp-2 purified from lysosomes of rat livers contains many sialyl *N*-linked oligosaccharide chains. To measure the rate of transport of NS-lamp-2 to the *trans*-Golgi, its carbohydrate processing was examined, since galactosyltransferase and sialyltransferase, which catalyze the terminal glycosylation of complex-type *N*-glycans reside mainly in the *trans*-Golgi area (*29*). The cells were pulse-labeled for 20 min at 37°C with [^{35}S]methionine, then incubated for 0-90 min in the presence of unlabeled methionine. Lamp-2 was isolated from the cell lysates by immunoprecipitation and analyzed by SDS-PAGE and fluorography.

As shown in Fig. 2, NS-lamp-2 migrated as an 87-kDa precursor form immediately after the pulse-labeling. A 96-kDa mature form of NS-lamp-2 was detected at 40 min, after which conversion from the precursor to the mature form continued, and was completed within 120 min. The half time ($t_{1/2}$) of the carbohydrate processing of NS-lamp-2 was calculated to be 32 min, which is twice that of NS-lamp-1 ($t_{1/2}$=13 min) determined previously in cultured rat hepatocytes.

Transport of NS-Lamp-2 to the Cell Surface—A small portion of NS-lamp-1 is transported to the cell surface before its delivery to lysosomes (Table I). We determined whether or not NS-lamp-2 appears on the plasma membrane, as is the case with NS-lamp-1. Hepatocytes were

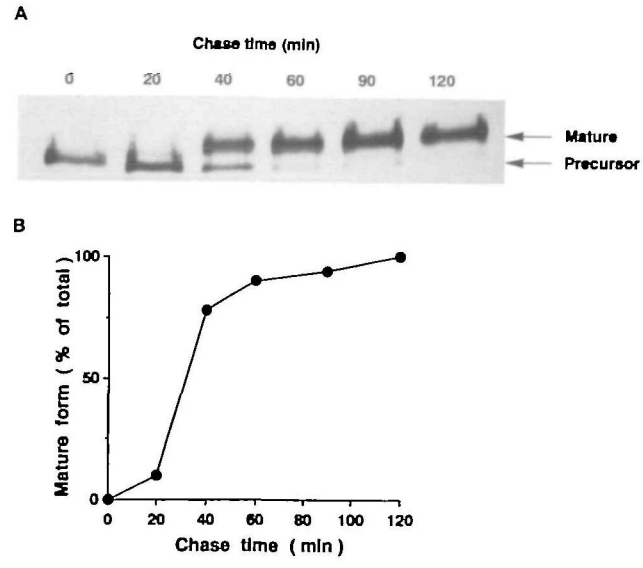

Fig. 2. **Carbohydrate processing of lamp-2 in cultured rat hepatocytes.** Cultured rat hepatocytes were pulsed with [^{35}S]methionine for 20 min and chased for the indicated times before solubilization with detergents and immunoprecipitation with anti-lamp-2 antibody. (A) The precipitated lamp-2 was resolved by SDS-PAGE and visualized by fluorography. Arrows show the migration positions of 87-kDa precursor and 96-kDa mature forms. (B) The fluorogram was quantified by densitometric tracing. The proportion of the mature form was calculated relative to the sum of both forms at each time point. The kinetic data were reproducible in three separate experiments. Values are the means of three determinations.

