Transport of the Lysosomal Membrane Glycoprotein lgp120 (lgp-A) to Lysosomes Does Not Require Appearance on the Plasma Membrane

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Abstract. We have used stably transfected CHO cell lines to characterize the pathway of intracellular transport of the lgp120 (lgp-A) to lysosomes. Using several surface labeling and internalization assays, our results suggest that lgp120 can reach its final destination with or without prior appearance on the plasma membrane. The extent to which lgp120 was transported via the cell surface was determined by two factors: expression level and the presence of a conserved glycine–tyrosine motif in the cytoplasmic tail. In cells expressing low levels of wild-type lgp120, the majority of newly synthesized molecules reached lysosomes without becoming accessible to antibody or biotinylation reagents added extracellularly at 4°C. With increased expression levels, however, an increased fraction of transfected lgp120, as well as some endogenous lgp-B, appeared on the plasma membrane. The fraction of newly synthesized lgp120 reaching the cell surface was also increased by mutations affecting the cytoplasmic domain tyrosine or glycine residues. A substantial fraction of both mutants reached the surface even at low expression levels. However, only the lgp120G→A7 mutant was rapidly internalized and delivered from the plasma membrane to lysosomes. Taken together, our results show that the majority of newly synthesized wild-type lgp120 does not appear to pass through the cell surface en route to lysosomes. Instead, it is likely that lysosomal targeting involves a saturable intracellular sorting site whose affinity for lgp's is dependent on a glycine–tyrosine motif in the lgp120 cytoplasmic tail.

Lysosomes contain a characteristic set of highly glycosylated membrane proteins. By cloning cDNAs from different species (Cha et al., 1990; Chen et al., 1988; Fambrough et al., 1988; Granger et al., 1990; Himeno et al., 1989; Howe et al., 1988; Noguchi et al., 1989; Viitala et al., 1988), they have been divided in two groups (lgp-A and lgp-B) that share a high degree of homology in their overall structure and sequence (Kornfeld and Mellman, 1989). Features common to all lgps include a large number of N-linked oligosaccharides in their luminal domain, a single transmembrane anchor, and a short 10–11-amino acid cytoplasmic tail. Despite their well-characterized biochemical properties, nothing is known about the functions of lgp's.

Another important but as yet unresolved issue concerns the pathways taken by lgp's from the Golgi complex to late endosomes and lysosomes. Transport to lysosomes might occur after the delivery of newly synthesized lgp's to the plasma membrane and subsequent endocytosis. Alternatively, lgp's might be sorted intracellularly and reach endosomes and/or lysosomes without appearing on the cell surface. Several considerations suggest that either or both possibilities may occur.

Kinetic studies of lgp120 (lgp-A) transport to lysosomes in normal rat kidney (NRK)[1] cells and mouse macrophages suggested directed delivery of lgps to lysosomes without initial transport to the plasma membrane (Green et al., 1987). This conclusion was based on the observation that lgp120 was transported from the Golgi complex to lysosomes as fast as plasma membrane proteins were transported from the Golgi complex to the cell surface; thus, the initial cohort of newly synthesized lgp molecules reached lysosomes too rapidly to have involved obligatory transport through the cell surface. A similar conclusion was reached by cell fractionation for mouse lamp-1 (lgp-A) in 3T3 cells (D'Souza and August, 1986). Conceivably, transport of lysosomal membrane components may follow the same pathway taken by newly synthesized lysosomal enzymes bound to the cation-independent mannose 6-phosphate receptor (MPR). In this case, several considerations indicate that enzyme–receptor complexes leave the *trans*-Golgi network (TGN) in clathrin-coated vesicles which deliver their contents to endosomes and, in turn, to lysosomes without reaching the cell surface (for review see Kornfeld and Mellman, 1989). A similar finding for lgp's would imply that these molecules too possess a sorting determinant that directs their transport upon exit from the TGN.

The alternative route, appearance on the cell surface followed by endocytosis, has been suggested to occur for at least a portion of the lgp molecules in chicken fibroblasts (LEP100, lgp-A; Lippincott-Schwartz and Fambrough, 1986), primary rat hepatocytes (LGP107, lgp-A; Furuno et al., 1989*a*,*b*), human leukemia cells (lamp-1, lgp-A; Mane et

1. *Abbreviations used in this paper*: LAP, lysosomal acid phosphatase; MPR, mannose 6-phosphate receptor; NRK cells, normal rat kidney cells; TGN, *trans*-Golgi network.

al., 1989), and MDCK cells (AC17 antigen; Nabi et al., 1991). Based on antibody binding, each of these studies found 2–3% of total lgp on the cell surface at steady state and that anti-lgp antibody could be internalized at 37°C. Accordingly, these studies did not, however, determine the fraction of newly synthesized lgp that reached lysosomes via the plasma membrane. While recent pulse-chase experiments suggest that in MDCK cells the bulk of at least one lgp-like molecule appears at the basolateral plasma membrane before reaching lysosomes, quantitative interpretation is limited by the fact that "surface appearance" was determined after antibody addition at 37°C and could not be distinguished from antibody binding in endosomes (Nabi et al., 1991).

Transport via the cell surface to lysosomes has also been observed in stably transfected BHK cells overexpressing human lysosomal acid phosphatase (LAP), a soluble lysosomal enzyme that is transported as a membrane protein precursor (Braun et al., 1989). However, kinetic data seem to indicate that LAP, which is not a member of the lgp120 family, is likely to reach lysosomes by a route distinct from lgp's. Whereas molecules such as lgp120 are transported to lysosomes with a $t_{1/2}$ of ~30 min, LAP reaches lysosomes with a $t_{1/2}$ of 5–6 h and is recycled repeatedly between the plasma membrane and endosomes en route.

A potential plasma membrane intermediate in lgp transport from the TGN to lysosomes is also indirectly implied by the fact that all lgp's, like many plasma membrane receptors (Davis et al., 1986; Jing et al., 1990; Lobel et al., 1989), contain conserved cytoplasmic domain tyrosine residues that may serve as signals for rapid endocytosis (Kornfeld and Mellman, 1989; Ktistakis et al., 1990). Thus, lgp-A or LAP mutants in which this tyrosine was altered accumulate on the cell surface and cannot be internalized (Peters et al., 1990; Williams and Fukuda, 1990). While the failure of rapid endocytosis might account for the inability of these mutants to reach lysosomes, it is also possible that the conserved tyrosine residue plays a role in intracellular sorting, perhaps reflecting the accumulation of lgp's in clathrin-coated buds in the TGN. Interestingly, all lgp's also contain a conserved glycine residue on the amino-terminal side of the tyrosine; the significance of this residue for targeting to lysosomes is unknown.

We have generated a series of stably transfected CHO cell lines selected for different expression levels of wild-type lgp120 or various cytoplasmic domain mutants. Using several independent assays to selectively monitor the kinetics of surface appearance vs. internalization of newly synthesized lgp's, we have been able to better define the extent to which lgp's pass through the plasma membrane en route to lysosomes and the factors that regulate cell surface transport. We have found that under normal conditions, most of the lgp is sorted intracellularly and reaches lysosomes without appearing on the plasma membrane. However, intracellular sorting can be rendered less efficient, and the degree of surface transport increased, by amplifying the level of lgp expression or by cytoplasmic domain mutations that more severely affect targeting from the TGN than endocytosis.

Materials and Methods

Cell Culture and Transfection

NRK fibroblasts were maintained in DME containing 5% FBS. CHO-K1 were grown in α-MEM supplemented with 10% FBS. CHO cell lines deficient in dihydrofolate reductase, DUKX-B11 and DG44, were obtained from Dr. L. Chasin (Columbia University, NY). The untransfected mutant cells were maintained in α-MEM supplemented with nucleosides and 10% FBS. All cells were grown at 37°C in 5% CO_2 in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin. CHO cells were stably transfected with 10 μg plasmid DNA/10-cm tissue culture dish of 30% confluency by the calcium phosphate precipitation technique (Wigler et al., 1979). On day 2 after transfection, cells were dissociated with trypsin/EDTA and plated in selective medium containing 600 μg/ml G418 (Gibco BRL, Gaithersburg, MD; CHO-K1) or in α-MEM without nucleosides supplemented with 10% dialyzed FBS (DUKX-B11 and DG44). To amplify expression, DUKX-B11 cells, transfected with pMT21 wild-type lgp120 DNA, were plated in selection medium containing 4 μg/ml folic acid and 0.1 μM methotrexate (Lederle Parenterals, Inc., West Grove, PA). Stable cell lines were obtained by subcloning of single colonies or by limiting dilution 14 d after transfection. For most experiments, cells were plated out 24–48 h before use.

Oligonucleotide-directed Mutagenesis and Plasmid Construction

Mutations in the cytoplasmic tail of lgp120 were generated by oligonucleotide-directed mutagenesis using the method of Kunkel (1985) as described in the Bio-Rad mutagene instruction manual (Bio-Rad Labs.-Chem. Div., Richmond, CA). Mutagenic 20-base oligonucleotides were used in which the codon for the cytoplasmic glycine residue located at position 7 was changed to an alanine residue or the codon for a tyrosine residue at position 8 was changed to a cysteine residue. The mutations were verified by dideoxynucleotide sequencing (Sanger et al., 1977) using the Sequenase kit (Un. States Biochem. Corp., Cleveland, OH) according to the manufacturer's instructions. EcoRI–HindIII fragments containing the cDNA coding for wild-type or mutant rat lgp120 were excised from the vectors M13mp19 or M13mp18. EcoRI linkers were added to the fragments which were then cloned into a single EcoRI site of the plasmid vector pFRCMneo (pFRSV derivative, map available upon request; Horwich et al., 1985) located downstream from the SV40 origin of replication or into pMT21 downstream from an adenovirus major late promoter (Kaufman, 1990*a*).

