Kinetics of Intracellular Transport and Sorting of Lysosomal Membrane and Plasma Membrane Proteins

Samuel A. Green,* Klaus-Peter Zimmer,* Gareth Griffiths,‡ and Ira Mellman*

*Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510; and ‡European Molecular Biology Laboratory, 6900 Heidelberg, Federal Republic of Germany

Abstract. We have used monospecific antisera to two lysosomal membrane glycoproteins, lgp120 and a similar protein, lgp110, to compare the biosynthesis and intracellular transport of lysosomal membrane components, plasma membrane proteins, and lysosomal enzymes. In J774 cells and NRK cells, newly synthesized lysosomal membrane and plasma membrane proteins (the IgG1/IgG2b Fc receptor or influenza virus hemagglutinin) were transported through the Golgi apparatus (defined by acquisition of resistance to endo-β-*N*-acetylglucosaminidase H) with the same kinetics ($t_{1/2}$ = 11–14 min). In addition, immunoelectron microscopy of normal rat kidney cells showed that lgp120 and vesicular stomatitis virus G-protein were present in the same Golgi cisternae demonstrating that lysosomal and plasma membrane proteins were not sorted either before or during transport through the Golgi apparatus. To define the site at which sorting occurred, we compared the kinetics of transport of lysosomal and plasma membrane proteins and a lysosomal enzyme to their respective destinations. Newly synthesized proteins were detected in dense lysosomes (lgp's and β-glucuronidase) or on the cell surface (Fc receptor or hemagglutinin) after the same lag period (20–25 min), and accumulated at their final destinations with similar kinetics ($t_{1/2}$ = 30–45 min), suggesting that these two lgp's are not transported to the plasma membrane before reaching lysosomes. This was further supported by measurements of the transport of membrane-bound endocytic markers from the cell surface to lysosomes, which exhibited additional lag periods of 5–15 min and half-times of 1.5–2 h. The time required for transport of newly synthesized plasma membrane proteins to the cell surface, and for the transport of plasma membrane markers from the cell surface to lysosomes would appear too long to account for the rapid transport of lgp's from the Golgi apparatus to lysosomes. Thus, the observed kinetics suggest that lysosomal membrane proteins are sorted from plasma membrane proteins at a post–Golgi intracellular site, possibly the *trans* Golgi network, before their delivery to lysosomes.

T HE lysosomal membrane contains a characteristic set of proteins that reflects its unusual properties. In addition to sequestering a high concentration of hydrolytic enzymes, the lysosomal membrane is responsible for the acidification of the lumen (Mellman et al., 1986), the selective transport of degradation products and metabolites from the lumen to the cytoplasm (Ehrenreich and Cohn, 1969; Cohn and Ehrenreich, 1969; Jonas et al., 1982; Maguire et al., 1983; Pisoni et al., 1985; Rosenblatt et al., 1985), and the regulation of fusion events between lysosomes and other organelles (Steinman et al., 1983). Several lysosomal membrane proteins (lgp's) have recently been identified and found to comprise a unique class of glycoproteins[1,2] (Reggio et al., 1984; Lewis et al., 1985; Chen et al., 1985; Lippincott-Schwartz and Fambrough, 1986; Barriocanal et al., 1986). In general, lgp's are characterized by relatively small am-

phipathic polypeptide chains (20–60 kD), a large number of NH₂-linked oligosaccharide chains (up to 18 per polypeptide), and extremely acidic isoelectric points (pI < 2–4) due to the presence of numerous sialic acid residues (Lewis et al., 1985). It is possible that the structure of the lgp's is related to their ability to survive in a hydrolase-rich environment.

Since all lgp's contain complex NH₂-linked oligosaccharides, it is clear that they, like plasma membrane proteins, are synthesized in the rough endoplasmic reticulum (RER)[3] and terminally glycosylated in the Golgi apparatus (Kornfeld and Kornfeld, 1985). At some point, however, lgp's must be sorted from plasma membrane proteins since they are generally undetectable on the cell surface and plasma membrane proteins are not found in lysosomes (Burnside and Schneid-

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1. Granger, B., S. Green, K.-P. Zimmer, A. Helenius, and I. Mellman, unpublished results.
2. Schmid, S., H. Plutner, and I. Mellman, manuscript in preparation.

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er, 1982; Lewis et al., 1985; Chen et al., 1985; Barriocanal et al., 1986; Marsh et al., 1987). Accordingly, the intracellular transport of newly synthesized lgp's must reflect a pathway normally avoided by proteins destined for the cell surface and indeed by most other membrane proteins in the cell. Even the 215-kD mannose 6-phosphate receptor, responsible for targeting most lysosomal enzymes, itself avoids transport to dense, hydrolase-rich lysosomes (Sahagian and Neufeld, 1983; von Figura et al., 1984; Brown et al., 1986).

To better define the pathway of lgp transport to lysosomes, we have compared the kinetics of biosynthesis and intracellular transport of two lysosomal and plasma membrane proteins in macrophage and epithelioid cell lines. Our results indicate that the sorting events that segregate lgp's from plasma membrane proteins occur after their transit through the Golgi apparatus but before arrival at the cell surface.

Materials and Methods

Cells

J774 mouse macrophages were maintained in suspension culture in DME containing 3.5% heat-inactivated (56°C, 30 min) FBS (K.C. Biological Inc., Lenexa, KS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Normal rat kidney (NRK) epithelioid cells were grown on tissue culture plates (Falcon Labware, Oxnard, CA) in DME containing 6% heat-inactivated FBS and antibiotics. Chinese hamster ovary (CHO) cells were maintained in suspension culture in alpha-modified Eagle's medium containing 5% heat-inactivated FBS and antibiotics.

Antibodies

Polyclonal rabbit antisera were prepared as described to lgp120 that had been affinity-purified from NRK cell lysates (Lewis et al., 1985) and to mouse Fc receptor (Mellman et al., 1983) and lgp110[1] that had been affinity-purified from J774 cell lysates. Goat anti-mouse β-glucuronidase, immunoglobulin fraction, was the generous gift of Roger Ganschow (University of Cincinnati). Affinity-purified goat anti-rabbit IgG and rabbit anti-goat IgG, for use in immunoprecipitation, were purchased from Tago Inc. (Burlingame, CA).

Metabolic Labeling and Immunoprecipitation

J774 cells were pulse-labeled in methionine-free MEM (Gibco, Grand Island, NY) containing 10% dialyzed heat-inactivated FBS, 10 mM Hepes pH 7.3, and 0.25–1.0 mCi/ml [^{35}S]methionine (>800 Ci/mmol, Amersham Corp., Arlington Heights, IL) as described previously (Green et al., 1985). NRK cells were labeled under similar conditions on plates when 80–95% confluent. For chase incubations, excess cold methionine was added such that the ratio of cold methionine to labeled methionine was at least 40,000:1.

After labeling, cells were washed with PBS, lysed in 1% Triton X-114, and the detergent phase isolated as described (Bordier, 1981; Green et al., 1985). Triton X-114 lysates of membranes recovered from density gradients (see below) were processed without the phase separation step. Immunoprecipitation of antigens from the lysates was carried out exactly as described (Green et al., 1985; Lewis et al., 1985).

Endoglycosidase Digestion of Immunoprecipitates

Endo-β-N-acetylglucosaminidase H (Endo H) was purchased from Boehringer-Mannheim Diagnostics, Inc. (Houston, TX) as a 1 U/ml stock. Washed immunoprecipitates were eluted into 50 µl of Endo H buffer (100 mM Na-acetate pH 5.6/0.1% SDS/1 mM phenylmethylsulfonyl fluoride) by resuspending and heating to 100°C for 3 min. Particles were removed by centrifugation in an Eppendorf microcentrifuge, and 25-µl aliquots of the supernatant were incubated 14–18 h at 37°C with or without 2 µl Endo H per sample. After incubation, samples were mixed with an equal volume of 2× concentrated electrophoresis sample buffer (Laemmli, 1970) and heated to 100°C for 3 min.

Percoll Density Gradient Fractionation of Cell Homogenates

J774 or NRK cells from one confluent 10-cm tissue culture dish (∼1 × 10^7 cells) were washed with PBS containing 1 mg/ml glucose at 4°C, harvested by scraping with a rubber policeman, and resuspended at 2–3 × 10^6 cells/ml in homogenization buffer (0.25 M sucrose/10 mM Hepes pH 7.2/1 mM EDTA). Cells were homogenized on ice in a steel Dounce homogenizer (Kontes Co., Vineland, NJ) and the homogenate centrifuged (750 g, 10 min, 4°C) to remove nuclei and unbroken cells (Galloway et al., 1983). The postnuclear supernatant was then mixed with additional homogenization buffer, Percoll (from a stock solution containing 90% Percoll [Sigma Chemical Co., St. Louis, MO] in 0.25 M sucrose), and 20% BSA in PBS to final concentrations of 27% (vol/vol) Percoll and 4 mg/ml BSA in a volume of 11.5 ml. The mixture was layered over a 1-ml cushion of 2.5 M sucrose and centrifuged (29,000 g, 90 min, 4°C) in a 50 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA). Fractions of ∼0.5 ml were collected from the bottom of the tube.

Organelle markers were assayed as described previously for trans Golgi elements (galactosyl transferase) (Brew et al., 1975) and for endosomes (FITC-dextran [Galloway et al., 1983] or horseradish peroxidase [Marsh et al., 1987], 6–10 mg/ml pulse-labeled for 5 min at 37°C). The lysosomal marker β-hexosaminidase was assayed fluorometrically in 50 µl 0.1 M citric acid/0.2 M dibasic sodium phosphate, pH 4.5 containing 0.1% NP-40 (Pool et al., 1983) using 2.3 mg/ml 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma Chemical Co.) as the substrate. The plasma membrane marker alkaline phosphodiesterase was assayed fluorometrically in 100 µl of 0.1 M Tris pH 9.0/25 mM CaCl$_2$ using 0.6 mg/ml 4-methylumbelliferyl-thymidine-5'-phosphate (Hawley et al., 1981) as the substrate. For both fluorimetric assays, the reactions were stopped by adding 2 ml 0.2 M sodium carbonate/0.11 M glycine pH 10. Samples were read at 365-nm excitation and 450-nm emission wavelengths in a fluorescence spectrophotometer. The distribution of organelle markers in the gradient fractions was highly reproducible over a wide range of cell loads (4 × 10^6 to 1 × 10^8 cells per gradient). The nuclear pellet contained 3–4% of all markers tested.

