Glycoproteins of the Lysosomal Membrane

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ABSTRACT Three glycoprotein antigens (120, 100, and 80 kD) were detected by mono- and/or polyclonal antibodies generated by immunization with highly purified rat liver lysosomal membranes. All of the antigens were judged to be integral membrane proteins based on the binding of Triton X-114. By immunofluorescence on normal rat kidney cells, a mouse monoclonal antibody to the 120-kD antigen co-stained with a polyclonal rabbit antibody that detected the 100- and 80-kD antigens as well as with antibodies to acid phosphatase, indicating that these antigens are preferentially localized in lysosomes. Few 120-kD–positive structures were found to be negative for acid phosphatase, suggesting that the antigen was not concentrated in organelles such as endosomes, which lack acid phosphatase. Immunoperoxidase cytochemistry also showed little reactivity in Golgi cisternae, coated vesicles, or on the plasma membrane. Digestion with endo-β-*N*-acetylglucosaminidase H (Endo H) and endo-β-*N*-acetylglucosaminidase F (Endo F) demonstrated that each of the antigens contained multiple N-linked oligosaccharide chains, most of which were of the complex (Endo H–resistant) type. The 120-kD protein was very heavily glycosylated, having at least 18 N-linked chains. It was also rich in sialic acid, since neuraminidase digestion increased the pI of the 120-kD protein from <4 to >8. Taken together, these results strongly suggest that the glycoprotein components of the lysosomal membrane are synthesized in the rough endoplasmic reticulum and terminally glycosylated in the Golgi before delivery to lysosomes. We have provisionally designated these antigens lysosomal membrane glycoproteins lgp120, lgp100, lgp80.

Lysosomes serve as the major digestive compartment of mammalian cells. They are responsible for the degradation of both extracellular material internalized by endocytosis and intracellular material delivered to lysosomes during autophagocytosis. Although the properties, biosynthesis, and targeting of lysosomal hydrolases have been studied in detail (1–3), less is known about the components of the lysosomal membrane. This membrane is, however, interesting in several respects. Among its most unique features are its apparent resistance to degradation by lysosomal hydrolases, its role in maintaining and generating an acidic intralysosomal environment (4, 5), its ability to selectively transport the products of lysosomal hydrolysis (amino acids, dipeptides, mono- and disaccharides) (6, 7), and the specificity with which it interacts and fuses with other membrane organelles of the vacuolar system including endosomes, phagosomes, and the plasma membrane (8, 9). The lysosomal membrane is also involved in complex regulatory events such as the transport and release of cobalamin (vitamin B_{12}) (10), cholesterol homeostasis (11), receptor down regulation (12–15), and host defense against certain phagocytized microorganisms (16).

As a first step in studying the properties of lysosomal membranes, we have in this paper used an immunological approach to identify three intrinsic lysosomal membrane proteins. One of these proteins is similar to a 100-kD lysosome-associated polypeptide recently described by Reggio et al. (17). Preliminary biochemical characterization has shown that each of these proteins is heavily N-glycosylated and/or rich in sialic acid.

MATERIALS AND METHODS

Cells: Normal rat kidney (NRK) fibroblasts were obtained from the American Type Culture Collection and maintained in monolayer culture in Dulbecco's modified Eagle's medium that contained 7% fetal calf serum (KC

[1] *Abbreviations used in this paper*: Endo F, endo-β-*N*-acetylglucosaminidase F; Endo H, endo-β-*N*-acetylglucosaminidase H; NRK, normal rat kidney; TX-114, Triton X-114.

Biological Inc., Lenexa, KA) and penicillin-streptomycin. Baby hamster kidney (BHK-21) cells and J774 mouse macrophages were grown as described previously (18, 19).

Cell Fractionation: Highly purified lysosomal membranes were prepared from rat liver by Percoll density gradient centrifugation and free-flow electrophoresis according to the method of Harms et al. (20).