pulsed with [^{35}S]methionine and chased for various periods. "Cell surface lamp-2" was allowed to bind to anti-lamp-2 antibody added to the culture medium at 4°C before extensive washing of the cells. After solubilizing the cells with detergents, lamp-2 and antibody complexes were precipitated with protein A-Sepharose beads. Immunocomplexes were analyzed by SDS-PAGE and fluorography (Fig. 3A). NS-lamp-2 was quantified by scanning densitometry of the fluorograms (Fig. 3B). A trace of NS-lamp-2 was detectable at the cell surface after chase for 20 min. Thereafter, the level of cell surface NS-lamp-2 increased and reached the maximum after 60 min of chase ($t_{1/2}$ = 47 min). At this point, about 3.5% of the total NS-lamp-2 was detected at the cell surface. NS-lamp-2 at the cell surface decreased from 60 min and was not significantly detected after 180 min of chase. When compared with the kinetics of cell surface transport of NS-lamp-1, NS-lamp-1 is transported to the cell surface faster than NS-lamp-2; $t_{1/2}$ for NS-lamp-1 is 32 min (Table I). However, the time required for the delivery of NS-lamp-2 from the *trans*-Golgi to the cell surface (= 15 min) is not different from that of NS-lamp-1 (19 min). NS-lamp-2 reaches the cell surface more slowly than NS-lamp-1, probably because it leaves the *trans*-Golgi later than NS-lamp-1.

Transport of NS-Lamp-2 to Early Endosomes—NS-lamp-2 was immunoprecipitated from the early endosomal fraction after various periods of chase. As shown in Fig. 4, NS-lamp-2 was detectable in early endosomes after a 20 min chase, and its level increased up to 60 min with a $t_{1/2}$ of 38 min. After the peak, the NS-lamp-2 level fell rapidly until 120 min, and then remained almost constant in early endosomes for 60 min. The kinetics of early endosomal transport of NS-lamp-2 is similar to that of NS-lamp-1 (*22*); the level of NS-lamp-1 in early endosomes peaked at 60 min (a $t_{1/2}$ of 33 min), declined and reached a plateau near 120 min. These kinetic data suggested that during the exit of the initially transported NS-lamp-2 from this compartment, the second wave of NS-lamp-2 arrived at early endosomes. An equilibrium between the inflow and outflow of NS-lamp-2 in early endosomes would result in a constant level later.

Quantitation of NS-Lamp-2 Delivered to the Cell Periphery—To determine the amount of NS-lamp-2 which appears at the cell periphery, NS-lamp-2 transported to the plasma membrane and peripheral early endosomes was continuously captured by incubating hepatocytes at 37°C in medium containing anti-lamp-2 antibody for 15 min before the end of the pulse-chase study (Fig. 5). Similar kinetic data were obtained when the pulse-chase experiments were carried out under the condition at chase times of 0, 30, 60, and 90 min; the level of NS-lamp-2 peaked at 60 min, and

TABLE I. **Summary of biosynthetic transport of lamp-1 and lamp-2 in cultured rat hepatocytes.**

Compartment	Transport rate ($t_{1/2}$) (min)		Maximum level (% of the total labeled)	
	NS-lamp-1[a]	NS-lamp-2	NS-lamp-1[a]	NS-lamp-2
Trans-Golgi	13	32	—	—
Plasma membrane	32	47	~5	~3.5
Early endosome	33	38	25	45
Late endosome	45	48	40	38
Lysosome	85	90	70	50

[a]Data cited from Ref. *22*.