Immunoprecipitation of labeled lysosomal and plasma membrane antigens from gradient fractions was performed using a modification of the method of Gieselmann et al. (1983). Gradient fractions were first pooled as follows: for J774 cells, fractions 4–10 (pool 1), fractions 11–16 (pool 2), and fractions 17–24 (pool 3, the remainder of gradient); for NRK cells, fractions 5–11 (pool 1), fractions 12–17 (pool 2), and fractions 18–24 (pool 3). The percentages of the marker enzymes measured in each pool varied <2% between experiments. The pooled fractions were diluted to equal volumes with homogenization buffer, and "carrier" membrane (postnuclear supernatant derived from 1 × 10^7 CHO cells) was added to each pool. The addition of carrier membrane (which does not contain immunoprecipitable lgp's) facilitated efficient recovery of labeled membranes and antigens particularly from the dense region of the gradients. The mixture was then centrifuged at 180,000 g for 3 h to pellet the Percoll. The membrane layer was collected from the top of the Percoll pellet and solubilized in Triton X-114 lysis buffer. The solubilized membranes were centrifuged (without phase separation) at 40,000 g for 15 min immediately before immunoprecipitation. The antigens in each pool were quantified by scanning densitometry of the autoradiographs (see below). A 10% aliquot of postnuclear supernatant was routinely removed and used as a standard to quantify the recovery of antigen from each Percoll gradient. This aliquot always contained ∼10% of the total antigen recovered from the three pools, indicating that the efficiency of immunoprecipitation was not affected by the presence of different amounts of Percoll in the pools. This was further demonstrated by adding equal trace amounts of [^{35}S]methionine-labeled membranes to gradient pools containing unlabeled membranes and finding that the recovery of labeled antigen from each pool was the same (data not shown).

Detection of Influenza Virus Hemagglutinin on the Plasma Membrane of NRK Cells

NRK cells were infected with the X:31 strain of influenza virus for 3.5 h and pulse-labeled with [^{35}S]methionine as described (Doms et al., 1985). After various durations of chase at 20° or 37°C, the transport of labeled hemagglutinin (HA) to the plasma membrane was measured by treating the cells with 100 µg/ml TPCK-trypsin (Sigma Chemical Co.) at 0°C to cleave precursor HA0 on the cell surface to HA1 and HA2, as described by Matlin and Simons (1983). The N2 monoclonal anti–HA antibody was used for immunoprecipitation (Copeland et al., 1986).

Electrophoresis and Fluorography

Immunoprecipitates were solubilized in electrophoresis sample buffer (125 mM Tris pH 6.95, 4% SDS, 15% sucrose, 2 mM EDTA, 0.001% bromphenol blue) by heating to 100°C for 3 min. Samples were then analyzed by electrophoresis on SDS-polyacrylamide gels (Laemmli, 1970) of 10 or 12% acrylamide. lgp120 and lgp110 were analyzed on 12% acrylamide gels to minimize the area of the bands, which were then quantitated by scanning densitometry. Destained gels were neutralized in PBS and soaked for 30 min in 0.5 or 1 M sodium salicylate, pH 7.1 in 30% (vol/vol) methanol (Chamberlain, 1979). Gels were then dried on filter paper and exposed to prefogged ($\Delta A_{540} = 0.12$) Kodak XOmat film at $-70°C$ (Laskey and Mills, 1975). Autoradiographs with maximum exposures of $\Delta A_{540} = 1.2$ were scanned on a Joyce-Loebl densitometer and the areas of the scans integrated using a Zeiss MOP-3 Image Analyzer.

Immunoelectron Microscopy

Immunocytochemistry was done on ultrathin frozen sections using the protein A-gold technique (Griffiths et al., 1983). NRK cells were infected with ts045 vesicular stomatitis virus (VSV) as previously described for BHK-21 cells (Griffiths et al., 1985) with minor modifications to be published elsewhere. Fractions from density gradients containing 4×10^7 unlabeled cells were pooled as described above, then mixed with an equal volume of 8% paraformaldehyde in 50 mM Hepes pH 7.4 and incubated for 30 min at room temperature. Membranes were collected by centrifugation (3,000 g, 10 min), fixed an additional 30 min, then embedded in 10% gelatin. Intact NRK cells were incubated for 4 h at 20°C in the presence of 12 μg/ml UV-inactivated Semliki Forest virus (Marsh et al., 1983), harvested by trypsinization at 0°C, and fixed in 5% paraformaldehyde for 60 min at room temperature. After infusion with 2.1 M sucrose and freezing in liquid nitrogen, ultrathin sections were cut at −110°C.

Double labeling of frozen sections (Griffiths et al., 1982) was carried out with protein A-gold particles prepared by the method of Slot and Geuze (1985), after determining the antibody dilutions yielding minimal background staining by single labeling with each antibody. Sections were labeled at room temperature for 45 min with polyclonal rabbit anti-lgp120 (NRK cells) or lgp110 (J774 cells) at a dilution of 1:60, followed by 9-nm protein A-gold. All free binding sites were then blocked with 0.1 mg/ml free protein A in PBS. The sections were then labeled with goat anti-mouse β-glucuronidase (1:100), followed by affinity-purified rabbit anti-goat IgG (Zymed, San Francisco, CA) at 20 μg/ml as a bridge antibody, and finally with 5-nm protein A-gold.

Results

Kinetics of Transport from the RER through the Golgi Apparatus

We first sought to determine the relative rates of lgp and plasma membrane protein transport from the RER through the Golgi apparatus. This was accomplished by comparing the conversion of oligosaccharide chains on newly synthesized lysosomal and plasma membrane proteins from their high-mannose Endo H–sensitive precursors to terminally glycosylated mature forms that are resistant to digestion by Endo H (Lewis et al., 1985; Green et al., 1985). Initial experiments concentrated on the biosynthesis of two immunologically distinct lysosomal membrane proteins of 110 and 120 kD (lgp110[1] and lgp120 [Lewis et al., 1985], respectively) in the J774 mouse macrophage cell line, in which these proteins are abundant. Cells were pulse-labeled for 5 min at 37°C with [35S]methionine and harvested after 0–80 min of chase in the presence of unlabeled methionine. lgp110 and lgp120 were sequentially immunoprecipitated; this was followed by a third round of immunoprecipitation of the IgG Fc receptor, a 60-kD plasma membrane glycoprotein (Green et al., 1985). Half of each immunoprecipitate was digested with Endo H, which cleaves only "immature" NH₂-linked
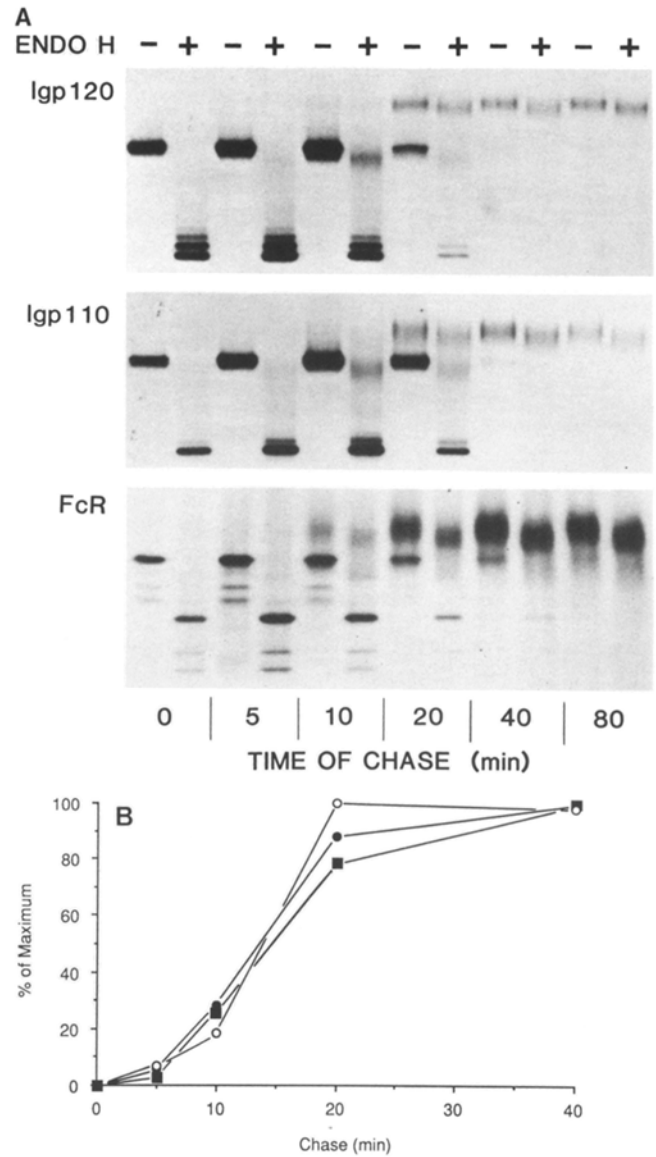
Figure 1. Kinetics of oligosaccharide processing. J774 cells labeled for 5 min with [35S]methionine were incubated in chase medium for the times indicated. At the end of the chase lgp120, lgp110, and Fc receptor (a plasma membrane protein) were sequentially immunoprecipitated from the detergent phase of Triton X-114 lysates. Immunoprecipitates were incubated without (−) or with (+) Endo H. (A) Autoradiographs of the SDS gels. (B) Quantitation of the mature molecular weight, Endo H–resistant antigens by scanning densitometry; ○, lgp120; ●, lgp110; ■, Fc receptor.

oligosaccharide chains that have not been processed in medial Golgi cisternae (Kornfeld and Kornfeld, 1985). The samples were then analyzed by SDS-PAGE and fluorography (Fig. 1 A).