Antibody Production: For the production of anti-lysosome monoclonal antibodies, female CD_2F_1 (Balb/c × DBA/2) mice were immunized by an intraperitoneal injection of purified lysosomal membranes in AluGel-S (Accurate Scientific, Westbury, NY). Typically, 25 μg of membrane protein in 0.5 ml sterile phosphate-buffered saline (PBS) was combined (by vortexing) with an equal volume of adjuvant just before use. Mice were boosted 3–5 wk later by an intravenous injection of an additional 25 μg of membranes in 0.5 ml sterile saline. 4 d later, the animals were sacrificed and their spleens removed and dissociated to single cells for fusion using the P3U1 myeloma cell line according to the procedure of Gefter et al. (21). Hybridomas were plated in 96-well microtiter dishes, and positive cell lines (identified by indirect immunofluorescence) were cloned twice in agarose and grown as ascites in pristane-primed syngeneic mice.

Polyclonal antisera against lysosomal membranes or against purified lysosomal membrane proteins were prepared in rabbits by injection of the antigen in complete Freund's adjuvant via the popliteal lymph nodes, as described by Reggio et al. (17). 25 μg of membranes or 5–10 μg of purified protein were used per injection.

Immunofluorescence: NRK cells were plated on round glass coverslips or on Teflon-masked glass microscope slides, and grown in medium that contained 1% fetal calf serum for at least 16 h before use. Fixation was in 3% paraformaldehyde-0.02% glutaraldehyde in PBS for 15 min at room temperature. For visualizing intracellular structures, cells were permeabilized after fixation by a brief (10 s) treatment with 100% methanol at −20°C. Unreacted aldehyde groups were quenched by incubation in PBS that contained 0.1% $NaBH_4$ for 10 min. Staining (30 min, room temperature) was performed using undiluted culture fluid, or ascites, serum, or purified antibodies diluted in PBS that contained 0.2% gelatin. After an extensive wash in PBS, bound antibodies were visualized using affinity purified $F(ab')_2$ second antibodies (diluted 1:40 in PBS-gelatin) coupled to rhodamine or fluorescein (Tago Inc., Burlingame, CA). Coverslips and slides were mounted in Moviol (the gift of Daniel Louvard, Pasteur Institute, Paris) and viewed under a Zeiss fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Antigen localization by electron microscopy was performed using the immunoperoxidase technique on fixed, saponin-permeabilized cells according to the method of Brown and Farquhar (22).

Immunoprecipitation: Cell pellets or subcellular fractions were lysed by vortexing in buffer that contained 1% Triton X-114 (TX-114, Fluka Chemicals, Happaugue, NY), 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride, and 0.23 U/ml Aprotinin (Sigma Chemical Co., St. Louis, MO) at 0°C. Lysates were clarified by centrifugation in the cold, first at 750 *g* (10 min) and then at 45,000 *g* (20 min). Clarified lysates were either used directly or incubated for 5 min at 37°C and centrifuged at room temperature (500 *g*, 5 min) to separate detergent and aqueous phases according to a modification of the method of Bordier (23). Detergent phases were diluted with the above-mentioned buffer to a final TX-114 concentration of 0.5–2.0% before use. Lysates from [^{35}S]methionine-labeled cells were pre-adsorbed by incubation with 2 μl preimmune rabbit (or mouse) serum and 40 μl of a 10% (wt/vol) solution of formaldehyde-fixed *Staphylococcus aureus* (Zysorbin, Zymed Laboratories, So. San Francisco, CA) (30 min, 4°C) followed by centrifugation in an Eppendorf microfuge (2 min, 4°C). For immunoprecipitation, lysates (0.2–0.8 ml) were incubated on ice with serum (1–3 μl), purified IgG's (5 μg/ml), or hybridoma culture fluid (25–50 μl) for 1 h; then, affinity-purified goat anti-rabbit or anti-mouse IgG was added (final concentration 15 μg/ml, Tago Inc.). 1 h later, 60 μl of *S. aureus* was added, and the incubation continued for an additional 30–60 min at 4°C with constant agitation. The immunoadsorbent was collected by centrifugation and washed by repeated sonication and pelleting as described previously (19). Immunoprecipitated proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) using 4–11% gel gradients (24). Fluorography was performed using salicylic acid (25).