Metabolic Labeling, Surface Biotinylation, and Immunoprecipitation

Cells grown in 35-mm dishes to a confluency of ~75% were metabolically labeled for 12–16 h with 0.5 mCi/ml of each ^{35}S-translabel (Trans^{35}S-label; ICN Biomedicals Inc., Costa Mesa, CA) and [^{35}S]cysteine (Amersham Corp., Arlington Heights, IL) in cysteine- and methionine-free MEM (MEM Select-Amine Kit, Gibco BRL) supplemented with 10% dialyzed FBS, 20 mM Hepes, pH 7.4, 1.5 μg/ml methionine, and 2.4 μg/ml cystine. Monolayers were washed six times over a period of 20 min with ice-cold PBS^{++} before adding sulfo-NHS-SS-biotin (Pierce Chemical Co., Rockford, IL) at a concentration of 1.5 mg/ml in PBS^{++} for 30 min on ice. After washing six times with 50 mM glycine in PBS^{++}, the cells were lysed with 1% Triton X-100 in 100 mM sodium phosphate, pH 8.0, containing protease inhibitors (0.3 U/ml aprotinin, 1 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin) for 30 min on ice. Postnuclear supernatants were obtained after two centrifugation steps of 10 min at 500 *g* and 30 min at 16,000 *g* and precleared with *Staphylococcus aureus* Wood 46 (Zymed Labs Inc., South San Francisco, CA). All immunoprecipitations were carried out in two steps. The first round was performed with a polyclonal rabbit anti-rat lgp120 antiserum and protein A–Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). After washing and dissociation of antibody–antigen complexes by boiling in 0.5% SDS in PBS for 10 min and quenching with Triton X-100 to a final concentration of 0.4% SDS and 2% Triton X-100, a second round of precipitation was performed. To determine the amount of surface-biotinylated lgp120 vs. total lgp120, one third of the eluted material was reprecipitated with the antibody and protein A–Sepharose; the other two thirds were precipitated with streptavidin-agarose (Sigma Chemical Co., St. Louis, MO). SDS-PAGE was carried out on 8% acrylamide gels under reducing conditions. Dried gels were exposed to x-ray film and protein bands were quantified by digitization using a Visage 2000 image processor (Eastman Kodak Co., Rochester, NY) supported by a Sun Microsystems workstation.

Cell Surface Transport Assays

CHO cells grown in 35-mm dishes to a confluency of 85–95% were starved

in MEM lacking cysteine and methionine (MEM Select-Amine Kit, Gibco BRL) for 30 min in a 37°C incubator. Cells were then pulse labeled with 3 mCi/ml ^{35}S-translabel (Tran^{35}S-label; ICN Biomedicals Inc., Costa Mesa, CA) and [^{35}S]cysteine (Amersham Corp.) for 15 min at 37°C. After washing twice with prewarmed complete medium containing a twofold excess of cysteine (0.3 mM) and methionine (0.3 mM), cells were chased for different periods of time. For surface biotinylation, the same protocol was used at each time point as described above. Cell surface immunoprecipitation was performed at each time point by incubating cells on ice for 2 h in a 1:50 dilution of anti-lgp120 antiserum in complete medium. Cells were then washed six times with PBS^{++} containing 1% BSA over a 30-min period, and twice with PBS without BSA followed by detergent lysis. After preclearing of the postnuclear supernatants with fixed, protein A–negative *S. aureus* Wood 46, the lysates were divided into two aliquots. lgp that appeared on the surface and thus bound antibody was precipitated from two thirds of the lysate by the addition of protein A–Sepharose. Total lgp was precipitated by supplementing one third of the lysate with additional anti-lgp120 antiserum and protein A–Sepharose. After washing, the antibody–antigen complexes were dissociated by boiling in SDS and a second round of immunoprecipitation was performed as described above. Surface precipitation and biotinylation procedures recovered surface lgp120 at equivalent efficiencies.

Endocytosis Assays

To monitor internalization by immunofluorescence, living cells grown on glass coverslips were incubated with hybridoma culture supernatant containing the mouse monoclonal anti-rat lgp120 antibody LylC6 IgG (Lewis et al., 1985) for 2 h on ice or at 37°C. Cells incubated on ice were washed three times with PBS containing 10% goat serum to remove excess antibody before warming to 37°C for 30 min or 1 h to allow internalization. Cells were then fixed with paraformaldehyde-lysine-periodate (McLean and Nakane, 1974) for 1 h at room temperature after permeabilization with 0.01% saponin in PBS for 7 min and labeling with an affinity-purified, Texas red–conjugated goat anti–mouse IgG (Jackson ImmunoResearch Labs., Inc., West Grove, PA). After extensive washing with PBS containing 10% goat serum, cells were mounted in Mowiol and viewed with an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY). Pictures were taken with Tri-X Pan film (Eastman Kodak Co.) developed at 1,600 ASA. Quantitative and kinetic analysis of internalization were performed using ^{125}I-labeled LylC6 IgG. Cells grown in 35-mm dishes were incubated with ^{125}I-LylC6 at a concentration of 1 μg/ml in complete medium for different periods of time at 37°C or for 2 h on ice. To remove excess antibody, cells were washed six times with ice-cold PBS containing 1% BSA over a period of 30 min. Cells incubated with ^{125}I-LylC6 on ice were subsequently warmed to 37°C for various times. Cells were then cooled on ice and treated twice with 0.5 M acetic acid in 0.15 M NaCl, pH ~2.5, for 7 min. This procedure removed 75–85% of the ^{125}I-LylC6 bound at 4°C without subsequent warming. Internalized antibody was defined as the fraction of antibody initially bound that was resistant to removal by the low pH wash. Specific internalization was determined by subtracting the amount of ^{125}I-LylC6 bound in the presence of a 150-fold excess of unlabeled LylC6 IgG. Endocytosis of metabolically labeled cells was analyzed using the protocol for surface biotinylation with sulfo-NHS-SS-biotin as described above. After quenching excess biotin, cells were incubated at 37°C in complete medium for 30 min or 1 h. Cells were then cooled on ice and treated three times for 10 min with 50 mM glutathione in 0.075 M NaCl, 0.01 M EDTA containing 1% BSA and 0.075 N NaOH at 4°C (Bretscher and Lutter, 1988). Excess glutathione was quenched with 5 mg/ml iodoacetamide in PBS before cell lysis. Glutathione treatment removed 95–100% of the biotinylated lgp120. Biotinylated lgp was precipitated as described above. Internalization was defined as the amount of lgp120 immunoprecipitated after glutathione treatment compared to lgp120 immunoprecipitated from a nontreated control. Quantitation of the glutathione-resistant lgp120 was performed by digital densitometry.

Immunofluorescence Microscopy

Cells were plated out on glass coverslips 2 d before the experiment and grown to a confluency of 30%. To detect rat lgp120, a polyclonal rabbit anti-rat lgp120 antiserum or the mouse monoclonal anti-rat lgp120 antibody LylC6 was used (Lewis et al., 1985). For labeling of hamster lgp95, the mouse monoclonal anti-hamster antibody E9A IgG was used (Schmid, S., R. Fuchs, and I. Mellman, unpublished procedure). An affinity-purified, polyclonal rabbit antipeptide antibody specific for the 11-amino acid cytoplasmic domain of wild-type lgp-A was used for double-labeling experiments. For surface labeling, living cells were incubated with the primary antibody diluted in medium containing 10% FBS and 20 mM Hepes, pH 7.4, or directly with the hybridoma culture supernatant. After washing off excess antibody with PBS containing 10% goat serum, cells were fixed with paraformaldehyde-lysine-periodate (McLean and Nakane, 1974) and incubated with an affinity-purified fluorescent secondary antibody. To localize intracellular antigen, cells were permeabilized with 0.01% saponin in PBS for 7 min at room temperature before adding the antibody. Coverslips were mounted in Mowiol and viewed with an Axiophot microscope. Pictures were taken with Tri-X Pan film and developed at 1,600 ASA.

Results

Since transient expression of cDNAs yields highly variable amounts of protein and can alter intracellular transport due to lytic effects of viral-based expression systems, we generated stable, lgp120-expressing cell lines using nonviral plasmid vectors. CHO cell lines expressing different amounts of the transfected protein were obtained by using two different expression vectors with or without drug-induced amplification. pFRCMneo, a cytomegalovirus-based derivative of pFRSV (Miettinen et al., 1989) containing the neomycin resistance gene, yielded expression levels of the transfected lgp similar to or lower than the amount of lgp120 produced endogenously by NRK cells. The second vector, pMT21, utilizes the adenovirus major late promoter (Kaufman, 1990*a*). Since both vectors contained a wild-type dihydrofolate reductase gene as a selectable marker, expression could be enhanced by growing the cells in the presence of increasing concentrations of methotrexate (Kaufman, 1990*b*; Miettinen et al., 1989). pMT21-transfected clones were chosen that expressed the transfected wild-type lgp120 over a 1 to >15-fold range compared to NRK lgp120. Expression was quantified by metabolic labeling, followed by quantitative immunoprecipitation, SDS gel electrophoresis, and computer-assisted digitization of the autoradiograms.

Wild-Type lgp120 Can Be Detected on the Cell Surface Only in High Expressing, Transfected Cells

The distribution of transfected rat wild-type lgp120 in CHO cells was first monitored by immunofluorescence microscopy and compared with endogenous rat lgp120 in NRK cells. For cell surface staining, live cells were incubated with a rat-specific anti-lgp120 antiserum on ice followed by fixation and labeling with a fluorescent secondary antibody. Intracellular lgp120 was visualized after fixation and permeabilization of the cells. As shown, NRK cells (Fig. 1, *A* and *B*), and transfected CHO cells expressing low levels of lgp120 (Fig. 1, *C* and *D*), did not have detectable amounts of the protein on the cell surface. However, in transfected CHO cells expressing higher amounts of the protein, cell surface staining was clearly visible (Fig. 1, *E* and *F*).