At chase times of 0–10 min, the 90-kD precursors of lgp120 and lgp110 were digested by Endo H to 42- and 45-kD polypeptides, respectively. At longer chase times, the 90-kD precursor bands became less prominent and the mature, Endo H–resistant 120- and 110-kD bands began to appear. Immature Endo H–resistant forms of the antigens exhibiting slightly higher mobilities than the precursors, presumably
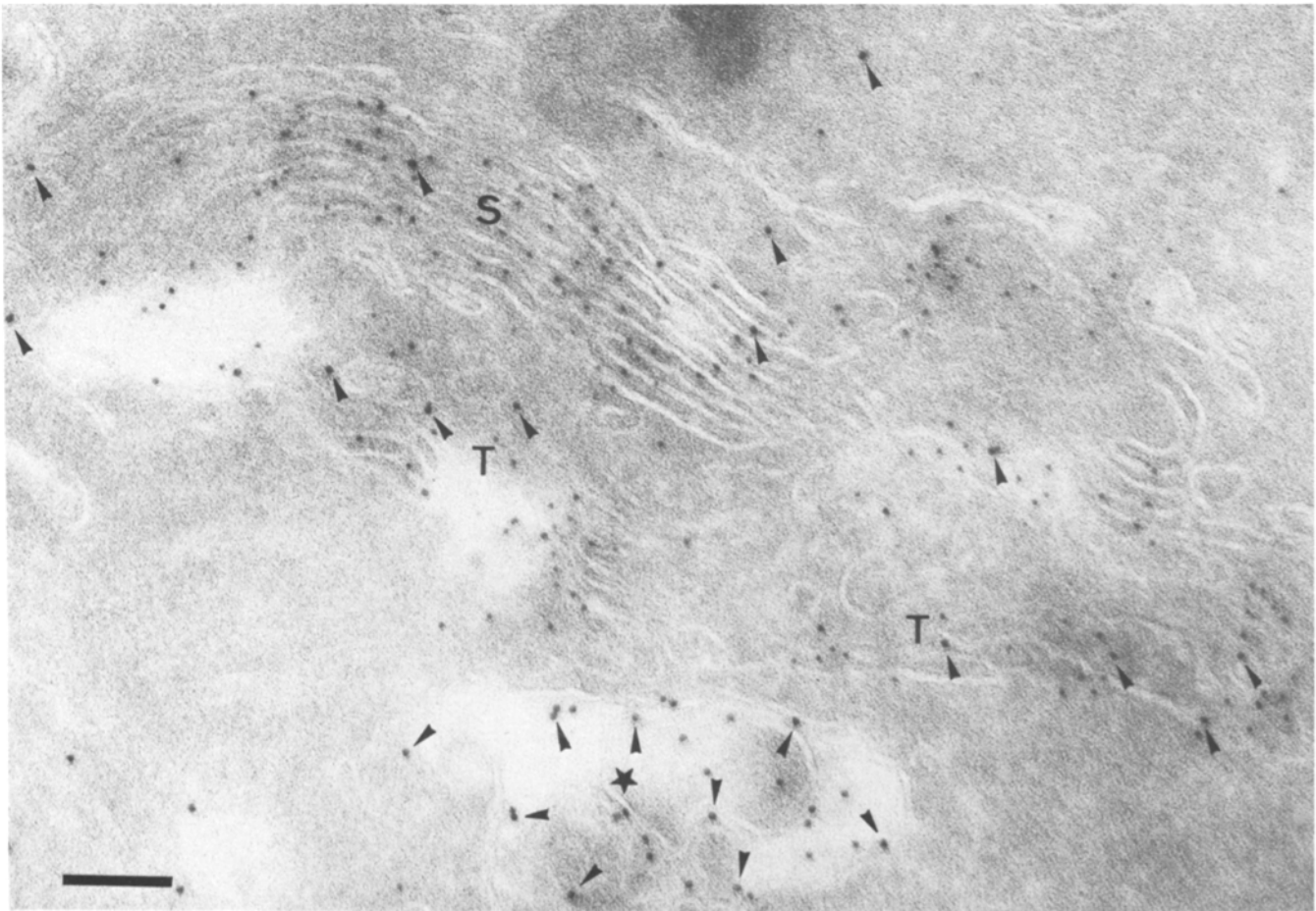
Figure 2. Localization of lgp120 and VSV G-protein in NRK cells. NRK cells were infected with the ts045 mutant of VSV for 4 h at 39.5°C, then shifted to 20°C for 2 h. Frozen thin sections were labeled with a monoclonal antibody against the cytoplasmic domain of VSV G-protein followed by rabbit anti–mouse IgG and 5-nm colloidal gold–protein A, and with antiserum against lgp120 followed by 9-nm colloidal gold–protein A *(arrowheads)*. The majority of the labeling is for VSV G-protein localized throughout the Golgi stacks *(S)* and the *trans* Golgi reticulum *(T)*. A structure which labeled exclusively with lgp120 antibody is indicated by the asterisk (*), and may represent a lysosome or lysosome precursor. Bar, 0.1 μm.

reflecting partially processed *medial* Golgi forms, were transiently detected (most prominent at 10 min of chase). Conversion of the 90-kD precursors to the 120- and 110-kD mature proteins was complete within 40 min of chase. Similar kinetics were observed for the Fc receptor, whose 53-kD Endo H–sensitive precursor was completely converted to the 60-kD mature form by 40 min of chase (Fig. 1 *A*).

These data were then quantified by scanning densitometry. To measure transport to the *trans* Golgi, we determined the appearance of labeled bands corresponding to the mature, galactose-containing species (Lewis et al., 1985; Green et al., 1985). Although both the precursor and mature forms of the Fc receptor were detected with equal efficiencies at all times of chase, the recoveries of mature lgp120 and lgp110 by immunoprecipitation decreased by 50%, suggesting that the antisera used had lower affinities for the mature antigens than for the precursors (or recognized a subpopulation of the mature antigens), or that a fraction of the molecules were rapidly degraded.[4] In either event, the mature lgp120 and

lgp110 molecules recognized by the antisera were quantitatively recovered in all experiments. Thus, to allow kinetic comparison of lgp and Fc receptor processing, we plotted the amount of fully mature antigen detected at various times of chase as a fraction of the maximum amount of mature antigen at the end of the experiment (80 min). As shown in Fig. 1 *B*, mature lgp110, lgp120, and Fc receptor began to appear after 5 min and then accumulated rapidly. The processing of each protein occurred with a half-time of 14 min and was nearly complete after 20 min.

In addition, we determined the rate of oligosaccharide processing in normal and influenza virus–infected NRK cells, using lgp120 and influenza HA as lysosomal and plasma membrane markers, respectively. The appearance of the mature forms of both proteins was found to be slightly faster

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4. We have subsequently found that antiserum against the lysosomal membrane protein LAMP-1 (Chen et al., 1985) recognizes lgp120 by immuno-

precipitation, and has equal affinity for the precursor and for the mature form (not shown). LAMP-1 antiserum immunoprecipitated 20 times more [3H]glucosamine-labeled antigen than lgp120 antiserum, but only twice as much [35S]methionine-labeled antigen (Green, S., C. Gabel, and I. Mellman, unpublished observations), suggesting that terminal glycosylation of this polypeptide yields two distinct populations of molecules, and that the lgp120 antiserum recognizes only one of them.
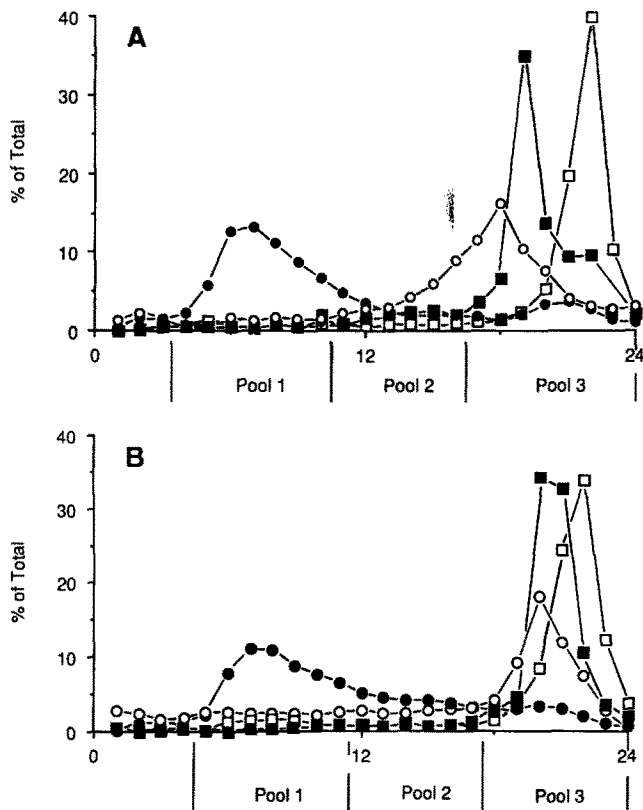
Figure 3. Subcellular fractionation of J774 cell and NRK cell organelles on Percoll density gradients. Fractions obtained after Percoll density gradient centrifugation of J774 cell or NRK cell homogenates were assayed for; ●, β-hexosaminidase (lysosomes); ○, fluid phase endocytic content (fluorescein-dextran in J774 cells, horseradish peroxidase in NRK cells) internalized during 5 min at 37°C (endosomes); ■, galactosyl transferase (*trans* Golgi apparatus); □, alkaline phosphodiesterase (plasma membrane). The fractions were combined as indicated below the marker profiles to obtain three pools for recovery of antigens by immunoprecipitation.

than in J774 cells, beginning within 5 min but with half-times of ∼11 min (not shown). These results demonstrate in two cell types that at least some lysosomal and plasma membrane proteins are transported from the RER through the Golgi apparatus at equivalent rates.