Glycosidase Digestion: Digestion with endo-β-*N*-acetylglucosaminidase F (Endo F) and endo-β-*N*-acetylgucosaminidase H (Endo H) was performed using slight modifications of published procedures (26, 27). Briefly, immunoprecipitated antigens were eluted from the *S. aureus* pellet by its resuspension in Endo F buffer (100 mM NaP_i pH 6.1, 50 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% β-mercaptoethanol) or Endo H buffer (100 mM sodium citrate, pH 5.5, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride) and heating to 100°C for 5 min. *S. aureus* was removed by centrifugation, and the supernatants digested with the respective enzyme for 2 h at 37°C. Digests were collected with cold 10% TCA, and the precipitates were washed with 1:1 ethanol/ether, then dissolved by heating to 100°C in SDS PAGE sample buffer. For neuraminidase digestion, immunoprecipitates were eluted into 50 mM Na acetate pH 5.5/2% SDS/2 mM $CaCl_2$ by boiling for 5 min. *S. aureus* particles were removed by centrifugation, and Nonidet P-40 was added to a final concentration of 2%. Aliquots of the eluate were incubated with or without 2 U of neuraminidase (Type X, Sigma Chemical Co.) for 4 h at 37°C. Samples were mixed with 1 mg/μl urea and made 20 mM in dithiothreitol and 0.5% CHAPS before they were loaded on an isoelectric focusing gel. Two-dimensional isoelectric focusing/SDS PAGE was performed essentially as described by O'Farrell (31). The isoelectric focusing gel contained 3.2% ampholytes (30% pH 3–10 and 0.2% pH 3–5), 1% Nonidet P-40, and 1% CHAPS. The SDS gel contained 12.5% acrylamide and 0.1% *N,N'*-methylenebisacrylamide.

Purification of Membrane Proteins: The one-step purification of a 120-kD lysosomal membrane protein was performed by immunoaffinity chromatography using the mouse monoclonal antibody Ly1C6. NRK cells were grown in plastic 850-cm^2 roller bottles, harvested, and lysed in 1% TX-114 as described above. A typical preparation used the cells from 12 confluent roller bottles and 80 ml of lysis buffer. To assist in the determination of recoveries, [^{35}S]methionine-labeled cells from a 100-mm dish were included at the lysis step. Detergent extracts were phase separated, diluted to their original volumes, and incubated with Ly1C6 coupled to CNBr-activated Sepharose 4B (2–4 mg purified IgG/ml of packed resin, 2–4 ml of resin per experiment). Incubation was performed batch-wise with constant agitation for 2 h at 4°C after which time the resin was collected by centrifugation (750 *g*, 2 min) and washed as described previously (28). The resin was then poured into a column, and the bound antigen was eluted using 0.5 M propionic acid. The eluate was immediately neutralized and dialyzed against 0.1 M ammonium bicarbonate. Purified proteins were concentrated by lyophilization, dissolved in distilled water, and used (with or without SDS denaturation) for immunization. Protein was determined using fluorescamine (Roche Diagnostics, Nutley, NJ) and by estimation from [^{35}S]methionine radioactivity. The efficiency of adsorption to the affinity support was assessed by immunoprecipitation of ^{35}S-labeled antigen from detergent lysates before and after incubation with the resin.

Other Methods: Cell surface iodination was performed at 4°C using carrier free $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) by the lactoperoxidase/glucose oxidase technique (29). Metabolic labeling was with either [^{35}S]methionine (>650 Ci/mmol, Amersham Corp.) in methionine-free medium or [^{3}H]mannose (20–30 Ci/mmol, ICN Pharmaceuticals, Inc., Irvine, CA) in low glucose (50 μM) medium that contained 10% dialyzed fetal calf serum. Purified proteins were iodinated using Iodogen (Pierce Chemical Co., Rockford, IL) (30) as described previously (12). All antibody preparations were judged >90% pure by SDS PAGE and cellulose acetate electrophoresis.