To quantify the fraction of total rat lgp on the plasma membrane at steady state, surface lgp molecules were selectively derivatized by the membrane-impermeant probe sulfo-NHS-SS-biotin. Cells were labeled to equilibrium with [^{35}S]cysteine and [^{35}S]methionine followed by surface biotinylation on ice. After cell lysis, total lgp was precipitated with a polyclonal antibody specific for rat lgp120 and the resulting immunoprecipitates were divided into two aliquots. Biotinylated "surface lgp" was precipitated from one aliquot corresponding to two thirds of the lysate using immobilized streptavidin; "total lgp" was precipitated from one third by a second round of immunoprecipitation. Total and

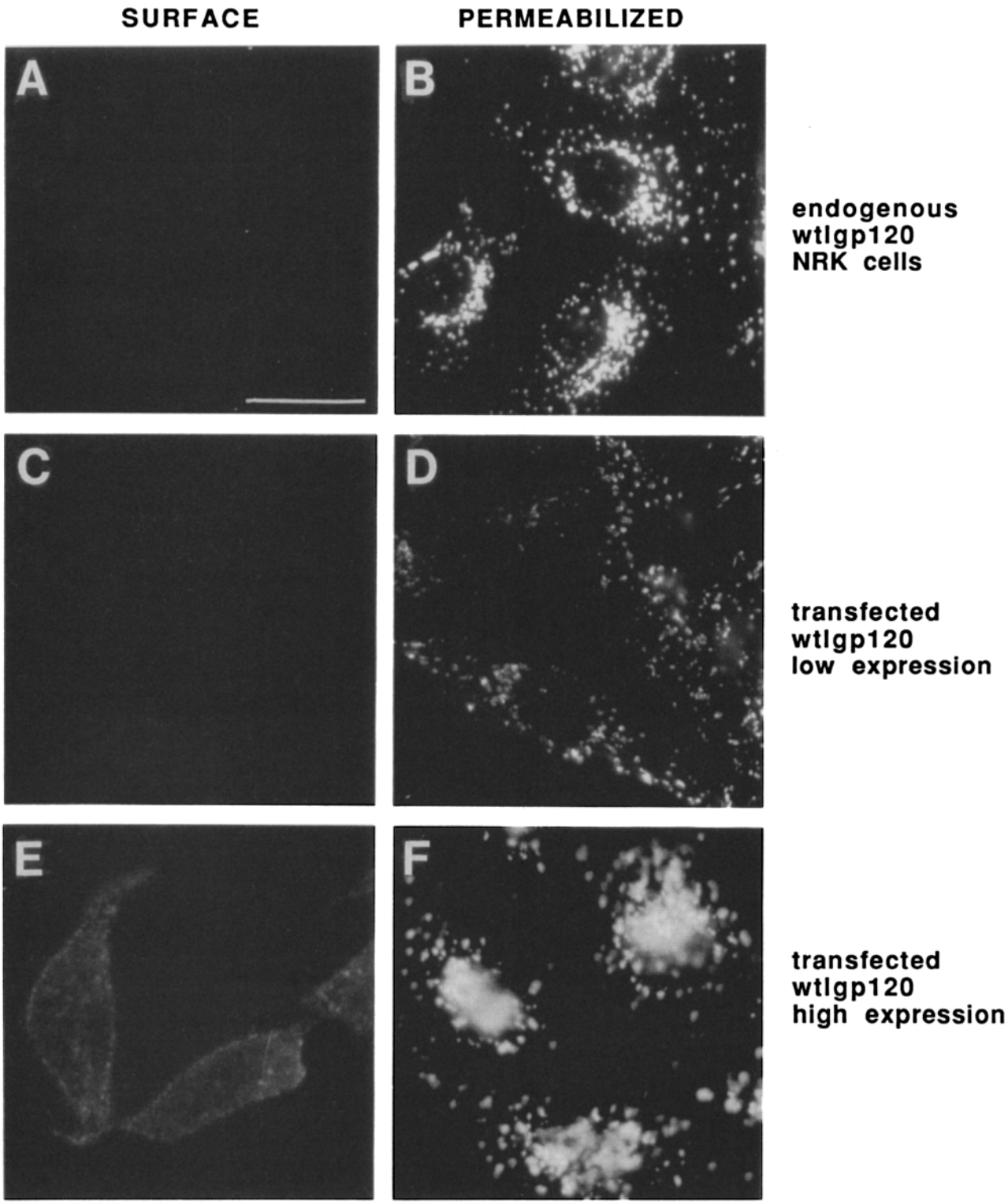

Figure 1. Immunofluorescence localization of rat wild-type lgp120 in NRK and transfected CHO cells. For surface labeling, living cells were incubated with a rabbit anti-rat lgp120 antiserum for 2 h on ice before fixation. For staining of total (surface + intracellular) rat lgp120, cells were fixed and permeabilized before labeling with a rabbit anti-rat lgp120 antiserum. Affinity-purified, Texas red-conjugated IgG was used as a secondary antibody. (*A*) Surface labeling of endogenous lgp120 in NRK cells. (*B*) Total lgp120 in permeabilized NRK cells. (*C*) Surface labeling of transfected, low expressing CHO cells. (*D*) Total rat lgp120 in transfected, low expressing CHO cells. (*E*) Surface labeling of transfected, high expressing CHO cells. (*F*) Total rat lgp120 in transfected, high expressing CHO cells. Relative levels of lgp120 expression are given in Fig. 2. Bar, 5 μm.

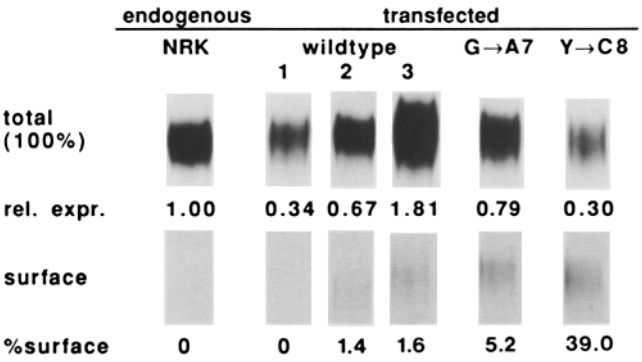

Figure 2. Cell surface distribution of wild-type and mutant lgp120. Equal numbers of cells were labeled with [^{35}S]methionine and [^{35}S]cysteine overnight, washed, and subjected to surface biotinylation using sulfo-NHS-SS-biotin for 30 min on ice. Biotinylated cells were lysed in Triton X-100, and total lgp120 was immunoprecipitated with anti-rat lgp120 antiserum, and the immune complexes were dissociated from the immunoadsorbent by boiling in SDS. Samples were divided in aliquots of one third for reprecipitation of total lgp120 with anti-lgp120 antiserum and immobilized protein A (*total*), and two thirds for precipitation of biotinylated lgps with streptavidin-agarose (*surface*). Quantification was done by computer-assisted digitization of autoradiograms using a Visage 2000 image processor (Eastman Kodak Co.). Total ^{35}S-labeled lgp120 is expressed relative to the amount of endogenous NRK lgp120 (defined as a relative expression level = 1). Surface biotinylated ^{35}S-labeled lgp120 was expressed as the percentage of total ^{35}S-labeled lgp120, after correcting for differences in aliquot size.

biotinylated lgp were quantified after SDS-PAGE and digital analysis of resulting autoradiograms. To provide a basis for comparison, total amounts of rat lgp120 in the transfected cell lines were expressed relative to endogenous NRK lgp120. These results are shown in Fig. 2.

In agreement with the fluorescence microscopy, no biotinylated rat lgp120 was detectable on the surface NRK cells or CHO cells expressing low levels (clone 1, relative expression = 034). However, in cell lines expressing higher amounts of the transfected lgp (clones 2 and 3), the biotinylated rat lgp120 accounted for 1.5% of the total immunoprecipitable ^{35}S-labeled lgp120.

Endogenous lgp-B Also Reaches the Cell Surface in CHO Cells Overexpressing Rat lgp120

The increased amounts of surface lgp120 on cells expressing higher levels of the transfected protein might reflect an enhanced ability to detect a small but constant fraction of protein on the plasma membrane. However, since surface lgp120 was not observed in NRK cells which expressed more of the protein than some of the CHO transfectants (e.g., Fig. 2, clone 2), sensitivity of the biotinylation assay was not a limiting factor. Alternatively, surface expression might have resulted from the saturation of a intracellular sorting site needed for delivery of lgp120 from the TGN to endosomes or lysosomes. If true, such a situation might also be expected to result in the partial missorting of endogenous lgp's in CHO cells which presumably compete for the same, limited capacity sorting apparatus. To test this possibility, we examined the distribution of lgp95 (lgp-B) in cells expressing different amounts of the transfected protein.

In nontransfected cells, lgp95 is normally undetectable on the cell surface by immunofluorescence or biochemical labeling (not shown). Similarly, in cells expressing low levels of transfected lgp120, neither the transfected lgp120 (Fig. 3 *A*) nor lgp95 was detected on the plasma membrane (Fig. 3 *B*); both transfected and endogenous lgp's colocalized intracellularly (Fig. 3, *C* and *D*). However, in cells expressing levels of rat lgp120 that lead to the appearance of the transfected protein on the cell surface, plasma membrane lgp95 was also detected (Fig. 3, *E* and *F*). Labeling of lgp95 on the cell surface was not the result of cross reaction with secondary antibodies, since similar results were obtained using single labeling. Moreover, the anti-lgp95 antibody was found not to react with the transfected rat lgp120, since it failed to detect rat lgp120 in permeabilized NRK cells (not shown) and did not detect the precise pattern observed using rabbit anti-lgp120 in transfected cells (Fig. 3, *G* and *H*). In summary, these results suggest that upon overexpression of lgp120 in transfected cell lines, both the transfected and endogenous lgp may be missorted to the cell surface.

Differential Effect of Cytoplasmic Tail Mutations on Lysosomal Transport

Whether lgp's are delivered to lysosomes directly from the TGN or after internalization from the cell surface, it is clear that they must contain one or more sorting determinants that specify their intracellular transport. At least one important determinant is in the lgp cytoplasmic domain, since replacement of the cytoplasmic tail tyrosine residue at position 8 (Y8) of human lamp-1 (lgp-A) has been shown to result in its transport to the cell surface in transfected COS cells (Williams and Fukuda, 1990). However, although this tyrosine is needed for endocytosis, it is unclear whether it also has a role in targeting from the Golgi complex. Another factor that may play a role in lgp transport is the glycine residue at position 7 (G7) that is also conserved in all known lgp's.

We investigated the role of the glycine–tyrosine motif in lysosomal targeting by generating stable CHO cell lines expressing the cytoplasmic tail mutants lgp120Y→C8 and lgp120→A7 at relatively low levels to exclude missorting due to overexpression. Nevertheless, in both cell types, the mutant lgp's were found at the plasma membrane after antibody labeling of live cells at 4°C by immunofluorescence (Fig. 4, *A* and *C*). While little of the tyrosine mutant was found in intracellular vesicles (Fig. 4 *B*), a significant fraction of the glycine mutant gave a typical lysosome-like staining pattern (Fig. 4 *D*). We next quantified the extent of plasma membrane expression of both mutants and found that lgp120Y→C8 was almost eightfold more accessible to surface biotinylation than lgp120G→A7, 39% vs. 5.2% of the amount of immunoprecipitated lgp120, respectively (Fig. 2). Both of these values were considerably higher than obtained for even high expressing wild-type lgp120 transfectants (1.5%).