To determine whether lgp's and plasma membrane markers were transported through the same Golgi stacks, vesicular stomatitis virus (VSV) G-protein, another plasma membrane marker, and lgp120 were localized by immunoelectron microscopy in VSV-infected NRK cells at 20°C (Fig. 2). The Golgi stacks (*S*) and the *trans* Golgi network (*T*) labeled heavily with the VSV G-protein antibody. lgp120 was detected in the same Golgi stacks and in the *trans* Golgi network in lower amounts. lgp120 was also detected in vacuoles on the *trans* aspect of the Golgi that did not label with the anti–G-protein antibody (indicated by the "*"), and were thus not part of the *trans* Golgi network per se. Together with the kinetic data, these findings suggest that transport of lysosomal and plasma membrane proteins from the RER to the Golgi apparatus is not selective and that the sorting of these proteins does not occur at the level of the Golgi cisternae.

Transport of lgp's from Golgi Apparatus to Lysosomes

Next, to measure the transport of newly synthesized lgp120 and lgp110 from the Golgi apparatus to lysosomes, a quantitative assay was developed based on the isolation of lysosomes from other organelles on isosmotic Percoll density gradients followed by immunoprecipitation of antigens from the gradient fractions. The distributions of organelle markers in the Percoll density gradient fractions are shown for J774 cells (Fig. 3 *A*) and NRK cells (Fig. 3 *B*). Most of the lysosomal marker (β-hexosaminidase) sedimented as a single heavy density peak which was well separated from markers for Golgi apparatus (galactosyl transferase), plasma membranes (alkaline phosphodiesterase), and endosomes (horseradish peroxidase or FITC-dextran). The fractions were then pooled (see Fig. 3) for immunoprecipitation as follows: pool 1, containing the peak of lysosomal enzyme activity; pool 2, intermediate density membranes containing small overlapping amounts of all markers tested; and pool 3, low density membranes containing endosomes, Golgi apparatus, plasma membrane, and RER (see below). While two-thirds of the lysosomal enzyme activity were recovered in the densest pool (pool 1), some β-hexosaminidase activity was also present in the light fractions.

To identify the structures containing both lysosomal enzymes and lgp's, ultrathin frozen sections were prepared from membranes recovered from J774 cell and NRK cell gradient pools. The sections were labeled with antibodies to lgp120 or lgp110 followed by 9-nm protein A–gold, then with antibodies to β-glucuronidase followed by 5-nm protein A–gold, and examined by electron microscopy. In J774 cells, typical lysosomes from pool 1 (densest pool) contained membranes and a dense matrix, as seen in lysosomes in vivo, and were positive for both lgp110 (large gold) and β-glucuronidase (small gold) (Fig. 4 *A*). Lysosomes from pool 1 from NRK cells were similar in appearance, but had finer, more lamellar contents than J774 lysosomes (Fig. 4 *B*). In both cells, lgp labeling was generally restricted to the limiting membrane, as opposed to the membranous content. Although less common, two other structures were found in pool 1, especially in J774 cell fractions; tubular elements, which were positive for both lgp110 and β-glucuronidase (Fig. 4 *C*), and clear vacuoles, negative for lysosomal antigens, which nevertheless appeared to be in continuity with the more characteristic lysosomal structures (Fig. 4 *D*, also seen in Fig. 4, *A* and *C*). The latter may represent endosomal membranes that had just fused with lysosomes. The only other structures observed in pool 1 were a few mitochondria, which were unlabeled. Pool 2 (intermediate density, overlap pool) (Fig. 4 *E*) contained most of the mitochondria, some RER and weakly labeling vesicles, and very few heavily labeled structures, which were similar in appearance to those in pool 1. Pool 3 (light fractions) contained a variety of structures, including recognizable Golgi elements (Fig. 4 *F*) and RER. The relatively weak lgp labeling seen in pool 3 was associated with small vesicles. Large, heavily labeled structures were absent. These observations confirm the biochemical data showing that pool 1 was enriched in dense, hydrolase-rich lysosomes and depleted in all other organelles except mitochondria, and that pool 3 contained most of the remaining membranes, including Golgi apparatus, RER, and small vesicles showing weak labeling of lgp's. Therefore, we inter-

A
CHASE

0

20'

40'

60'

2h

4h

12h

1 2 3 i

POOLS

B
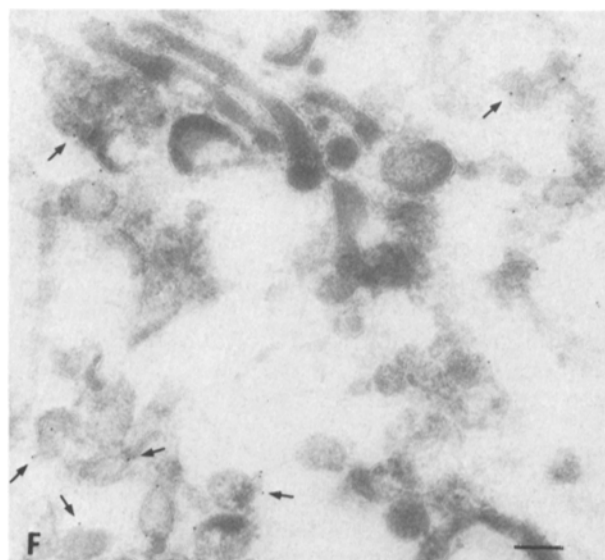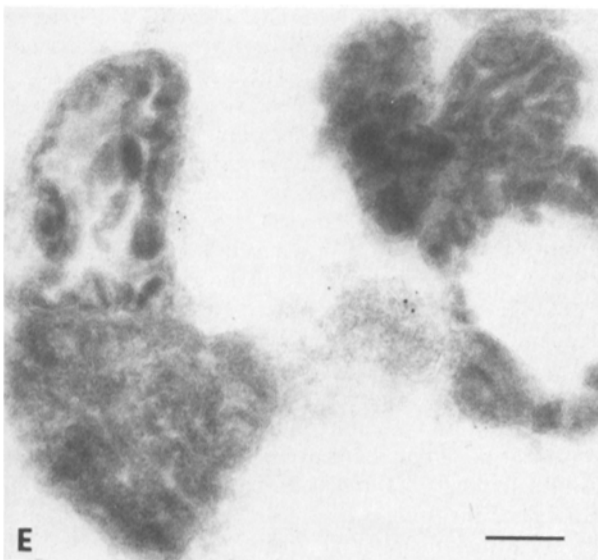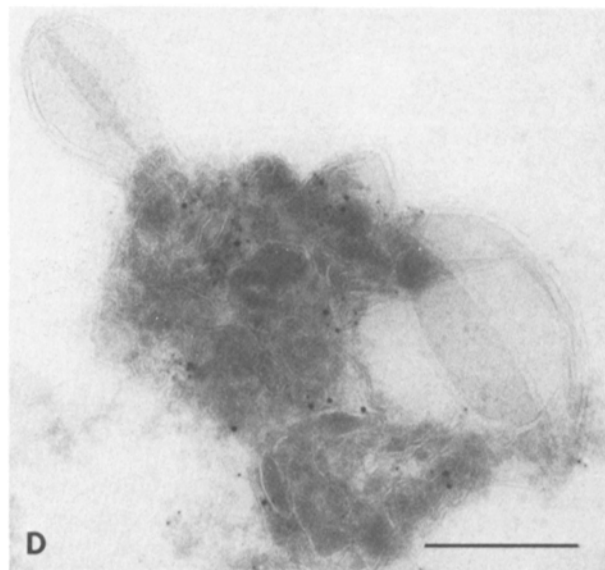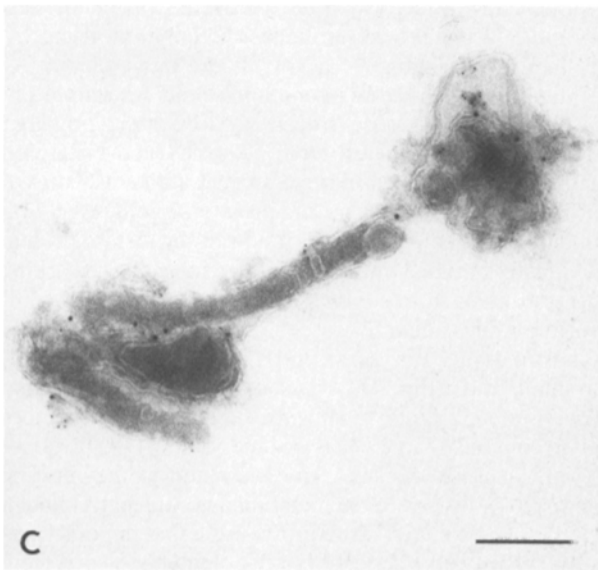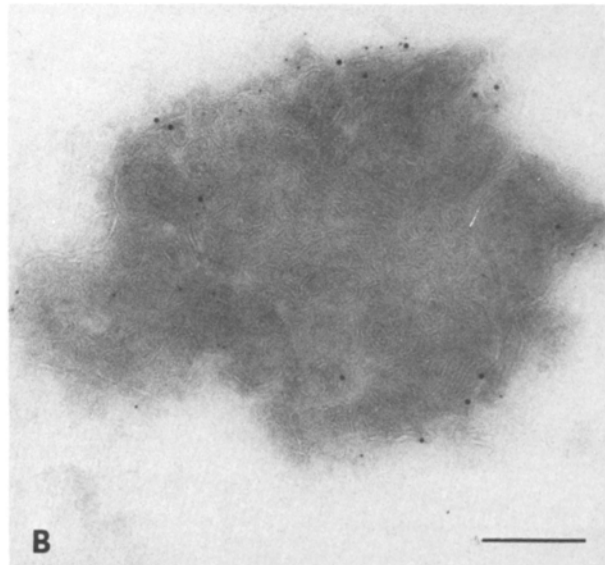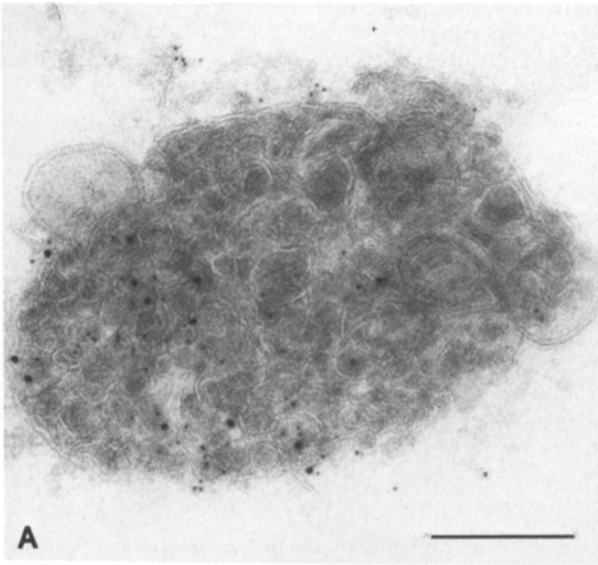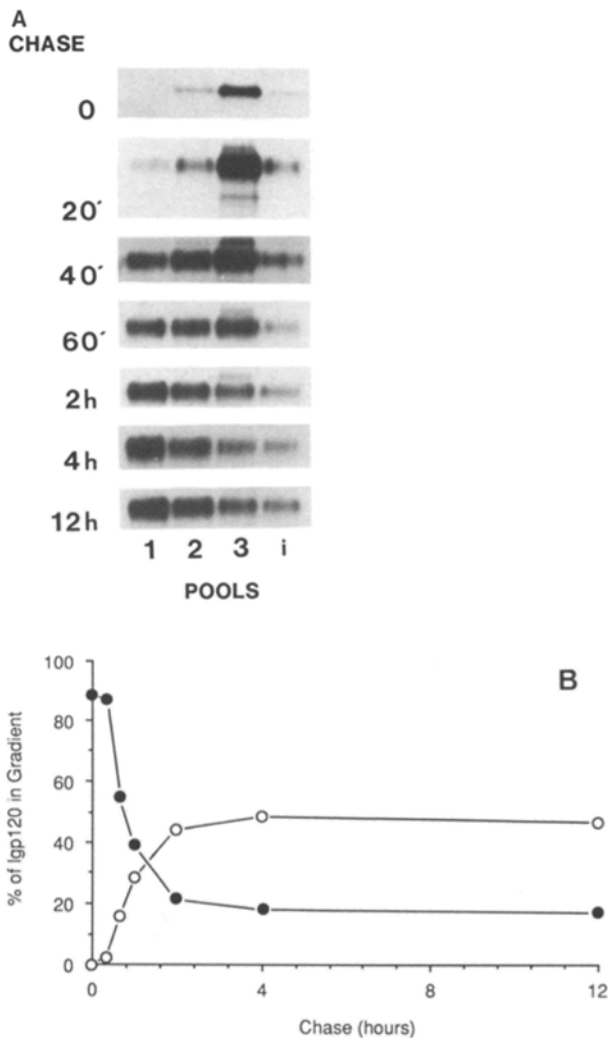
Figure 5. Distribution of lgp120 in NRK cell density gradient fractions. (A) NRK cells were pulse labeled for 10 min with [³⁵S]methionine and chased for the times indicated before fractionation on Percoll gradients. Gradient fractions were pooled as indicated in Fig. 3; pool 1 was the densest (lysosomal) pool, pool 2 the intermediate (overlap) pool, and pool 3 the least dense (peaks of all nonlysosomal markers). An internal control (i, 10% of the post nuclear supernatant) was included to measure antigen recovery from each gradient. The membranes in each pool were isolated by centrifugation in the presence of unlabeled carrier membranes, solubilized, and subjected to immunoprecipitatoin of lgp120 from NRK cells. Autoradiographs of the SDS gels are shown. (B) The antigens in the experiments shown in Fig. 5 A were quantified by scanning densitometry of the autoradiographs. The amount of antigen present in each gradient pool was calculated as a percentage of total antigen recovered from each gradient. The percentages of lgp120 in pool 3 (●), the least dense (nonlysosomal) pool, and in pool 1 (o), the densest (lysosomal) pool, are shown.