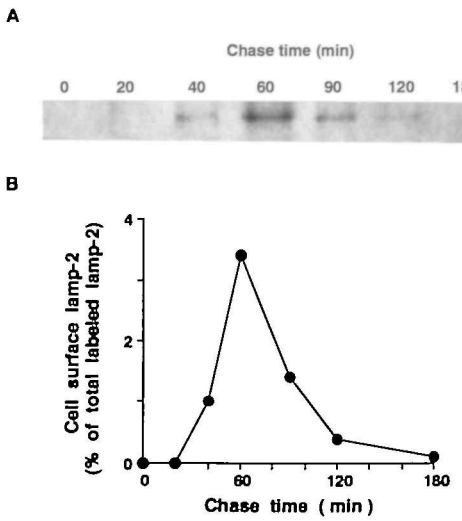

Fig. 3. **Transport of newly synthesized lamp-2 (NS-lamp-2) to the cell surface.** (A) Hepatocytes were pulsed with [^{35}S]methionine for 20 min and incubated in unlabeled medium for the indicated periods at 37°C. Immediately after the chase, the cells were incubated with anti-lamp-2 antibody at 4°C for 30 min. They were washed extensively with Hanks' buffer, then antibody-bound, surface lamp-2 was precipitated by lysing the monolayer with detergents, then adding protein A-Sepharose CL-4B to 75% of the cell lysate. Total ^{35}S-labeled lamp-2 was immunoprecipitated from the remainder of the cell lysate with anti-lamp-2 antibody. (B) The fluorogram was quantified by densitometric tracing. The ratio of surface to total lamp-2 was calculated and is expressed as a percentage of the total, corrected for differences in sample volumes. The kinetic data were reproducible in two separate experiments. Values are the means of two determinations.

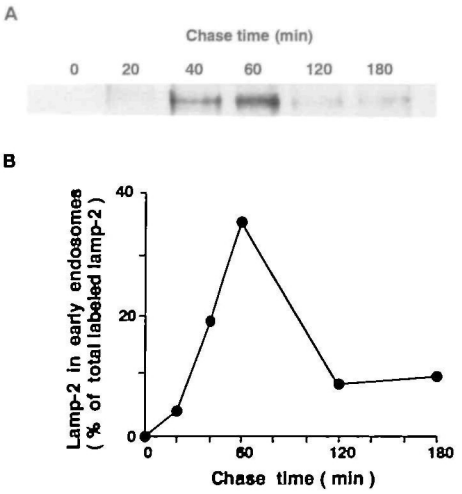

Fig. 4. **Transport of NS-lamp-2 to early endosomes.** (A) After the chase, 80% of the PNS of the hepatocytes was separated by Percoll density centrifugation, and fractions containing early endosomes were collected (see Fig. 1). Lamp-2 was immunoprecipitated after lysis in detergents, then resolved by SDS-PAGE and visualized by fluorography. Total ^{35}S-labeled lamp-2 was immunoprecipitated from the remainder of the PNS with anti-lamp-2 antibody. (B) The fluorogram was quantified by densitometric tracing. The ratio of early endosomal to total lamp-2 was calculated and is expressed as a percentage of the total, corrected for differences in sample volumes. The kinetic data were reproducible in two separate experiments. Values are the means of two determinations.

then declined to half of the maximum at 90 min. At the maximum, about 45% of the total NS-lamp-2 (at 60 min of chase) was recovered by cell surface immunoprecipitation under these conditions. Since NS-lamp-2 appearing on the plasma membrane is less than 5% of the total, as stated above, it is likely that ~45% of total NS-lamp-2 is transported directly to early endosomes from the *trans*-Golgi. Concomitantly, at 60 min, 35% of the total NS-lamp-2 is recovered in the early endosomes after cell fractionation (Fig. 4).

The $t_{1/2}$ for exit of NS-lamp-2 from the cell periphery was calculated to be 40 min, less than that of NS-lamp-1 ($t_{1/2}$ = ~100 min) (*22*). Lamp-1 and lamp-2 have been shown to shuttle continuously between the cell peripheral region and late endosomes (*10-12*). During the exit of the initially transported NS-lamp-1 and NS-lamp-2 from the cell peripheral region, second waves of NS-lamp-1 and NS-lamp-2 enter this region from the late endosomes. The apparent rate of exit of NS-lamp molecules from the cell periphery is determined mainly by their inflow to and outflow from the cell periphery. Since the percentage of NS-lamp-2 transported to the cell peripheral region is 1.8 times greater than that of NS-lamp-1 (Table I), the ratio of outflow to inflow for NS-lamp-2 seems to be higher than that for NS-lamp-1. Thus, NS-lamp-2 leaves the cell periphery at a higher rate than NS-lamp-1.