To determine whether the intracellular lgp120G→A7 colocalized with endogenous lgp-A, permeabilized cells were stained using the rat-specific mouse monoclonal antibody Ly1C6 IgG and an affinity-purified rabbit antitail peptide lgp-A antibody. While the antipeptide antibody detected endogenous lgp-A in CHO cells, it did not react with either cytoplasmic domain mutant. As shown in Fig. 5, the intracellular staining due to lgp120G→A7 (anti-rat lgp120) exhibited significant colocalization with staining due to endogenous CHO cell lgp-A (antitail lgp-A). A fraction of lgp120G→A7

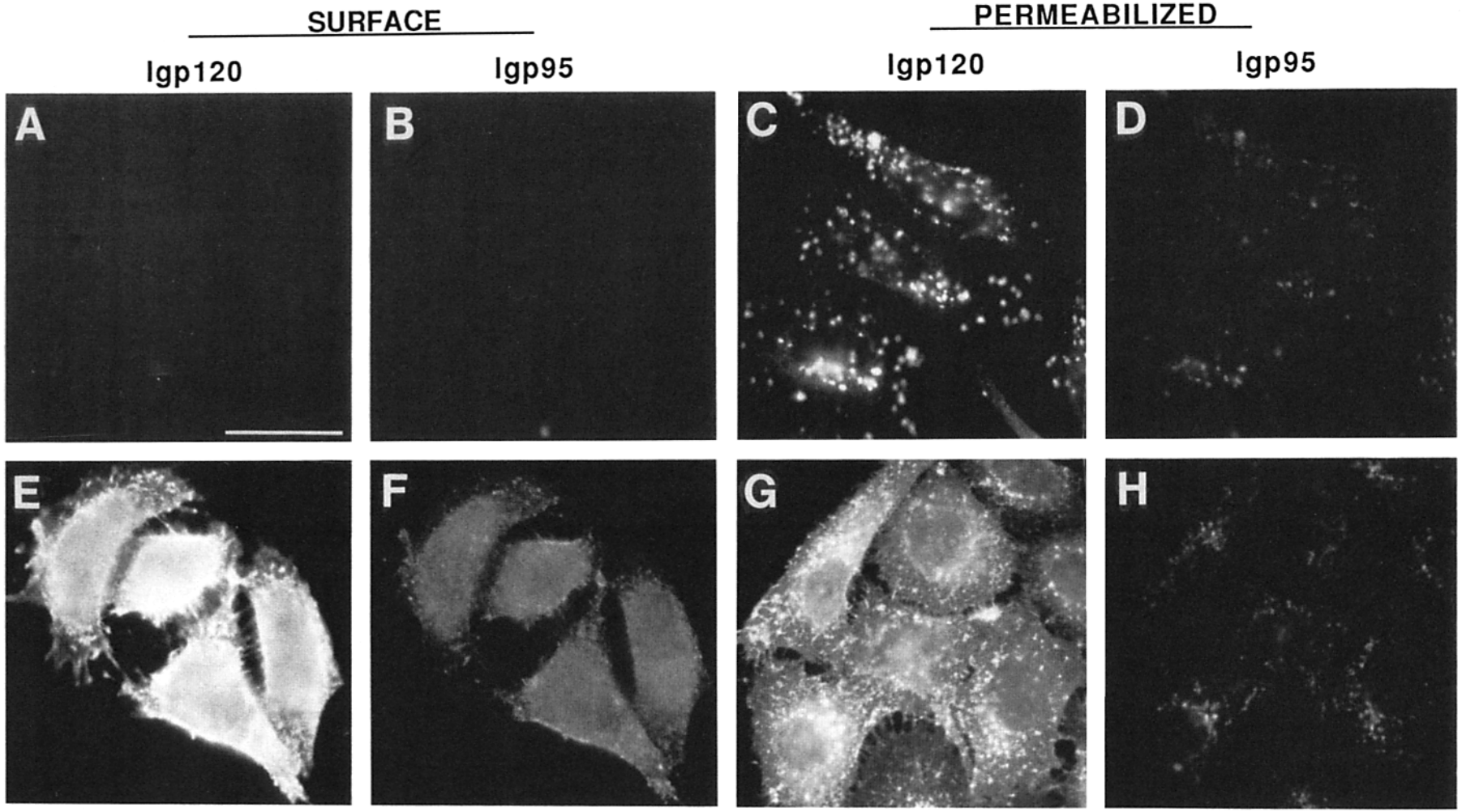

Figure 3. Distribution of endogenous hamster lgp95 (lgp-B) and transfected rat lgp120 in CHO cells. CHO cells producing either low (*A–D*) or high (*E–H*) levels of rat lgp120 were analyzed for the surface vs. intracellular distributions of the transfected rat lgp120 (lgp-A) and endogenous hamster lgp95 (lgp-B). To detect lgp's present on the plasma membrane, live cells were double labeled at 0°C with a rat-specific rabbit anti-lgp120 antiserum (*A* and *E*) and a hamster-specific mouse monoclonal anti-lgp95 antibody (E9a IgG) (*B* and *F*). Surface lgp120 and lgp95 was only detected on CHO cells overexpressing the transfected rat lgp120 (*E* and *F*). For labeling of total lgp's, cells were fixed and permeabilized before incubation with the anti-rat (*C* and *G*) or hamster (*D* and *H*) antibodies. Affinity-purified fluorescein-conjugated goat anti-mouse and Texas red–conjugated goat anti-rabbit IgGs were used as secondary antibodies. In control experiments in which the anti-lgp120 vs. anti-lgp95 were used alone and/or in combination with the incorrect second antibody, we established that neither the first nor second antibodies exhibited cross-reactivity under the conditions used here. Bar, 5 μm.

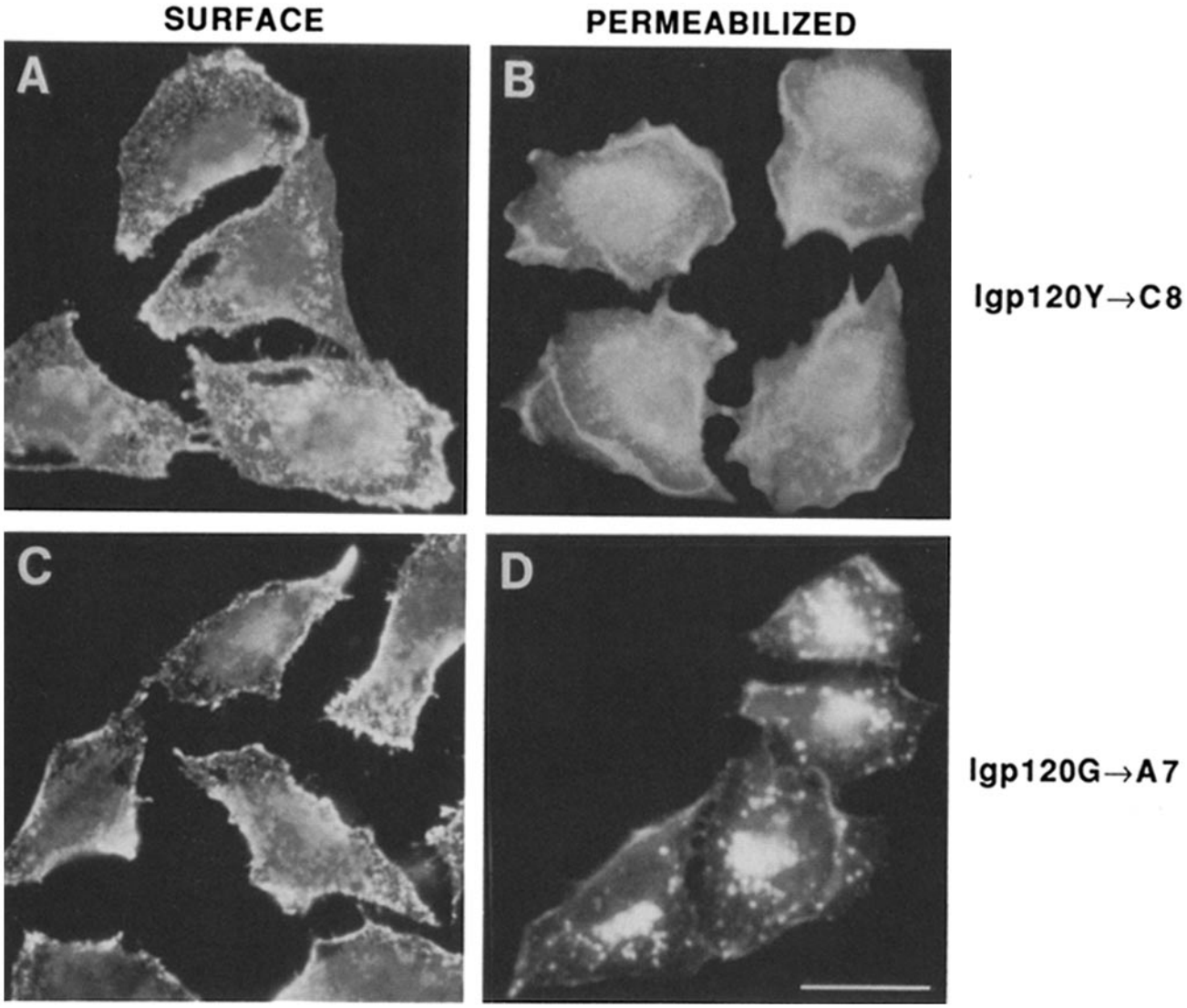

Figure 4. Immunofluorescence localization of lgp120Y→C8 and lgp120G→A7 in transfected CHO cells. Cells expressing either the lgp120Y→C8 (*A* and *B*) or lgp120G→A7 (*C* and *D*) mutants were stained for cell surface (*A* and *C*) or intracellular (*B* and *D*) rat lgp120 using a rabbit anti-rat lgp120 antiserum as described in the legend to Fig. 1. Bar, 5 μm.

mutant was also detected at the cell surface that was not detected by the antitail peptide antibody. Virtually all of the lgp120Y→C8 mutant exhibited a diffuse surface staining pattern that was entirely distinct from the pattern of endogenous lgp-A distribution. The fact that the two patterns were so different emphasizes the distinct specificities of the two antibodies.