pret any lgp detected biochemically in pool 1 as having been present in lysosomes.

Transport of Newly Synthesized Lysosomal Membrane Proteins to Lysosomes

To obtain a kinetic description of the transport of newly synthesized lgp's to lysosomes, cells were pulse labeled for 10 min with [³⁵S]methionine, chased for 0–12 h, and fractionated on the Percoll density gradients described above. 10% of the postnuclear supernatant was used as a standard for determining the recovery of antigens from each gradient. The membranes in the pooled fractions were recovered by centrifugation with unlabeled carrier membranes, and labeled lgp120 and β-glucuronidase were sequentially immunoprecipitated.

When labeled NRK cells were analyzed with no chase incubation, lgp120 was recovered as the 90-kD precursor (presumably in the RER), primarily in the lowest density gradient pool (pool 3) (Fig. 5 A). After only 20 min of chase, mature lgp120 began to appear in lysosomes as judged by its recovery from the high density lysosomal fractions (pool 1). The fraction of lgp120 in the lysosomal pool increased with longer chase times, while the amount in the low density fractions (pool 3) decreased. Quantitation of these results (Fig. 5 B) indicated that delivery of lgp120 to lysosomes began after a 20-min lag and occurred with a half-time of 50 min. The appearance of labeled lgp120 in the high density fractions reached a plateau within 4 h of chase and corresponded exactly to the loss of antigen from the low density fractions.

Even after 12 h, the maximum percentage of labeled lgp120 found in pool 1 was no greater than 55% (Fig. 5 B), less than the percentage in pool 1 of β-hexosaminidase activity (66%) (Fig. 3 B). While this discrepancy may reflect a slow component in the rate of lgp120 transport to dense lysosomes, a more accurate estimate of the rate and extent of lgp delivery must take into account the rate of lgp degradation, since the duration of the experiment was long relative to the measured half-life of the protein. Assuming that lgp120 is degraded in lysosomes, the distribution of pulse-labeled antigen will not reflect the steady-state distribution of the entire antigen population. In both NRK and J774 cells, degradation of pulse-labeled lgp120 and lgp110 exhibited first order kinetics, with a half-time of 18–20 h for lgp120 in NRK cells and a half-time of 10 h for both antigens in J774 cells (not shown). The actual delivery of all of the pulse-labeled antigen to lysosomes (versus distribution of the remaining label) was thus estimated by adding back the percentage of the pulse degraded at each chase time to each of the three pools in proportion to their content of lysosomal enzyme activity (66% in pool 1). After making this correction, delivery of the pulse-labeled lgp120 to lysosomes in NRK cells was judged to be complete within 5 h.

Figure 4. Frozen thin sections of membranes recovered from J774 cell gradient fractions. Membranes from gradient pools obtained from unlabeled cells were fixed with 4% paraformaldehyde and recovered by centrifugation. Frozen thin sections were labeled with anti–lgp110 antibodies (J774 membranes) or anti–lgp120 antibodies (NRK membranes) followed by 9-nm protein A–gold, then with anti-β-glucuronidase antibodies followed by 5-nm protein A–gold. (A) A typical lysosome from J774 cell pool 1; (B) a typical lysosome from NRK cell pool 1; (C) a tubular lysosomal element from J774 cell pool 1; (D) a lysosome from J774 cell pool 1 with contiguous clear unlabeled membranes; (E) J774 cell pool 2, showing a small, weakly labeled vacuole. Note the low background staining over the mitochondria; F, single labeling for lgp110 in J774 cell pool 3 showing Golgi cisternae and weakly labeled vesicles (arrows). Bar, 0.2 μm.
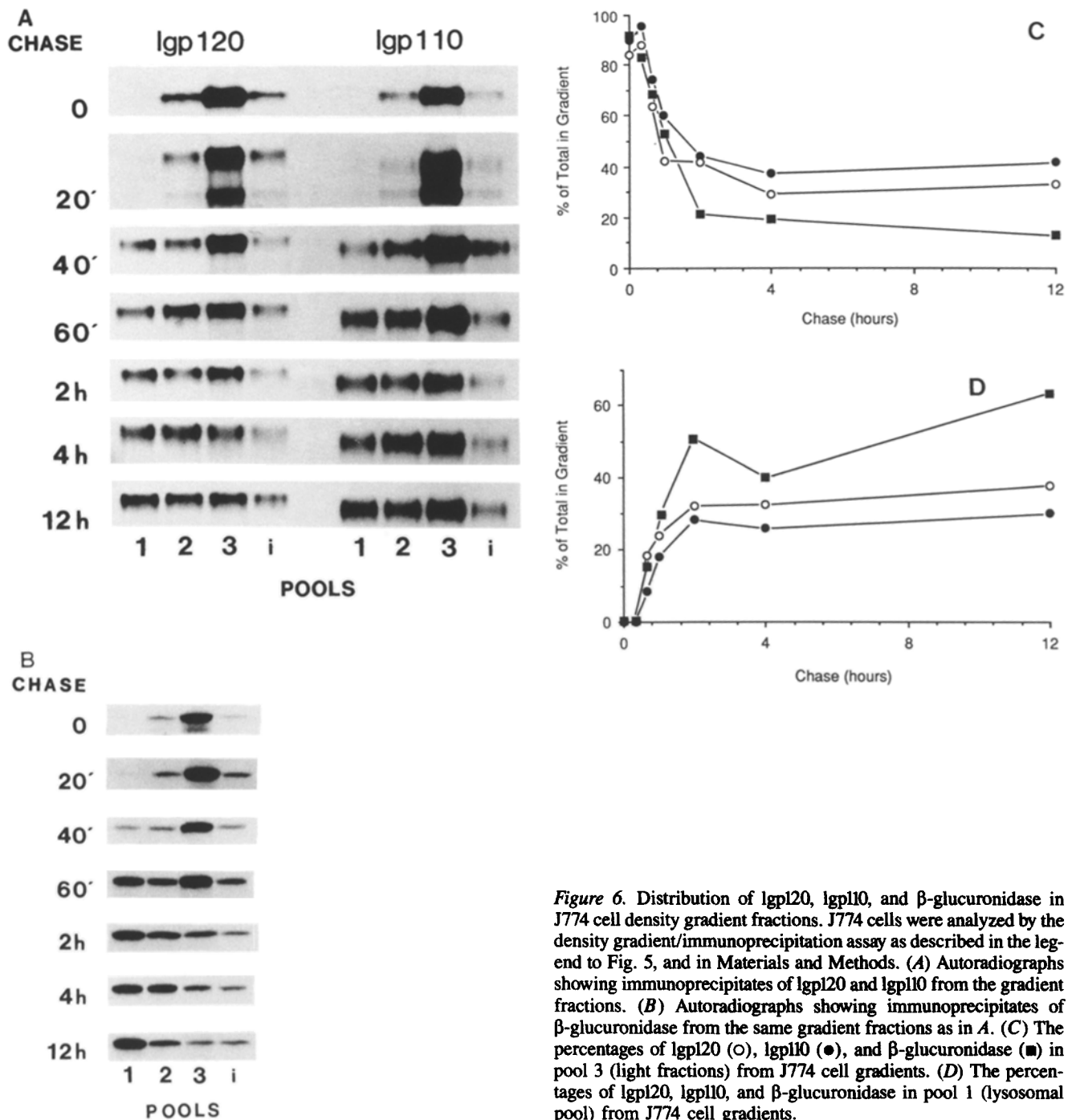
Figure 6. Distribution of lgp120, lgp110, and β-glucuronidase in J774 cell density gradient fractions. J774 cells were analyzed by the density gradient/immunoprecipitation assay as described in the legend to Fig. 5, and in Materials and Methods. (*A*) Autoradiographs showing immunoprecipitates of lgp120 and lgp110 from the gradient fractions. (*B*) Autoradiographs showing immunoprecipitates of β-glucuronidase from the same gradient fractions as in *A*. (*C*) The percentages of lgp120 (o), lgp110 (●), and β-glucuronidase (■) in pool 3 (light fractions) from J774 cell gradients. (*D*) The percentages of lgp120, lgp110, and β-glucuronidase in pool 1 (lysosomal pool) from J774 cell gradients.