Biochemical Reagents: Lactoperoxidase was obtained from Calbiochem-Behring Corp. (LaJolla, CA); glucose oxidase, neuraminidase (Type X), protein A, and Percoll were from Sigma Chemical Co.; Endo H was obtained from the New York State Department of Health Division of Research Laboratories (Albany, NY); and Endo F was prepared and kindly provided by Steve Rosenzweig and Jim Jamieson of the Department of Cell Biology (Yale University).

RESULTS

Antibodies to Lysosomal Membranes

To prepare polyclonal antibodies against lysosomal membrane proteins, rat liver lysosomes were isolated and depleted of their contents by repeated sonication and freeze-thaw cycles in distilled water. Washed membranes were collected by centrifugation and injected into rabbits via the popliteal lymph nodes. Monoclonal antibodies were produced from mice immunized with the same membrane preparations. Both the rabbit antisera and several of the hybridoma supernatants exhibited a punctate, largely perinuclear fluorescent staining pattern on fixed, permeabilized NRK cells (Fig. 1). Whereas a high percentage (35/300) of the hybridoma clones were initially positive, all but one ceased producing specific antibody during expansion of these cell lines. This clone was designated Ly1C6 and was analyzed in detail.

Electron microscopy using the immunoperoxidase technique on saponin-permeabilized NRK cells revealed that both the rabbit antiserum and Ly1C6 intensely stained regularly shaped vacuoles preferentially localized in the perinuclear region (Fig. 2). The diaminobenzidine reaction product was