These observations thus indicate that the conserved glycine and tyrosine residues of the rat lgp120 cytoplasmic domain contributed differentially to lysosomal targeting. While removing the tyrosine residue abolished lysosomal transport and resulted in cell surface localization, substitution of the glycine residue only partially interfered with lysosomal targeting.

Endocytosis of lgp120 Requires Only the Cytoplasmic Tail Tyrosine Residue

If the normal pathway of lgp120 transport to lysosomes required transit through the plasma membrane, then the dual localization of lgp120G→A7 on the cell surface and in lysosomes might be explained by a much slower rate of internalization due to the mutation of the cytoplasmic tail glycine. To test this possibility, we next compared the kinetics of endocytosis of lgp120G→A7 and wild-type lgp120.

Internalization was first monitored by immunofluorescence. Cells were incubated for 2 h at 37°C in medium containing the anti-lgp120 mAb Ly1C6 IgG. As shown in Fig. 6, antibody bound to cells overexpressing wild-type lgp120 (Fig. 6 *A*) or expressing lgp120G→A7 (Fig. 6 *B*) was internalized and delivered to intracellular vesicles typical of late endosomes and lysosomes. Uptake was similar for both cell lines, although a small amount of surface staining remained in the case of the cells expressing the glycine mutant. In contrast, cells expressing the lgp120Y→C8 mutant were incapable of delivering anti-lgp120 antibody to intracellular vesicles, with all cell-associated Ly1C6 remaining at the plasma membrane (Fig. 6 *C*). Importantly, even after prolonged incubations at 37°C, neither binding nor internalization was observed in CHO cells expressing low levels of wild-type

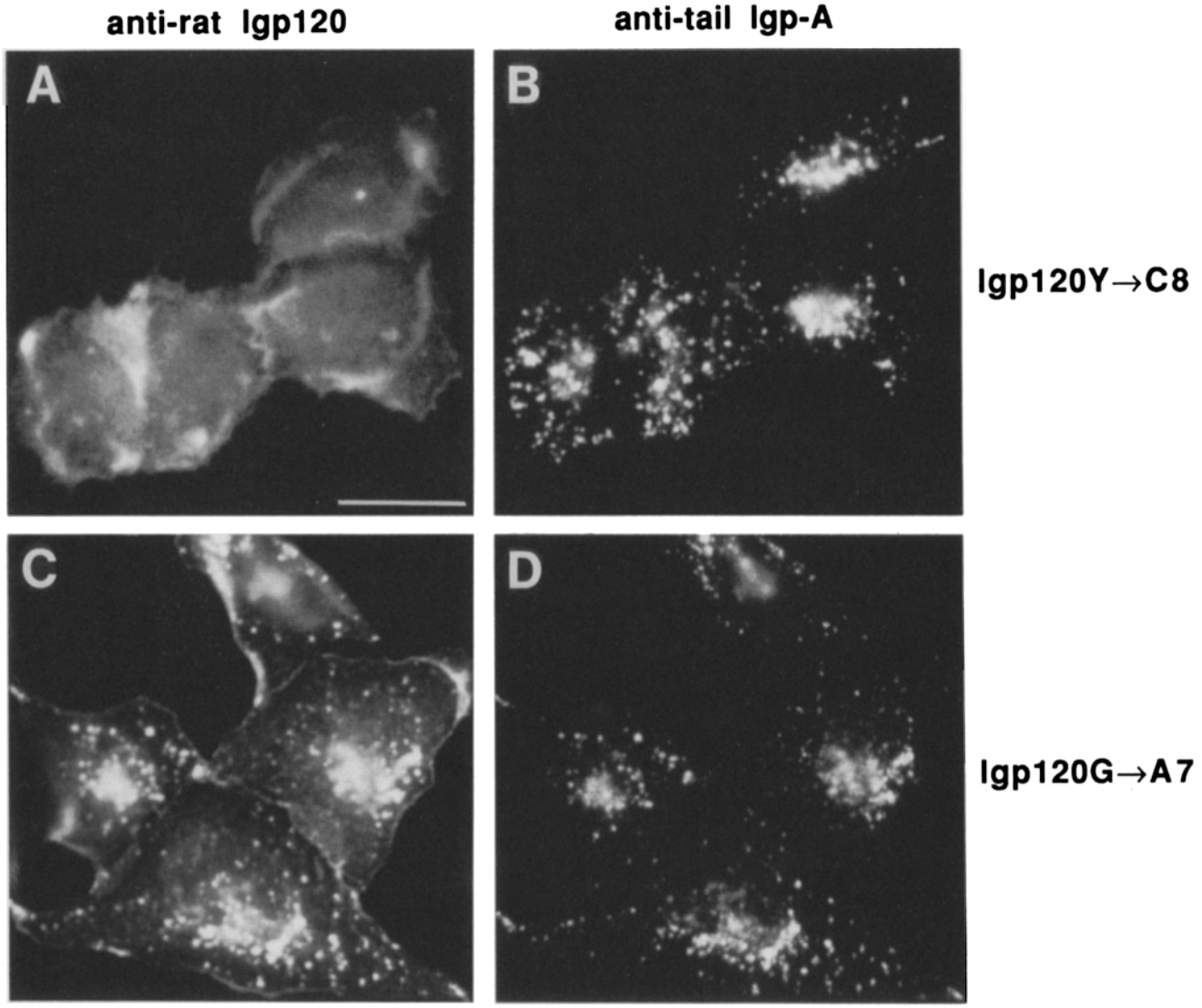

Figure 5. Colocalization of lgp120Y→A8 or lgp120G→A7 with endogenous lgp-A. Permeabilized CHO cells expressing lgp120Y→A8 (*A* and *B*) and lgp120G→A7 (*C* and *D*) mutants were double labeled with the rat-specific mouse monoclonal anti-lgp120 antibody Lyl C6 IgG (*A* and *C*) or with a rabbit antitail lgp-A peptide antiserum (*B* and *D*) followed by staining with the appropriate fluorescein-conjugated anti-rabbit and Texas red-conjugated anti-mouse secondary antibodies. Although peripheral surface staining was observed for both mutant transfectants using Lyl C6 IgG, this pattern was not visualized using the antitail peptide antibody which was thus judged to react only with endogenous wild-type CHO lgp-A in endosomes and lysosomes. Bar, 5 μm.

lgp120 (clone 1) (Fig. 6 *D*); similar results were obtained for nontransfected CHO cells (not shown) and NRK cells (not shown). Thus, lgp120G→A7 bound and internalized externally added antibody in a manner qualitatively similar to overexpressed wild-type lgp120, suggesting that the increased expression of lgp120G→A7 at the cell surface did not reflect an endocytosis defect. Moreover, the failure of NRK cells and low expressing wild-type transfectants to internalize antibody suggested that lgp120 reached lysosomes in these cells without becoming accessible to externally added antibody.

To confirm the immunofluorescence results quantitatively and to obtain actual rates of endocytosis, we next determined the kinetics of internalization. Cell monolayers were incubated in ^{125}I-Lyl C6 IgG at 37°C for various lengths of time in the presence or absence of excess unlabeled antibody and internalized (acid-resistant) antibody determined. As shown in Fig. 7, cells expressing the wild-type protein or lgp120G→A7 internalized equivalent percentages of ^{125}I-Lyl C6 IgG after 30 min, although the initial rate of uptake for the wild-type protein was somewhat faster than for the glycine mutant. Both cell lines internalized ^{125}I-Lysl C6 IgG far more rapidly than did cells expressing the lgp120Y→C8 mutant (Fig. 7).

While the rate of internalization of ^{125}I-Lyl C6 IgG was comparable for both the overexpressed wild-type lgp120 and the lgp120G→A7 mutant, when normalized to the different levels of lgp120 expression in each cell line (Fig. 2), the absolute amount of antibody bound and taken up by cells expressing lgp120G→A7 was significantly higher. From the actual amounts of antibody measured in Fig. 7, cells expressing lgp120G→A7 bound and internalized 4.4×10^3 and $45.0 \times$

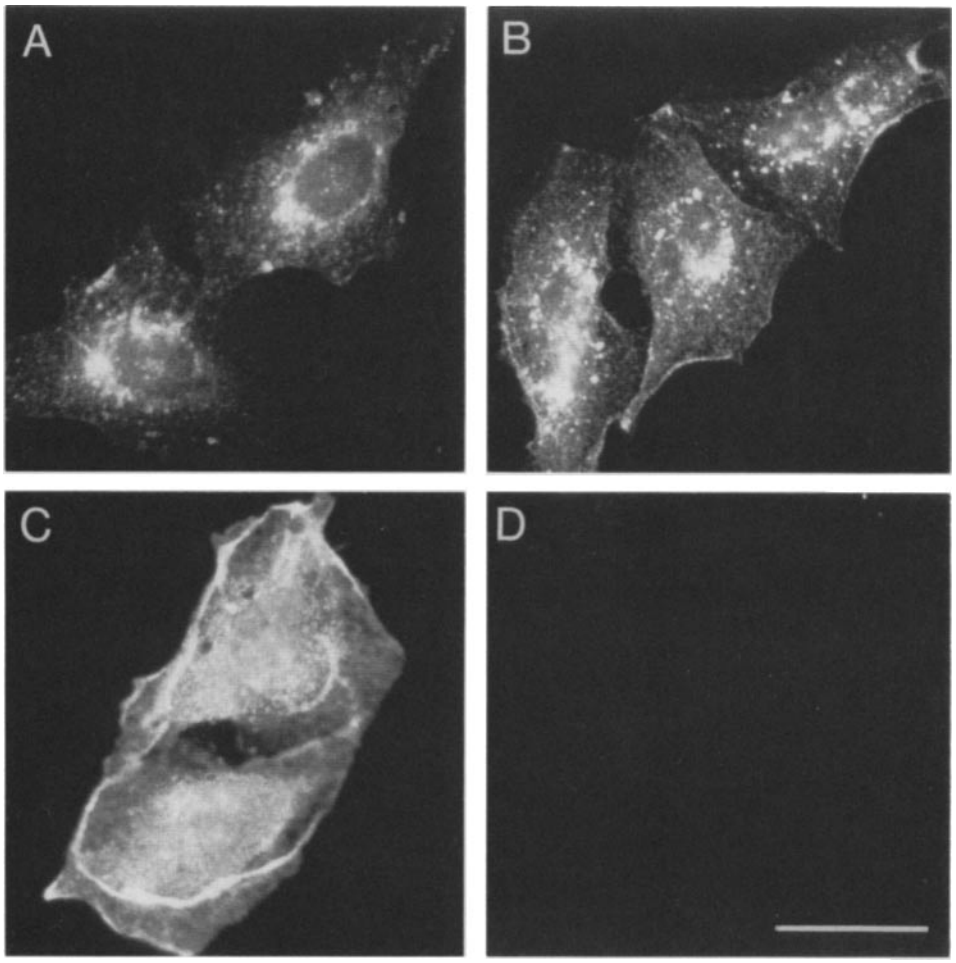

Figure 6. Immunofluorescence of internalized anti-rat lgp120 antibody by CHO cells transfected with wild-type and mutant lgp120. Transfected cells expressing wild-type lgp120, lgp120G→A7, and lgp120Y→C8 were incubated in medium containing the anti-rat lgp120 antibody Lyl C6 IgG (15 μg/ml) for 2 h at 37°C. The cells were then washed on ice, fixed, permeabilized, and stained with a Texas red-conjugated anti-mouse antibody. (*A*) Wild-type CHO transfectants at high expression level. (*B*) lgp120G→A7 CHO transfectants. (*C*) lgp120Y→C8 CHO transfectants. (*D*) Wild-type CHO transfectants at low expression level.