The delivery of lysosomal enzymes to dense lysosomes in normal peritoneal macrophages (Brown and Swank, 1983) and fibroblasts (Gieselmann et al., 1983) takes considerably longer than the delivery of lgp120 to lysosomes in NRK cells. Although the distribution of immunoprecipitable β-glucuronidase in NRK cell fractions was similar to the distribution of lgp120, too little radioactivity was incorporated into the enzyme to permit a quantitative comparison of lgp and β-glucuronidase transport to lysosomes. Thus, lgp120, lgp110, and β-glucuronidase were sequentially immunoprecipitated from gradient fractions prepared using labeled J774 cells, which have a high content of lysosomal enzymes (Fig. 6, *A*

and *B*). Quantitation of the antigens from these autoradiographs showed that, as in NRK cells, the kinetics of transport of lgp's and β-glucuronidase were very similar (Fig. 6, *C* and *D*). We estimate by extrapolation of the curves in Fig. 6 *D* that first arrival of lysosomal proteins in pool 1 (lysosomal pool) occurred after a lag of slightly more than 20 min. The percentages of lgp120, lgp110, and β-glucuronidase all declined in the lowest density pool (pool 3) (Fig. 6 *C*) and increased in the lysosomal pool 1 (Fig. 6 *D*) with similar kinetics (half-time of ~45 min).

The fraction of lgp120 in pool 1 approached a lower plateau in J774 cells than in NRK cells. One possible reason for

this difference may be, as mentioned above, that lgp's are degraded more rapidly in J774 cells than in NRK cells. In addition, however, J774 cells (and certain other cells) exhibit large numbers of tubular lysosomes that are stabilized by association with microtubules (Robinson et al., 1986; Swanson et al., 1987). Breakage of tubular lysosomes by homogenization would presumably result in the formation of small vesicles with a very high surface to volume ratio relative to vacuolar lysosomes, and may therefore have a low buoyant density. These vesicles would also have a much higher ratio of lgp (membrane) to lysosomal enzyme activity (content) than vacuolar lysosomes. Indeed, treatment of J774 cells with nocodazole to disrupt microtubules before homogenization affected the distribution of lgp120 in J774 cell gradients but not in NRK cell gradients, without altering the distribution of lysosomal enzyme markers in either cell type (not shown). This suggests that the presence of tubular lysosomes in J774 cells contributes to the difference observed in the distribution of lgp120 between NRK and J774 cell gradients.

Kinetics of Membrane Protein Transport to Lysosomes and the Plasma Membrane

While the inability to detect lgp110 and lgp120 on the cell surface (Lewis et al., 1985) suggests that these proteins are not transported to the plasma membrane en route to lysosomes, a transient but obligatory passage cannot be eliminated. To help determine the pathway followed by newly synthesized lysosomal membrane proteins after leaving the Golgi apparatus, we next compared the kinetics of delivery of lysosomal and plasma membrane proteins to their final destinations.

Since both lysosomal and plasma membrane proteins studied here were transported from the RER to the *trans* Golgi apparatus at the same rate (Fig. 1), their kinetics of post-Golgi transport were conveniently compared simply by determining the rates of their arrival at lysosomes or the plasma membrane after pulse labeling. To detect the arrival of newly synthesized Fc receptor (in J774 cells) and influenza virus HA (in NRK cells) at the cell surface, the accessibility of pulse-labeled antigens to trypsinization of intact cells was measured (Green et al., 1985; Matlin and Simons, 1983). These two proteins have previously been shown to be transported from the RER to the cell surface as rapidly as any other examples studied thus far. The delivery of both the Fc receptor and HA to the plasma membrane was quantified by scanning densitometry and compared with the delivery of lgp120 to the densest (lysosomal) gradient pool (Fig. 7, *A* and *B*). The delivery of Fc receptor to the cell surface and of lgp120 to lysosomes in J774 cells both exhibited half times of ∼45 min (Fig. 7 *A*), while in NRK cells the accumulation of HA on the plasma membrane was slightly faster ($t_{1/2}$ = 30 min) than the accumulation of lgp120 in lysosomes ($t_{1/2}$ = 50 min) (Fig. 7 *B*). In addition, the lag times preceding the appearance of the labeled antigens at their respective destinations (determined by extrapolation of the curves in Fig. 7) were very similar for lgp120 and the plasma membrane markers in both cell types: slightly less than 20 min in NRK cells (Fig. 7 *B*) and 20–30 min in J774 cells (Fig. 7 *A*). Since both lysosomal membrane and plasma membrane antigens began to arrive at their final destinations after similar lag times and/or with similar half-times, it appears likely that
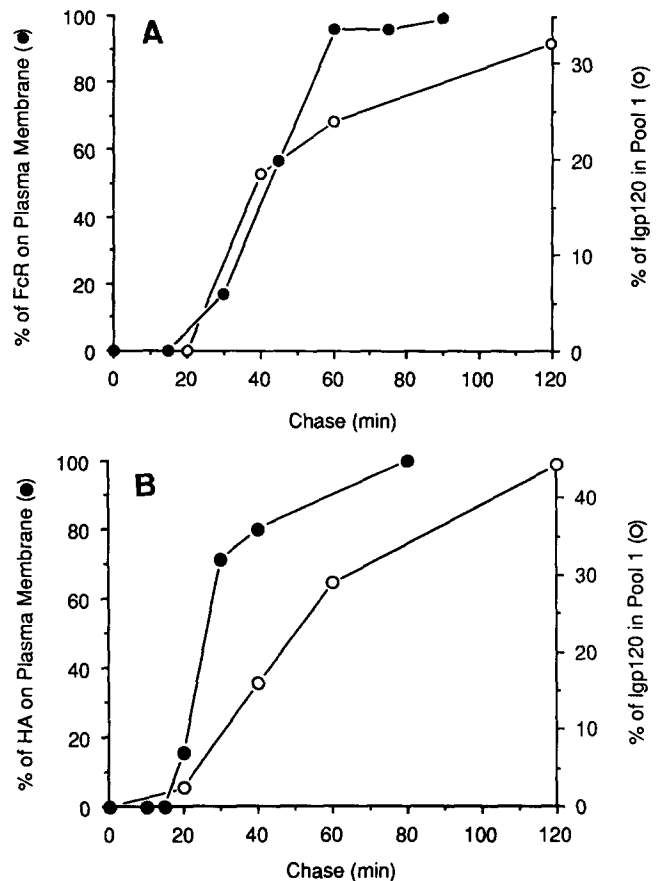

Figure 7. Kinetic comparison of the delivery of plasma membrane and lysosomal membrane proteins to their destinations in J774 and NRK cells. J774 cells or influenza virus–infected NRK cells were labeled for 5 min with [^{35}S]methionine and chased for the times indicated before trypsinization of the cell surface (see Green et al., 1985; Matlin and Simons, 1983; and Materials and Methods). Fc receptor immunoprecipitated from J774 cells, and influenza HA immunoprecipitated from NRK cells were analyzed by SDS gel electrophoresis and fluorography. The products of trypsin cleavage were quantitated by scanning densitometry of the autoradiographs, and the data obtained from each cell type plotted with the data from Fig. 5 and 6 for the appearance of lgp120 in the densest (lysosomal) gradient pool. (*A*) Quantitation of Fc receptor on the plasma membrane (●), and lgp120 in the lysosomal gradient pool (○) in J774 cells. (*B*) Quantitation of HA on the plasma membrane (●), and lgp120 in the lysosomal gradient pool (○) in NRK cells.

most of the newly synthesized lgp's are delivered to lysosomes without first being transported to the cell surface.