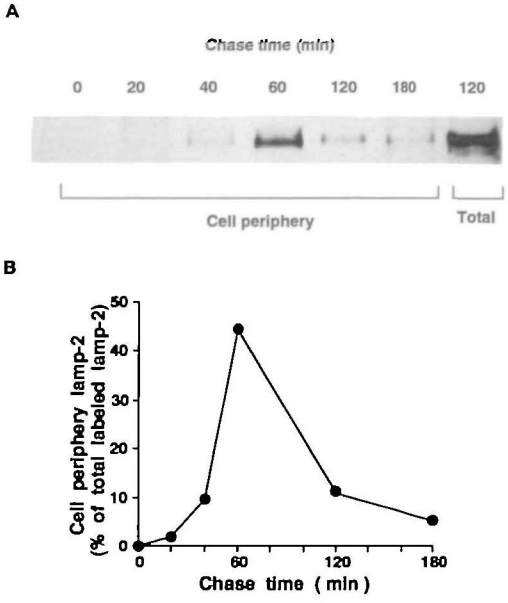

Fig. 5. **Quantitation of NS-lamp-2 delivered to the cell periphery.** (A) Hepatic cells were pulsed with [^{35}S]methionine for 10 min and chased at 37°C for the indicated periods. Anti-lamp-2 antibody was added to the culture medium 15 min before the chase was terminated. Antibody and lamp-2 complexes were precipitated, after washing of the cells, by lysing the monolayer with detergents, then adding protein A-Sepharose CL-4B to 75% of the cell lysate. Total ^{35}S-labeled lamp-2 was immunoprecipitated from the remainder of the cell lysate with anti-lamp-2 antibody. Radio-labeled lamp-2 immunoprecipitated from the whole cell lysates obtained after a 120 min chase was electrophoresed in the right lane labeled "total" for comparison. (B) The fluorogram was quantified by densitometric tracing. The ratio of the cell peripheral to total lamp-2 was calculated and is expressed as a percentage of the total, corrected for differences in sample volumes. The kinetic data were reproducible in two separate experiments. Values are the means of two determinations.

Transport of NS-Lamp-2 to Late Endosomes—NS-lamp-2 isolated from the late endosomal fraction (fractions

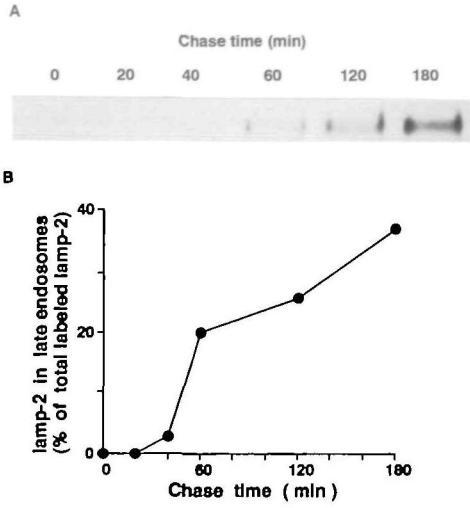

Fig. 6. **Transport of NS-lamp-2 to late endosomes.** (A) After the chase, two-thirds of the PNS of the hepatocytes was separated by Percoll density centrifugation, and the late endosomal fraction was collected (see Fig. 1). Lamp-2 was immunoprecipitated after lysis with detergents, then resolved by SDS-PAGE and visualized by fluorography. Total ^{35}S-labeled lamp-2 was immunoprecipitated from the remainder of the PNS with anti-lamp-2 antibody. (B) The fluorogram was quantified by densitometric tracing. The ratio of late endosomal to total lamp-2 was calculated and is expressed as a percentage of the total, corrected for differences in sample volumes. The kinetic data were reproducible in three separate experiments. Values are the means of three determinations.