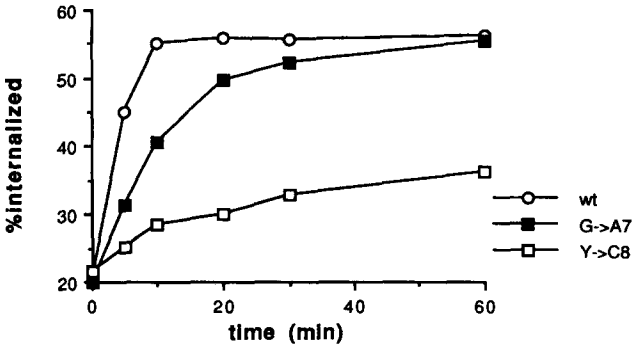

Figure 7. Kinetics of internalization of ^{125}I-Lyl C6 IgG by cells expressing wild-type or mutant lgp120. Transfected cell lines were incubated with ^{125}I-Lyl C6 IgG (1 μg/ml) for 2 h at 37°C in the presence or absence of 150-fold excess unlabeled antibody. The monolayers were then cooled on ice and washed to remove unbound antibody. Surface-bound antibody (acid-sensitive) was eluted by washing at low pH, followed by cell lysis to determine internalized (acid-resistant) radioactivity. Specific internalization was determined after subtracting ^{125}I-IgG bound and/or internalized in the presence of unlabeled Lyl C6. All data points represent the average of duplicate measurements of two independent experiments (±10% variation).

10^3 cpm of ^{125}I-Lyl C6 IgG (respectively) as compared with 0.9×10^3 cpm bound and 15.9×10^3 cpm internalized for the wild-type transfectants. Thus, cells expressing the lgp120G→A7 mutant were more efficient at binding and endocytosis of ^{125}I-Lyl C6 IgG, suggesting that a greater fraction of the mutant reached the cell surface than did the wild type.

Finally, to ensure that wild-type and glycine mutant lgp120 were internalized comparably even in the absence of bound antibody, we measured lgp endocytosis after surface biotinylation with sulfo-NHS-SS-biotin. After derivatization on ice, cell monolayers were warmed to 37°C for 30 or 60 min. Cells were then cooled and treated with glutathione to remove any biotin groups remaining on the cell surface. After cell lysis, total vs, biotinylated lgp120 was determined. As illustrated in Fig. 8, comparable amounts of biotinylated lgp120 were internalized in cells expressing the wild-type lgp120 or lgp120G→A7 mutant; relatively little protein was protected from reduction in cells expressing the lgp120Y→C8 mutant. Quantitation yielded results similar to those obtained by antibody internalization: 23.4% of lgp120G→A7 and 34% of wild-type lgp120 were found intracellularly while the value for the lgp120Y→C8 mutant was at the limit of detection of the assay (5.4%).

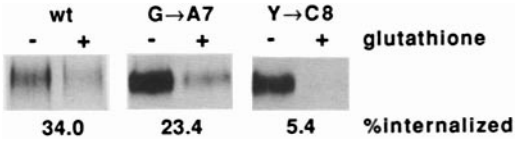

Figure 8. Internalization of surface biotinylated wild-type and mutant lgp120. Transfected cell lines were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine overnight and subjected to cell surface biotinylation with sulfo-NHS-SS-biotin at 4°C. After quenching excess biotin reagent, cells were warmed to 37°C for 30 min to allow internalization. Cell surface–bound biotin was then removed by treatment of the cells with reduced glutathione. ^{35}S-labeled, biotinylated lgp120 was precipitated with anti-rat lgp120 antibody and protein A–Sepharose, followed by reprecipitation with streptavidin-agarose; precipitates were then analyzed by SDS-PAGE and autoradiography. Quantitation was performed using computer-assisted digitization of autoradiograms. The fraction of glutathione-resistant (*% internalized*) lgp is expressed as percentage of precipitated lgp from a control dish not subjected to glutathione treatment.

Together, these results show that lgp120G→A7 is internalized somewhat more slowly than, but at an efficiency comparable with, wild-type lgp120 in the presence or absence of antibody. Such a slight difference in endocytosis does not seem to be sufficient to account for the significant increase in the amount of surface lgp120G→A7. Rather, the difference in surface expression implied that a greater fraction of newly synthesized lgp120G→A7 than wild-type lgp120 reached the plasma membrane after leaving the Golgi complex.

Newly Synthesized Wild-Type and Mutant lgp's Reach the Plasma Membrane with Different Efficiencies

To determine directly the fraction of newly synthesized wild-type and mutant lgp's arriving at the plasma membrane, we performed pulse–chase labeling combined with surface biotinylation or immunoprecipitation. Cells were pulse labeled for 15 min with [^{35}S]cysteine and [^{35}S]methionine and then chased for different periods of time. At each time point,

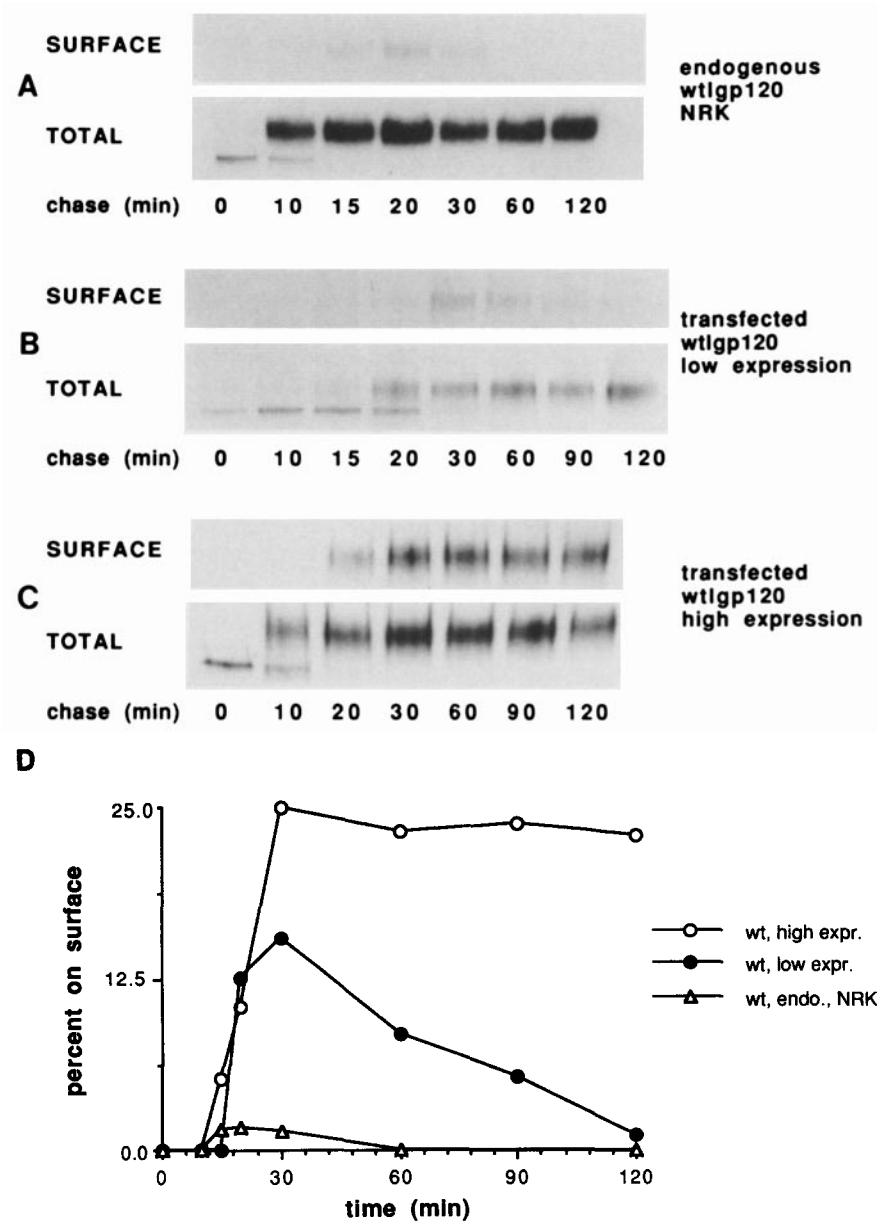

Figure 9. Surface appearance of newly synthesized wild type rat lgp120 in NRK cells and transfected CHO cells. Cells were pulse labeled with [^{35}S]methionine and [^{35}S]cysteine for 15 min and then chased for the indicated times at 37°C. At each time point, the cells were placed on ice and labeled lgp-120 that had appeared on the surface derivatized using sulfo-NHS-SS-biotin or by the addition of a rabbit anti-rat lgp120 antiserum at 4°C. Surface-biotinylated and total cell lgp120 were precipitated from detergent lysates as described in the legend to Fig. 2. Antibody-labeled surface lgp was precipitated after washing the cells to remove unbound antibody, by lysing the monolayers in Triton X-100 and then adding protein A–Sepharose to four fifths of the cell lysate; total lgp was immunoprecipitated from one fifth by adding additional anti-lgp120 antiserum. All precipitates were analyzed by SDS-PAGE and autoradiography; identical results were obtained using either the biotinylation or surface-bound antibody procedures. (*A*) Cell surface appearance of endogenous wild-type rat lgp-120 in NRK cells. (*B*) Transfected CHO cells expressing low levels. (*C*) Transfected CHO cells expressing rat lgp120 at high levels. (*D*) Percentage of lgp's detected on the cell surface relative to the total amount of immunoprecipitated lgp's during the chase period as determined by computer-assisted digitization of autoradiograms and after correcting for differences in sample size. The results from two or three independent experiments were used for quantitation.