Kinetics of Transport from the Cell Surface to Lysosomes

Further kinetic evidence against a plasma membrane intermediate in the transport of lgp110 and lgp120 derives from the fact that once on the cell surface, lgp's would require still additional time to reach lysosomes via endocytosis. For example, the kinetics of Fc receptor-mediated endocytosis in J774 cells are such that receptor-bound ligands are first detected in lysosomes after a lag of 5–10 min and are delivered with a half-time of 90 min, as measured by recovery of ligand from density gradient fractions, by degradation of ligand, or by electron microscopy (Ukkonen et al., 1986). Analogous
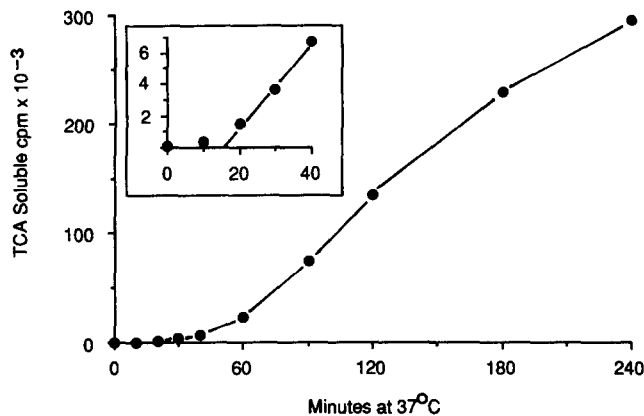
Figure 8. Delivery of Semliki Forest virus to lysosomes in NRK cells. NRK cells were incubated in the presence of [^{35}S]methionine-labeled Semliki Forest virus for 1 h at 0°C, washed, then warmed to 37°C. The appearance of TCA soluble radioactivity in the medium was measured at the times indicated. (*Inset*) Expanded scale of the first 40 min.

data were obtained for NRK cells using [^{35}S]Semliki Forest virus as a membrane-bound endocytic marker. As shown in BHK-21 cells (Marsh et al., 1983), transport to lysosomes is the rate-limiting step in this assay, since degradation of the viral proteins is extremely rapid. Degradation of viral proteins in NRK cells was first detected after a 15-min lag after internalization, and proceeded with a half-time of >120 min (Fig. 8).

Assuming that these values represent the fastest possible rates of transport to lysosomes via endocytosis, the observed rate of transport of newly synthesized lgp120 molecules to dense lysosomes ($t_{1/2}$ = 45–50 min) is inconsistent with obligatory transport to the plasma membrane and through the endocytic pathway. If lgp's could be transported as rapidly as the Fc receptor, HA, or VSV G-protein, reaching the cell surface would occur with a half-time of no less than 30–45 min (Fig. 7). From there, delivery of each cell surface cohort of lgp120 to lysosomes would exhibit a half-time of 90–120 min. Consequently, lgp's would reach lysosomes very slowly, with half-times on the order of several hours. These results strongly suggest that lgp120 was sorted from plasma membrane proteins at an intracellular site.

Intracellular Transport of lgp's at 20°C

Incubation of cells at reduced temperature selectively blocks specific membrane transport events, including the delivery of internalized endocytic tracers to lysosomes (Dunn et al., 1980; Marsh et al., 1983; Ukkonen et al., 1986) and the exit of viral membrane glycoproteins from the *trans* Golgi apparatus (Matlin and Simons, 1983; Griffiths et al., 1985). If lgp's were delivered to lysosomes via the plasma membrane and endosomes, incubation at reduced temperature should result in their accumulation in nonlysosomal structures containing plasma membrane proteins, endocytic tracers, or both.

To determine whether reduced temperature would inhibit delivery of lgp's to lysosomes, we examined the transport of newly synthesized lgp120 in NRK cells at 20°C, using the density gradient/immunoprecipitation assay (Fig. 9). Most

of the lgp120 was recovered as the mature form after 4 h at 20°C, indicating that it had been transported through the Golgi apparatus. However, only a small quantity of lgp120 (3%) was detected in the lysosomal pool (pool 1). The percentage of lysosomal enzyme activity in pool 1 was only slightly reduced in this experiment. Thus, reduced temperature almost completely inhibited the delivery of lgp120 to dense lysosomes for 4 h in NRK cells.

We next used immunocytochemistry to determine where lgp120 accumulated in NRK cells after 4 h at 20°C. Semliki Forest virus internalized at 20°C served to identify endosomal vacuoles in the sections (Marsh et al., 1983). As shown in Fig. 10, lgp120 was not detected in the vacuoles filled with virus particles, but was often concentrated in adjacent vacuoles lacking dense content, indicating that lgp120 did not accumulate in endosomes under these conditions. This further supports the hypothesis that lgp's are not normally transported to lysosomes via the cell surface, which would be expected to result in their accumulation in endosomes at 20°C.

Finally, we examined the transport of influenza HA to the cell surface at 20°C, to determine whether the block in the transport of viral membrane glycoproteins to the cell surface at 20°C is eventually overcome in NRK cells, as it is in BHK-21 cells (Griffiths et al., 1985). While HA was not accessible to extracellular trypsinization after 1 h at 20°C, by 2 h some HA was detectable on the cell surface, and by 4 h most of the HA synthesized during the 10-min pulse had been transported to the cell surface (Fig. 11). Thus, in contrast to the total inhibition of lgp120 transport to lysosomes, the inhibition of HA transport to the cell surface at 20°C was overcome in NRK cells in <2 h. The inability to detect lgp120 in endosomes by immunocytochemistry was apparently not due to a lack of transport of newly synthesized proteins to the cell surface at reduced temperature. These data further suggest that the pathways followed by HA from the Golgi apparatus to the plasma membrane, and by lgp120 from the Golgi apparatus to lysosomes are distinct, and that these pathways diverge at an intracellular site.

Discussion

In both the secretory and endocytic pathways, most membrane proteins avoid transport to lysosomes (Steinman et al., 1983), and if redirected to lysosomes by binding ligands or antibodies, are rapidly degraded (Mellman et al., 1987). Therefore, the recent discovery of a discrete class of mem-
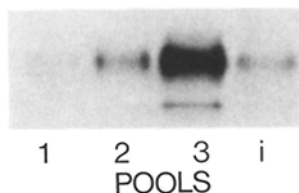

Figure 9. Distribution of lgp120 in density gradient fractions from NRK cells incubated at 20°C. NRK cells labeled with [^{35}S]methionine for 10 min were chased for 4 h at 20°C. The distribution of lgp120 synthesized during the pulse was analyzed using the density gradient/immunoprecipitation assay, as in Fig. 5. Pool 1 was the densest (lysosomal), pool 3 the least dense (nonlysosomal), and *i* indicates the internal control which contained 10% of the antigen loaded on the gradient. Approximately 3% of the labeled antigen was detected in the lysosomal pool.
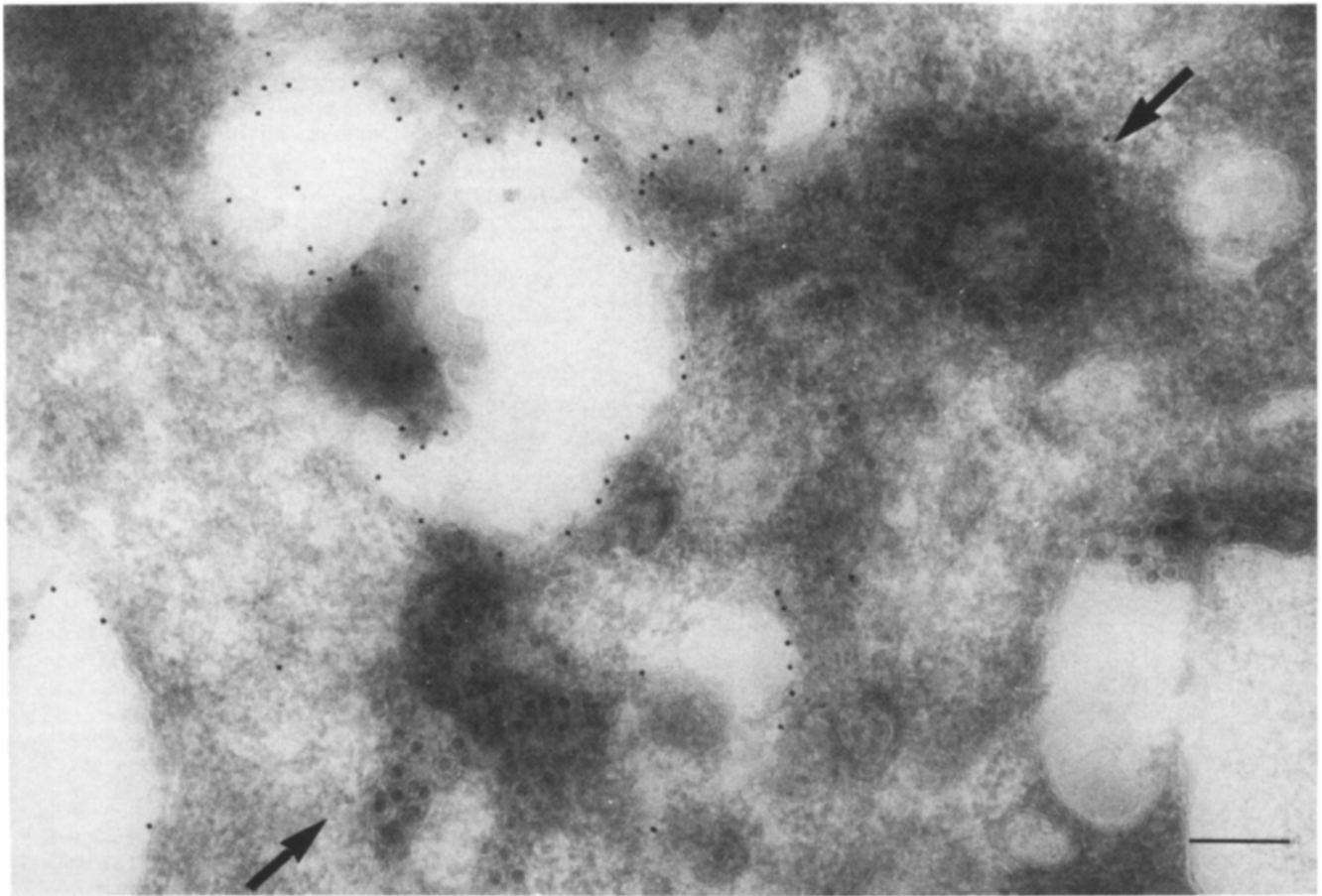
Figure 10. Localization of lgp120 and endocytic tracers in NRK cells incubated at 20°C. NRK cells incubated at 20°C for 4 h in the presence of 12 µg/ml Semliki Forest virus before fixation in 5% paraformaldehyde. Frozen thin sections were labeled with anti-lgp120 antibodies followed by protein A-gold (9 nm). lgp120 was not found in endosomes (*arrows*), which were heavily labeled by virus particles internalized at 20°C. Bar, 0.2 µm.

brane glycoproteins which preferentially[2] (Tougard et al., 1985; Lippincott-Schwartz and Fambrough, 1986) or exclusively[1] (Lewis et al., 1985; Chen et al., 1985; Barriocanal et al., 1986) accumulate in lysosomes raises new questions:
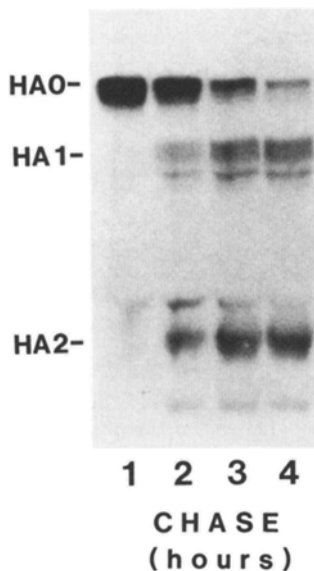
Figure 11. Transport of HA to the plasma membrane of NRK cells at 20°C. NRK cells infected with influenza virus for 3.5 h were labeled for 10 min with [³⁵S]methionine. The cells were chased at 20°C for the times indicated before trypsinization of the cell surface at 0°C, lysis, and immunoprecipitation of HA. An autoradiograph of the SDS gel is shown.