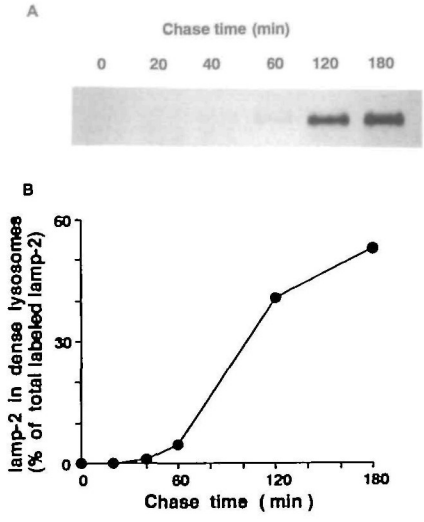

Fig. 7. **Transport of NS-lamp-2 to lysosomes.** (A) At each time point, two-thirds of the PNS of the hepatocytes was fractionated by Percoll density centrifugation. Fractions containing dense lysosomes were collected (see Fig. 1). Lamp-1 was immunoprecipitated after lysis with detergents, then resolved by SDS-PAGE and visualized by fluorography. Total ^{35}S-labeled lamp-2 was immunoprecipitated from the remainder of the PNS with anti-lamp-2 antibody. (B) The fluorogram was quantified by densitometric tracing. The ratio of lysosomal to total lamp-2 was calculated and is expressed as a percentage of the total, corrected for differences in sample volumes. The kinetic data were reproducible in three separate experiments. Values are the means of three determinations.

13 and 14 of the Percoll gradient shown in Fig. 1) was resolved by SDS-PAGE and fluorography (Fig. 6). NS-lamp-2 was detectable in late endosomes after a 40 min chase. The NS-lamp-2 level increased up to 180 min in a biphasic manner, after which it reached a plateau (data not shown). As estimated from an initial phase of the increase (~60 min), NS-lamp-2 is delivered directly from the *trans*-Golgi with a $t_{1/2}$ of 48 min. The biphasic increase of NS-lamp-2 in late endosomes seems to be reflected by transport of a significant fraction of NS-lamp-2 from early endosomes as described above.

Transport of NS-Lamp-2 to Dense Lysosomes—NS-lamp-2 was immunoprecipitated from lysosomes recovered in the densest fraction of the Percoll gradient (fractions 17 and 18). As shown in Fig. 7, the level of NS-lamp-2 in dense lysosomes increased up to 180 min chase after the initial detection at 60 min and then NS-lamp-2 reached a constant level (data not shown). The $t_{1/2}$ for lysosomal transport of NS-lamp-2 was estimated to be 90 min.

DISCUSSION

The route of NS-lamp-2 from the *trans*-Golgi to lysosomes was investigated in cultured rat hepatocytes. Lamp-2 synthesized in rough endoplasmic reticulum was transported to the *trans*-Golgi where modification of its *N*-linked carbohydrate chains was accomplished. A notable difference was observed in the carbohydrate processing rates of lamp-2 ($t_{1/2}$ = 32 min) and lamp-1 ($t_{1/2}$ = 13 min) in cultured rat hepatocytes (Table I). The rates of carbohydrate processing for lamp-1 and lamp-2 are reportedly very similar to each other in other cell types (*15, 18*). Lamp-1 and lamp-2 that occur in certain cell types possess *N*-linked oligosaccharide chains bearing polylactosamines (*5*). Nabi and Rodriguez-Boulan (*30*) have shown that the rate of lamp-2 transport to the *trans*-Golgi is affected by the amount of polylactosamines in its *N*-linked oligosaccharide chains. In rat hepatocytes lamp-2 might have more complex forms of *N*-linked oligosaccharides than lamp-1, resulting in the slower carbohydrate processing of lamp-2 than lamp-1. Additionally, the slower transit of lamp-2 to the *trans*-Golgi seems to be related to the occurrence of *O*-linked glycosylation on the protein core of lamp-2. Lamp-2 purified from rat liver contains *N*-acetylgalactosamine residues sufficient for ~10 mucin-type *O*-glycans, but lamp-1 has no such carbohydrate residue (*23*). Thus, accomplishment of *O*-glycosylation on lamp-2 protein might delay its passage through the Golgi-complex.