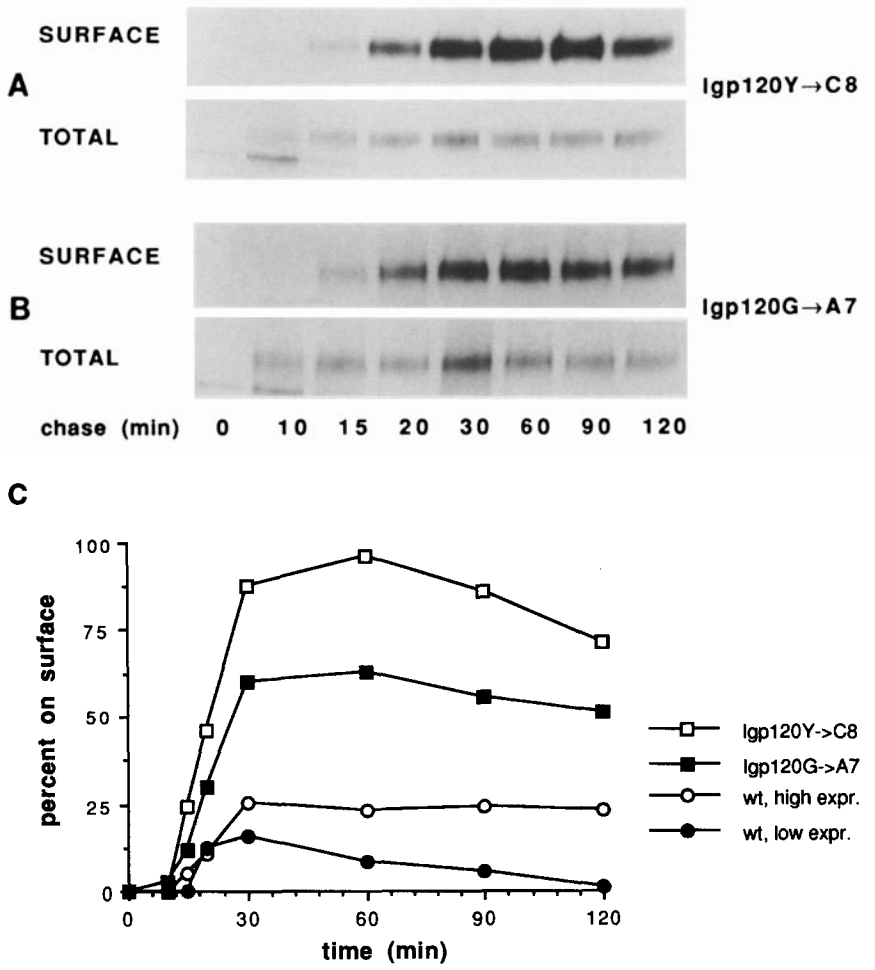

Figure 10. Surface appearance of lgp120Y→C8 and lgp120G→A7 in transfected CHO cells. Cells were treated as described in the legend to Fig. 9. Cell surface appearance of cells expressing lgp120Y→C8 (*A*) or lgp120G→A7 (*B*) was determined using cell surface biotinylation or antibody addition (not shown). (*C*) Percentage of lgp's detected on the cell surface relative to the total amount of immunoprecipitated lgp's during the chase period as determined by computer-assisted digitization of autoradiograms. Values for percent on surface were obtained after correcting for differences in sample size. The results from four (lgp120Y→C8) or three (lgp120G→A7) independent experiments were used for the quantitative analysis. Since 95% of newly synthesized lgp120Y→C8 was found as having reached the plasma membrane, this mutant is likely to be transported almost quantitatively to the cell surface. Accordingly, we estimated that the biotinylation procedure was at least 95% efficient at recovering cell surface lgp120.

surface appearance was detected by biotinylation of the cells at 0°C. As a control for the biotinylation procedure, each cell line was also examined by surface immunoprecipitation, in which cells were incubated on ice with a rat-specific rabbit anti-lgp120 antibody followed by cell lysis and precipitation with protein A-Sepharose. Identical results were obtained from both procedures. After biotinylation or surface precipitation from four fifths of the initial lysate, total lgp120 was determined by quantitative immunoprecipitation from an aliquot corresponding to one fifth of the cell lysate. The ratio of surface to total rat lgp120 was determined by digitization of autoradiograms obtained after SDS-PAGE of the precipitates and surface lgp expressed as a percentage of total after correcting for the differences in sample size.

In contrast to the results obtained after steady-state labeling (Fig. 2), pulse-chase labeling of NRK cells and transfected low expressing CHO cells demonstrated that a small, but reproducible fraction of newly synthesized wild-type lgp120 was transported to the plasma membrane. Surface appearance in NRK cells was transient, reaching a maximum of 1.8% on the surface after 20 min, disappearing by 60 min (Fig. 9, *A* and *D*). A small but significantly greater fraction was detected transiently at the cell surface in CHO cells expressing low levels of rat lgp120 (Fig. 9 *B*), reaching a maximum of 15% after 30 min of chase (Fig. 9 *D*). In cells expressing higher levels of lgp120, an even greater fraction of newly synthesized protein was transported to the cell surface reaching a maximum of 25% (Fig. 9, *C* and *D*). The kinetics of appearance were similar to the low expressing cells, but the fraction at the cell surface remained constant for 2 h, possibly reflecting the recycling of lgp120 in overexpressing cells. In any event, these results suggested that a relatively small fraction of the total amount of newly synthesized lgp120 reached the cell surface in cells expressing the wild-type protein. In transfected CHO cells, this fraction appeared to correlate with the total amount of lgp120 made.

A different situation was observed for cells expressing either of the two mutant lgp's. As shown in Fig. 10 *A*, lgp120Y→C8 was rapidly and efficiently transported to the cell surface, becoming almost completely (95%) accessible to cell surface biotinylation or externally added antibody after 30 min (Fig. 10 *C*). Although not quite as efficient, most of the newly synthesized lgp120G→A7 also reached the plasma membrane (Fig. 10 *B*), reaching a maximum of 65% (Fig. 10 *C*). This represented a much greater fraction of lgp120G→A7 reaching the cell surface than was observed in the same cells expressing wild-type lgp120 at levels comparable to or greater than the level of lgp120G→A7 expression (Fig. 10 *C*). Moreover, since 95% of newly synthesized lgp120Y→C8 was detected as having reached the plasma membrane, we conclude that the biotinylation procedure must be almost completely efficient at recovering all lgp120 forms from the cell surface.

The kinetics of surface appearance of the mutant proteins

was similar to the wild-type protein, lgp first being detected by biotinylation or antibody addition after a lag of 10–15 min and reaching a maximum after ~30 min. As was the case for the overexpressed wild-type protein, a considerable fraction of the mutant lgp's reaching the cell surface was not cleared from the plasma membrane. In the case of the lgp120Y→C8 mutant, this probably reflected the relatively low rate of endocytosis. Since the overexpressed wild-type lgp120 and lgp120G→A7 were subject to rapid internalization (Figs. 6–8), their slow disappearance from the plasma membrane possibly reflected continuous recycling between the cell surface and one or more intracellular compartments.

Discussion

The issue of whether, or to what extent, lysosomal membrane glycoproteins such as lgp120 are transported from the TGN to lysosomes via an intracellular route or following endocytosis after insertion into the plasma membrane represents a critical unknown in our understanding of lysosomal biogenesis and Golgi apparatus function. Since the bulk of newly synthesized lysosomal enzymes bound to MPR appear to reach lysosomes without reaching the cell surface, it would appear likely that lysosomal membrane components take the same intracellular route (Kornfeld and Mellman, 1989). If lgp's indeed are targeted intracellularly, then they must contain one or more sorting determinants that prevent their transport to the cell surface and/or mediate their inclusion onto a specific pathway to lysosomes. Since lgp's do not contain mannose 6-phosphate residues (Howe et al., 1988), and since they bear no obvious sequence homology with MPR, it would be of clear importance to identify the nature of such sorting determinants.

In this paper, we have characterized several essential features controlling the transport of lgp120 which may help clarify some of the superficially conflicting data concerning the pathways of lgp to lysosomes. We have used a series of biochemical assays to specifically and quantitatively determine the fraction of lgp120 that appears on the cell surface. Our results show that only a very small fraction of the wild-type protein ever becomes accessible to labeling or antibody binding at the plasma membrane. This was true for rat lgp120 expressed endogenously in NRK cells or after transfection into CHO cells. However, surface appearance could be increased in one of two ways: first, by elevating the amount of lgp120 expression; and second by altering to the lgp120 cytoplasmic domain.

Most importantly, mutation of either the conserved glycine or tyrosine residues resulted in the expression of proteins the majority of which appeared on the plasma membrane after exit from the Golgi complex. These results demonstrate that the assays used were capable of distinguishing efficient vs. inefficient transport of lgp120 to the plasma membrane. They also suggest that the lgp120 cytoplasmic domain contains a determinant that is important for directing transport to lysosomes via an intracellular route. Since the lgp120G→A7 mutant was capable of endocytosis from the plasma membrane, such a determinant is not identical to that required for rapid internalization nor is the increased surface appearance of lgp120G→A7 likely to be secondary to an internalization defect.