How are these proteins targeted to lysosomes? How do they resist degradation once they are there? What are their functions? We have begun to define the pathway of lysosomal membrane biogenesis by comparing the transport of newly synthesized plasma membrane and lysosomal membrane proteins through the cell.

Lysosomal membrane proteins could be transported to lysosomes via the cell surface then through the endocytic pathway, similar to plasma membrane receptors redirected to lysosomes by ligand or antibody binding. In this case, no segregation from other proteins in the biosynthetic pathway would be necessary. Their observed concentration in lysosomes would be a consequence of efficient internalization (or prevention of recycling once internalized) and resistance to degradation. Alternatively, transport could occur via a more direct intracellular route, suggesting a selective segregation, or sorting event, during biosynthetic transport. We have measured the rates of transport of lysosomal and plasma membrane proteins between the major vacuolar compartments. Our results indicate that sorting of newly synthesized lysosomal and plasma membrane proteins is an intracellular event.

Localization of the Sorting Event

There is no selectivity in the transport of lysosomal and

plasma membrane proteins before reaching the *trans* aspect of the Golgi apparatus. Transport of these proteins from the RER through the Golgi apparatus occurred with the same rapid kinetics. Immunoelectron microscopy revealed that lgp120 and VSV G-protein were found throughout the same Golgi stacks in NRK cells. Several considerations indicate, however, that lgp's and plasma membrane proteins are segregated from each other intracellularly before delivery to the cell surface.

Firstly, comparing the kinetics of transport of plasma membrane and lysosomal membrane proteins provides evidence that sorting of these two classes of proteins does occur before their transport to their final destinations. The lag times observed for the arrival of the first newly synthesized lgp's and plasma membrane proteins at their final destinations were very similar, while the transport of endocytic tracers to lysosomes from the cell surface shows a lag of at least 5 min in J774 cells (Mellman and Plutner, 1984; Ukkonen et al., 1986) and at least 15 min in NRK cells. If lysosomal proteins all passed through the plasma membrane en route to lysosomes, the lag times for delivery to lysosomes should have been longer than the lag times for delivery to the cell surface.

Second, the half-times of transport measured for lysosomal and plasma membrane proteins, and for endocytic markers, also indicate that transport of the bulk of lysosomal membrane proteins to lysosomes also does not include an obligatory trip to the plasma membrane. The half-time of transport of plasma membrane proteins to the cell surface was 30 min in NRK cells, 45 min in J774 cells. The half-time of delivery of membrane-bound endocytic probes to lysosomes was at least 90 min. If newly synthesized lgp's followed these two transport pathways in sequence, the half-time of delivery to lysosomes would be several hours, even assuming that each lgp120 molecule was internalized immediately after its appearance on the cell surface. The observed rate of delivery of lgp120 to lysosomes (half-time of 45–50 min) is inconsistent with this prediction. While it is possible that lysosomal membrane proteins may be transported more efficiently than plasma membrane proteins, it should be emphasized that the rates of HA and Fc receptor transport to the cell surface (and of Fc receptor-bound ligands and Semliki Forest virus to lysosomes) are as rapid as any plasma membrane proteins studied thus far. It is interesting, however, that the transport of newly synthesized plasma membrane proteins appears to be more synchronous than transport of lgp's (Fig. 7) and could mean that the pathway from the Golgi apparatus to the cell surface involves fewer vesicular intermediate steps than the pathway to lysosomes (see below).

In addition, D'Souza and August (1986) have measured the redistribution of newly synthesized lysosomal membrane protein (LAMP-1, immunologically related to lgp120[1]) on sucrose density gradients. They did not detect LAMP-1 in the low density region of the sucrose gradients containing plasma membranes at any time. Immunocytochemical studies have shown only a very small quantity of lgp120 on the plasma membrane (Zimmer, K.-P., unpublished observations), although lgp120 cannot be detected on the cell surface biochemically (Lewis et al., 1985). Taken together with our kinetic data, these studies further support the conclusion that the primary transport route followed by lgp110 and lgp120

does not pass through the cell surface. We, of course, cannot eliminate the possibility that a relatively small fraction of these two antigens are transported to the plasma membrane before reaching lysosomes. Interestingly, other lysosomal membrane proteins, notably a 100-kD glycoprotein, have been detected on the plasma membrane in significant quantities[2] (Tougard et al., 1985; Lippincott-Schwartz and Fambrough, 1986) in addition to lysosomes and endosomes. While the 100-kD protein is found on the ruffled border of osteoclasts, a portion of the plasma membrane that encloses an acidic extracellular space containing lysosomal hydrolases, lgp120 is detected only in intracellular lysosomes in these cells (Baron et al., 1985; Baron, R., personal communication). Thus, there may be two distinct classes of lysosomal membrane proteins that could follow different biosynthetic pathways.

The probable sorting site for biosynthetic traffic is the *trans*-most Golgi compartment (Farquhar and Palade, 1981), recently described as the *trans* Golgi network (Griffiths and Simons, 1986). Regulated secretory products are packaged into vesicles (granules) derived from this region (Farquhar and Palade, 1981; Kelly, 1985; Tooze and Tooze, 1986). Also, influenza HA and VSV G-protein, membrane glycoproteins that are targeted to the apical and basolateral plasma membrane domains, respectively, in polarized epithelial cells, are found in the same *trans* Golgi compartments (Rindler et al., 1984; Fuller et al., 1985), but are sorted before insertion in the plasma membrane (Matlin and Simons, 1984). Our results are consistent with the hypothesis that the sorting and targeting of lysosomal membrane proteins also occurs at the level of the *trans* Golgi network.

Lysosomal Biogenesis

By definition, a secondary lysosome contains the combined input of both the endocytic and biosynthetic pathways (Steinman et al., 1983). Determining the point at which the two pathways converge is presently a topic of considerable interest and debate (Geuze et al., 1985; Brown et al., 1986; Griffiths and Simons, 1986). Lysosomal enzymes are transported by mannose 6-phosphate receptors from the Golgi apparatus to a nonlysosomal acidic compartment before delivery to lysosomes (Gartung et al., 1985; Geuze et al., 1984, 1985; Brown et al., 1986). The receptor itself is recycled to the Golgi apparatus (Brown et al., 1986), thereby avoiding transport to lysosomes (Sahagian and Neufeld, 1983). In fact, the receptor appears to be rapidly degraded if it is rerouted to lysosomes by binding antibodies (von Figura et al., 1984).

Unlike most lysosomal enzymes, lgp110 and lgp120 have been found not to contain phosphate residues,[1] indicating that the mannose-6-phosphate recognition system is not involved in the targeting of these proteins. The observation that lgp120 and β-glucuronidase were delivered to dense lysosomes at the same rate is consistent with the hypothesis that lysosomal enzymes and membrane proteins are transported through the biosynthetic pathway in the same vesicular carriers. This would require sorting of lgp's and mannose 6-phosphate receptors at a post-Golgi, prelysosomal stage, since the receptors are not found in dense lysosomes (Sahagian and Neufeld, 1983; von Figura et al., 1984; Brown et al., 1986). Lysosomal enzyme precursors have been detected in clathrin-coated vesicles (Schulze-Lohoff et al., 1985), sug-

gesting that clathrin plays a direct role in transporting the mannose 6-phosphate receptor-bound enzymes from the *trans* Golgi network, similar to the internalization of receptors at the cell surface (Steinman et al., 1983; Brown et al., 1984). Perhaps clathrin-coated vesicles also participate in the retrieval of mannose 6-phosphate receptors from prelysosomal vesicles and their recycling to the Golgi apparatus.

Although by immunofluorescence mannose 6-phosphate receptors and lgp120 do not co-localize (Brown et al., 1986), preliminary electron microscopic immunocytochemical results indicate that lgp120 and mannose 6-phosphate receptors can be co-localized in a post-Golgi compartment that does not label with endocytic tracers at 20°C (Griffiths, G., H. Geuze, J. Slot, S. Kornfeld, and I. Mellman, unpublished observations), and is, by this criterion, not endosomal. Since mannose 6-phosphate receptors are not found in mature lysosomes, and newly synthesized lgp120 is not delivered to lysosomes at 20°C (Fig. 9), this low temperature biosynthetic compartment (containing both lgp120 and mannose 6-phosphate receptors) is apparently also not lysosomal. These structures are also negative for plasma membrane markers, and thus are not part of the *trans* Golgi network. Perhaps this compartment represents the delivery site for both biosynthetic and endocytic traffic to lysosomes, and does not fuse with endosomes (negative for lgp120 reactivity, Fig. 10) or lysosomes (negative for mannose 6-phosphate receptor reactivity) at reduced temperature. It is also possible that passage through this compartment is at least partly responsible for the relative asynchrony observed for lgp transport to lysosomes. Simultaneous ultrastructural and biochemical localization of lysosomal membrane proteins, lysosomal enzymes and their receptors, and endocytic tracers will be required to address this question.

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