After NS-lamp-2 left the *trans*-Golgi, it was distributed to three distinct endocytic compartments; the cell surface, early endosomes and late endosomes, as observed for NS-lamp-1. At the maximum, ~3.5% of NS-lamp-2 was detectable at the cell surface, which is comparable with the case of NS-lamp-1. It has been debated whether or not newly synthesized lysosomal membrane proteins are transported to lysosomes after their appearance on the cell surface (*3, 5-7*). Taking into account quantitative studies thus far performed (*15, 17-22*), the amounts of endogenous NS-lamp-1 and NS-lamp-2 transported to the cell surface may be less than 5% of the total. These data strongly suggest that the cell surface transport of NS-lamps is a minor pathway.

NS-lamp-1 that appears on the plasma membrane early in the chase time comes directly from the *trans*-Golgi since NS-lamp-1 is delivered to these two compartments with the same half times (Table I). In contrast, the transit time of NS-lamp-2 to early endosomes from the *trans*-Golgi is about 10 min less than that to the plasma membrane from the *trans*-Golgi (Table I). Additionally, the amount of NS-lamp-2 delivered to early endosomes is about 10 times higher than that of NS-lamp-2 delivered to the cell surface. These kinetic and quantitative data appear to be consistent with the unique model for the biosynthetic trafficking of lamp molecules proposed Rohrer *et al.* (*34*), in which a major fraction of NS-lamp is delivered from the *trans*-Golgi to early endosomes before reaching late endosomes, and a small percentage of lamp escapes to the plasma membrane from early endosomes *via* recycling endosomes which serve for reutilization of cell surface receptors. However, we can not rule out the direct transport of NS-lamp-2 from the *trans*-Golgi to the plasma membrane.

TABLE II. **Amino acid sequence of the cytosolic domain of lamp-1 and lamp-2 from different species.** Amino acids are shown in the one letter code. Sequences of the tyrosine-based lysosomal targeting motif are underlined.

Human lamp-1	R-K-R-S-H-A-G-Y-Q-T-I
Mouse lamp-1	R-K-R-S-H-A-G-Y-Q-T-I
Rat lamp-1	R-K-R-S-H-A-G-Y-Q-T-I
Chicken lamp-1	R-K-R-S-H-A-G-Y-Q-T-I
Human lamp-2A	L-K-H-H-H-A-G-Y-E-Q-F
Human lamp-2B	R-R-K-S-Y-A-G-Y-Q-T-L
Mouse lamp-2	L-K-R-H-H-T-G-Y-E-Q-F
Rat lamp-2	L-K-R-H-H-T-G-Y-E-Q-F
Chicken lamp-2a	K-K-H-H-N-T-G-Y-E-Q-F
Chicken lamp-2b	R-R-K-S-R-T-G-Y-Q-S-V
Chicken lamp-2c	R-R-K-S-Y-A-G-Y-Q-T-L

Many investigations have been performed to identify the molecular signal(s) that targets lamp-1 to lysosomes (*20, 21, 31-34*). It is now accepted that the targeting signal is contained in the short cytoplasmic tail of lamp-1, and a G-Y-X-X-I sequence is the signal for lysosomal targeting of lamp-1 (Table II). Lamp-1 has been shown to be included into the clathrin-coated pits in the *trans*-Golgi network, and then leaves the Golgi complex in the clathrin-coated vesicles, which move to late endosomes (*7*). The cytoplasmic tail peptide of lamp-1 was found to bind to γ-adaptin, a component of adaptors associated with the Golgi-derived coated vesicles (*35*). Isoleucine, the COOH-terminal amino acid residue of the five-amino-acid motif, has been shown to be critical for γ-adaptin binding. Phenylalanine in place of isoleucine is present at the COOH-terminus of the motif in rat lamp-2's cytoplasmic tail (Table II). Therefore, it is likely that lamp-1 and lamp-2 bind to γ-adaptin with different affinities and then are concentrated into the Golgi-derived coated vesicles with different efficiencies, resulting in the different transport ratios of NS-lamp-1 and NS-lamp-2 to late endosomes.