Table I. Summary of Cell Surface Expression, Rate of Plasma Membrane Insertion, and Endocytosis for Wild-Type and Mutant lgp120

Cell line	lgp on cell surface at equilibrium*	Newly synthesized lgp reaching surface‡	Surface lgp internalized after 30 min§
	%	%	%
NRK	0	1.8	0
CHO-wt, low	0	15.0	0
CHO-wt, high	1.5	25.0	35
CHO-G→A7	5.2	65.0	32
CHO-Y→C8	39.0	95.0	12

* Determined by overnight labeling with [^{35}S]methionine/cysteine followed by surface biotinylation; data taken from Fig. 2.
‡ Determined by pulse-labeling with [^{35}S]methionine/cysteine followed by surface biotinylation and surface precipitation at various times of chase; data taken from Figs. 9 and 10.
§ Determined from the amount of ^{125}I-Ly1C6 IgG internalized after 30 min at 37°C; data taken from Fig. 7.

Intracellular Transport of lgp to Lysosomes

We used a variety of independent approaches to determine whether lgp's can take a direct route from the TGN to lysosomes or must make obligatory passage through the plasma membrane. First, although not definitive, the fact that at steady state little wild-type lgp120 was found on the surface suggested that the plasma membrane was not a major intermediate on the pathway to lysosomes. Virtually no surface lgp was detected in NRK cells or in CHO transfectants expressing low levels of lgp. Even in high expressing transfectants, only a small fraction (estimated at ~1.5%) of the total rat lgp120 was found on the plasma membrane. In contrast, far greater fractions of lgp120G→A7 and lgp120Y→C8 localized at the plasma membrane of even low expressing cells (estimated at ~5.4 and 39%, respectively) (Table I).

Since wild-type lgp120 and lgp120G→A7 were internalized to the same extents and at comparable rates, equal amounts of ^{125}I-anti-lgp120 IgG should have been taken up at 37°C if both passed through the cell surface to similar extents. However, no antibody uptake over background was measured in NRK cells or low expressing wild-type transfectants (Table I). Specific antibody internalization was detected in cells expressing high levels of the wild-type protein. However, the amount of antibody internalized was less than that internalized by cells expressing a two- to threefold lower level of lgp120G→A7. When normalized for expression levels, cells overexpressing wild-type lgp120 were only 30% as efficient at internalizing externally added anti-lgp120 antibody as cells expressing the lgp120G→A7 mutant, in spite of the fact that both cell lines internalized cell surface lgp120 at comparable rates (Table I). This result suggests that the majority of wild-type lgp120 never reaches the plasma membrane, even in overexpressing cells.

Finally, we were able to directly determine the fraction of newly synthesized lgp120 appearing at the cell surface after pulse labeling. If all lgp's were transported to lysosomes by endocytosis after insertion in the plasma membrane, we would have expected a substantial fraction of pulse labeled lgp120 to be transiently accessible to either of the two cell surface labeling procedures used to detect surface appearance. This proved not to be the case. In NRK cells, only a very small cohort (<2%) of endogenous lgp120 molecules

reached the plasma membrane after leaving the Golgi complex. A somewhat greater fraction was observed in transfected CHO cells, ranging from ~15% of the total in cells expressing low levels of rat lgp120 to ~25% in cells expressing higher amounts of the protein (Table I). Moreover, much of the labeled lgp120 reaching the surface in high expressing cells was not rapidly cleared from the plasma membrane, suggesting that this pool of lgp120 was not efficiently transferred to lysosomes. The slow clearance from the plasma membrane is difficult to reconcile with previous observations in NRK and J774 cells that lgp120 is transported from the Golgi complex to lysosomes with a $t_{1/2}$ of <30 min (Green et al., 1987). Moreover, since even in the high expressing cells only a small fraction of the total lgp120 synthesized ever appeared on the plasma membrane, it was unlikely that the cell surface served as an obligatory intermediate on the pathway to lysosomes.

The significance of these results was emphasized by the behavior of the two lgp120 cytoplasmic domain mutants, lgp120G→A7 and lgp120Y→C8. Almost all of the newly synthesized lgp in cells expressing the tyrosine mutant appeared on the surface within 30 min after pulse labeling (Table I). As shown previously (Williams and Fukuda, 1990), we found that this mutant accumulates on the plasma membrane and is incapable of rapid endocytosis. More importantly, a similarly large fraction of newly synthesized lgp120G→A7 mutant (~70%) was also detected on the plasma membrane. Since the lgp120G→A7 was internalized at an efficiency comparable to wild-type lgp120, its increased appearance on the cell surface could not be explained by a markedly decreased rate of endocytosis. Besides, as was found for wild-type lgp120 in overexpressing cells, much of the lgp120G→A7 mutant was not rapidly cleared from the cell surface. These results suggest that lgp's on the cell surface may not always be rapidly transported to lysosomes. Instead, especially when expressed at high levels, plasma membrane lgp120 may recycle several times as shown for cell surface LAP (Braun et al., 1989).

Taken together, these experiments strongly suggest that the majority of newly synthesized lgp120 molecules reach their final destinations without requiring prior insertion into the plasma membrane. This conclusion is further supported by our finding that in transfected MDCK cells only a small fraction of wild-type lgp120 localized at the basolateral surface from which it was efficiently internalized (Hunziker et al., 1991). To a first approximation, this conclusion is different from that reached for the transport of an endogenous lysosomal protein in MDCK cells (Nabi et al., 1991). A substantial fraction of this protein, possibly an lgp-B (Nabi, I., personal communication), was found to appear at the basolateral surface of MDCK cells before arrival in lysosomes. While this difference might be explained by differences in cell type or protein, it seems more likely to reflect the fact that these experiments detected surface appearance using antibody added at 37°C as opposed to 0°C. Thus, as the authors pointed out, their experiments could not quantitatively distinguish between surface appearance and insertion of newly synthesized lgp into endosomes. In any event, final proof of actual pathway taken by lgp120 must await identification of the actual carrier vesicles involved in its transport from the TGN.

Transport to Lysosomes May Involve a Saturable Intracellular Sorting Element

The correlation between expression level and cell surface appearance of lgp120, combined with the missorting of endogenous hamster lgp-B in transfectants expressing high concentrations of lgp120, suggest that existence of an intracellular mechanism of limited capacity that is responsible for the proper targeting of lgp molecules. While the nature of this putative sorting element is unknown, one attractive possibility is that it reflects the necessity for newly synthesized lgp120 to enter a specific population of TGN-derived vesicles that target transport to endosomes and lysosomes. Conceivably, these vesicles may be the clathrin-coated vesicles thought to mediate the transport of MPR-bound lysosomal enzymes from the TGN to endosomes (Kornfeld and Mellman, 1989). Thus, in the presence of excess lgp120 in overproducing cells, an increasing fraction of transfected and endogenous lgp's would be transported to the plasma membrane as a consequence of increased competition for a limited number of sorting sites.

These results are analogous, but not identical to, earlier observations in yeast cells in which the eightfold overexpression of the vacuolar protease carboxypeptidase Y led to secretion of up to 50% of the newly synthesized enzyme (Stevens et al., 1986). However, carboxypeptidase Y overexpression did not lead to the missorting of other vacuole proteases. Since in CHO cells, overexpression of a transfected lgp-A was associated with the plasma membrane appearance of both the transfected protein and an endogenous lgp-B, it is conceivable that both classes of closely related lgp molecules compete for a single sorting element. All lgp-A and lgp-B molecules sequenced thus far contain a glycine–tyrosine motif in their cytoplasmic domains (Kornfeld and Mellman, 1989). It is now apparent that both of these residues play a role in determining the proper intracellular targeting of newly synthesized lgp molecules.

Cytoplasmic Tails and Sorting Signals

While a tyrosine in the cytoplasmic domain of lgp120 is clearly important for lysosomal targeting, it is not yet clear why this is the case. The majority of newly synthesized lpg120 molecules did not appear to be inserted into the plasma membrane before reaching lysosomes. Therefore, it is unlikely that tyrosine mutants are unable to be delivered to lysosomes solely because they cannot be rapidly internalized – in spite of the fact that the tyrosine is also required for rapid internalization. Instead, it is more likely that the tyrosine is involved in specifying an intracellular sorting event.

By analogy to the extensively studied role of cytoplasmic domain tyrosine residues in the localization of plasma membrane receptors at clathrin-coated pits (Davis et al., 1986; Jing et al., 1990; Lobel et al., 1989), it is conceivable that the lgp120 cytoplasmic tail tyrosine performs a similar function in the TGN, allowing newly synthesized proteins to accumulate along with MPR at clathrin-coated buds which yield coated vesicles targeted for delivery to endosomes (Kornfeld and Mellman, 1989). Thus far, there is no direct evidence that lgp's exit the TGN via clathrin-coated vesicles. Nor is it clear whether coated buds in the Golgi complex even recognize tyrosine-containing cytoplasmic domain de-

terminants. Indeed, in vitro binding studies suggest that adaptor proteins in Golgi-derived coated vesicles, thought to interact with receptor cytoplasmic domains, do not interact with the tyrosine-containing portions of the MPR cytoplasmic tail (Glickman et al., 1989; Pearse, 1988). The conserved glycine-tyrosine motif characteristic of lgp120 has not yet been examined for adaptor protein interaction in vitro, nor is it clear that the same mechanism will apply in intact cells. Alternatively, the glycine-tyrosine-containing lgp120 cytoplasmic domain might indirectly specify entry into TGN coated buds, or may reflect the formation of a nonclathrin-coated vesicle (Duden et al., 1991; Serafini et al., 1991) which may mediate lysosomal transport by an as yet uncharacterized pathway.

It is apparent, however, that the lgp120 cytoplasmic domain tyrosine is involved in at least one additional biosynthetic sorting event. In MDCK cells transfected with the lgp120G→A7 mutant, a large fraction of newly synthesized molecules was found to appear on the basolateral surface (Hunziker et al., 1991). Mutation of the cytoplasmic domain tyrosine completely reversed the polarity of surface expression resulting in the transport of the lgp120Y→C8 mutant to the apical plasma membrane. Whether basolateral transport reflects inclusion of lgp120 into TGN-derived clathrin-coated vesicles in MDCK cells is unknown, but it is clear that sorting of newly synthesized molecules in the Golgi complex is dependent on the lgp120 cytoplasmic domain tyrosine. Conceivably, the mechanism leading to basolateral insertion in polarized cells, as well as to transport to lysosomes, may depend on common elements. If, in either polarized or nonpolarized cells, lgp120 exits the TGN in clathrin-coated vesicles, then their possible role in the transport of even plasma membrane receptors containing coated pit localization domains should be considered.

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