Recent studies have shown that avian and human lamp-2 occur in three and six variants, respectively (*36, 37*) (Table II). The avian variants have different amino acid sequences in the transmembrane and cytoplasmic domains (*36*). If such variants of lamp-2 exist in rat hepatocytes, the protein variation might be involved in the multiple biosynthetic pathways of lamp-2 as observed in our studies.

REFERENCES

1. Holtzman, E. (1989) *Lysosomes*, Plenum Press, New York
2. Hubbard, A.L. (1989) Endocytosis. *Curr. Opin. Cell Biol.* **1**, 675-683
3. Kornfeld, S. and Mellman, I. (1989) The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* **5**, 483-525
4. Gruenberg, J. and Howell, K.E. (1989) Membrane traffic in endocytosis: Insights from cell-free assays. *Annu. Rev. Cell Biol.* **5**, 453-481
5. Fukuda, M. (1991) Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. *J. Biol. Chem.* **266**, 21327-21330
6. Peters, C. and von Figura, K. (1994) Biogenesis of lysosomal membranes. *FEBS Lett.* **346**, 108-114
7. Hunziker, W. and Geuze, H.J. (1996) Intracellular trafficking of lysosomal membrane proteins. *Bioessays* **18**, 379-389
8. Lippincott-Schwartz, J. and Fambrough, D.M. (1986) Lysosomal membrane dynamics: Structure and interorganellar movement of a major lysosomal membrane glycoprotein. *J. Cell Biol.* **102**, 1593-1605
9. Lippincott-Schwartz, J. and Fambrough, D.M. (1987) Cycling of the integral membrane glycoprotein, LEP100, between plasma membrane and lysosomes: Kinetic and morphological analysis. *Cell* **49**, 669-677
10. Furuno, K., Ishikawa, T., Akasaki, K., Yano, S., Tanaka, Y., Yamaguchi, Y., Tsuji, H., Himeno, M., and Kato, K. (1989) Morphological localization of a major lysosomal membrane glycoprotein in the endocytic membrane system. *J. Biochem.* **106**, 708-716
11. Furuno, K., Yano, S., Akasaki, K., Tanaka, Y., Yamaguchi, Y., Tsuji, H., Himeno, M., and Kato, K. (1989) Biochemical analysis of the movement of a major lysosomal membrane glycoprotein in the endocytic membrane system. *J. Biochem.* **106**, 717-722
12. Akasaki, K., Fukuzawa, M., Kinoshita, H., Furuno, K., and Tsuji, H. (1993) Cycling of two endogenous lysosomal membrane proteins, lamp-2 and acid phosphatase, between the cell surface and lysosomes in cultured rat hepatocytes. *J. Biochem.* **114**, 598-604
13. Hellström, I., Garrigues, H.J., Garrigues, U., and Hellström, K.E. (1990) Highly tumor-reactive, internalizing, mouse monoclonal antibodies to Le^y-related cell surface antigens. *Cancer Res.* **50**, 2183-2190
14. Garrigues, J., Anderson, J., Hellström, K.E., and Hellström, I. (1994) Anti-tumor antibody BR96 blocks cell migration and binds to a lysosomal membrane glycoprotein on cell surface microspikes and ruffled membranes. *J. Cell Biol.* **125**, 129-142
15. Green, S.A., Zimmer, K.-P., Griffiths, G., and Mellman, I. (1987) Kinetics of intracellular transport and sorting of lysosomal membrane and plasma membrane proteins. *J. Cell Biol.* **105**, 1227-1240
16. D'Souza, M.P. and August, J.T. (1986) A kinetic analysis of biosynthesis and localization of a lysosome-associated membrane glycoprotein. *Arch. Biochem. Biophys.* **249**, 522-532
17. Carlsson, S.R. and Fukuda, M. (1992) The lysosomal membrane glycoprotein lamp-1 is transported to lysosomes by two alternative pathways. *Arch. Biochem. Biophys.* **296**, 630-639
18. Nabi, I.R., Le Bivic, A., Fambrough, D., and Rodriguez-Boulan, E. (1991) An endogenous MDCK lysosomal membrane glycoprotein is targeted basolaterally before delivery to lysosomes. *J. Cell Biol.* **115**, 1573-1584
19. Braun, M., Waheed, A., and von Figura, K. (1989) Lysosomal acid phosphatase is transported to lysosomes *via* the cell surface. *EMBO J.* **8**, 3633-3640
20. Mathews, P.M., Martinie, J.B., and Fambrough, D.M. (1992) The pathway and targeting signal for delivery of the integral membrane glycoprotein LEP100 to lysosomes. *J. Cell Biol.* **118**, 1027-1040
21. Harter, C. and Mellman, I. (1992) Transport of the lysosomal membrane glycoprotein lgp120 (lgp-A) to lysosomes does not require appearance on the plasma membrane. *J. Cell Biol.* **117**, 311-325
22. Akasaki, K., Michihara, A., Mibuka, K., Fujiwara, Y., and Tsuji, H. (1995) Biosynthetic transport of a major lysosomal membrane glycoprotein, lamp-1: Convergence of biosynthetic and endocytic pathways occurs at three distinctive points. *Exp. Cell Res.* **220**, 464-473
23. Akasaki, K., Yamaguchi, Y., Furuno, K., and Tsuji, H. (1991) Purification, some properties, and tissue distribution of a major lysosome-associated membrane glycoprotein (r-lamp-2) of rat liver. *J. Biochem.* **110**, 922-927
24. Seglen, P.O. (1976) Preparation of isolated rat liver cells. *Methods Cell Biol.* **13**, 29-83
25. Robins, E., Hirsch, H.E., and Emmons, S.S. (1968) Glycosidases in the nervous system. I. Assay, some properties, and distribution of β-galactosidase, β-glucuronidase, and β-glucosidase. *J. Biol. Chem.* **243**, 4246-4252
26. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
27. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
28. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354
29. Griffiths, G. and Simons, K. (1986) The trans Golgi network: sorting at the exit site of the Golgi complex. *Science* **234**, 438-443
30. Nabi, I.R. and Rodriguez-Boulan, E. (1993) Increased LAMP-2 polylactosamine glycosylation is associated with its slower Golgi transit during establishment of a polarized MDCK epithelial monolayer. *Mol. Biol. Cell* **4**, 627-635
31. Williams, M.A. and Fukuda, M. (1990) Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. *J. Cell Biol.* **111**, 955-966
32. Guarnieri, F.G., Arterburn, L.M., Penno, M.B., Cha, Y., and August, J.T. (1993) The motif Tyr-X-X-hydrophobic residue mediates lysosomal membrane targeting of lysosome-associated membrane protein 1. *J. Biol. Chem.* **268**, 1941-1946
33. Honing, S. and Hunziker, W. (1995) Cytoplasmic determinants involved in direct lysosomal sorting, endocytosis, and basolateral targeting of rat lgp120 (lamp-1) in MDCK cells. *J. Cell Biol.* **128**, 321-332
34. Rohrer, J., Schweizer, A., Russell, D., and Kornfeld, S. (1996) The targeting of lamp1 to lysosomes is dependent on the spacing of its cytoplasmic tail tyrosine sorting motif relative to the membrane. *J. Cell Biol.* **132**, 565-576
35. Ohno, H., Stewart, J., Fournier, M.-C., Bosshart, H., Rhee, I., Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T., and Bonifacino, J.S. (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science* **269**, 1872-1875
36. Hatem, C.L., Gough, N.R., and Fambrough, D.M. (1995) Multiple mRNAs encode the avian lysosomal membrane protein LAMP-2, resulting in alternative transmembrane and cytoplasmic domains. *J. Cell. Sci.* **108**, 2093-2100
37. Konecki, D.S., Foetisch, K., Zimmer, K.-P., Schlotter, M., and Lichter-Konecki, U. (1995) An alternatively spliced form of the human lysosome-associated membrane protein-2 gene is expressed in a tissue-specific manner. *Biochem. Biophys. Res. Commun.* **215**, 757-767