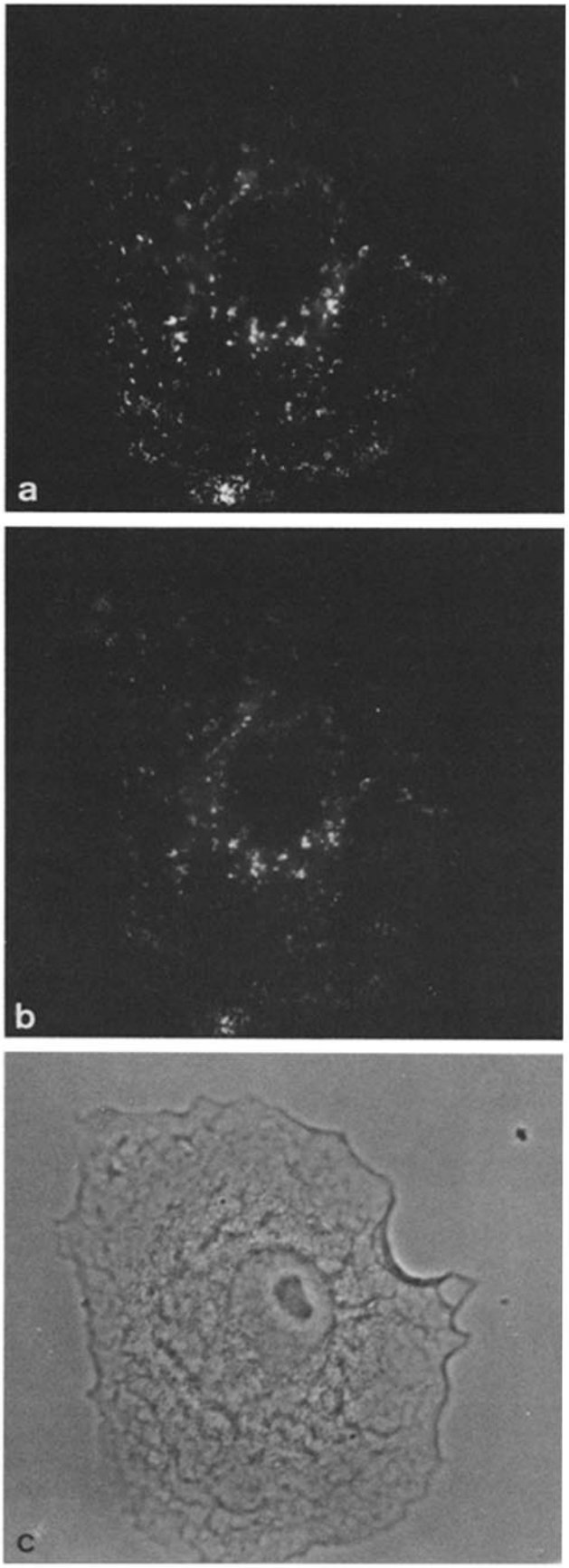

FIGURE 1 Co-localization of immunofluorescent staining using polyclonal and monoclonal antibodies to lysosomal membranes. NRK cells were fixed, permeabilized with cold methanol, and stained using a rabbit anti-lysosome antiserum (1:250 dilution) (*a*) or with the mouse anti-rat lysosome monoclonal antibody Ly1C6 (1:200 dilution of ascites fluid) (*b*). Rabbit and mouse IgG's were visualized using affinity-purified second antibodies coupled to rhodamine and fluorescein, respectively. Examination of the double immunofluorescent staining patterns reveals a striking co-localization of the antigens recognized by the polyclonal and monoclonal antibodies. (*c*) The corresponding phase-contrast micrograph of the cell shown in *a* and *b*. × 3,000.

largely confined to the interior of the positive vacuoles, suggesting that the antigenic determinants may be located on the luminal surface of the vacuole membrane. The cell surface, peripheral coated pits, and coated vesicles were negative, and Golgi cisternae were rarely (and, if so, only weakly) stained. Vesicular and tubular structures characteristic of endosomes were also negative.

The lysosomal nature of the positive vacuoles was confirmed by double immunofluorescent staining using rhodamine- and fluorescein-labeled second antibodies. Ly1C6 and the rabbit antiserum were found to label the same structures (Fig. 1, *a* and *b*). Moreover, Ly1C6 also stained the same organelles as did an affinity purified antibody to lysosomal acid phosphatase (Fig. 3, *a* and *b*). The fact that most Ly1C6-positive vesicles were also acid phosphatase-positive indicated that acid phosphatase-negative organelles such as endosomes and Golgi cisternae did not contain detectable amounts of the antigens recognized by the anti-lysosomal antibodies, in agreement with the immunoperoxidase experiments.

Indirect immunofluorescence of intact cells (either fixed or viable, at 0°C) did not reveal the presence of any of the antigens on the cell surface. This result was confirmed quantitatively by determining the extent of ^{125}I-labeled Ly1C6 binding to unfixed NRK cells, or by measuring the binding of the rabbit antibodies using ^{125}I-labeled protein A. Significant specific binding was only observed after permeabilization of the cells by methanol. Lactoperoxidase-catalyzed surface iodination of intact cells followed by immunoprecipitation (see below) also failed to detect the presence of antigens on the NRK plasma membrane.

Taken together, these results show that the antigens recognized by both the monoclonal and polyclonal antibodies were largely restricted to lysosomes in NRK cells.

Antigens Recognized by Anti-lysosome Antibodies

To identify the antigens recognized by Ly1C6 and the rabbit anti-lysosome antibodies, NRK cells were labeled with [^{35}S]-methionine, lysed in TX-114, and analyzed by immunoprecipitation. As shown in Fig. 4 (lane *1*), Ly1C6 precipitated a single labeled protein that migrated at 120 kD on SDS polyacrylamide gels. The migration was similar under both reducing or nonreducing conditions. In contrast, the rabbit antiserum immunoprecipitated two proteins of 100 and 80 kD (Fig. 4, lane *2*). The relative intensity of the two bands was variable, with some experiments showing greater labeling of the 100-kD band (as in Fig. 4), and others showing more equal labeling of the two species (e.g., Fig. 6).

The 120-, 100-, and 80-kD proteins were each judged to be integral membrane proteins because they partitioned almost quantitatively with the detergent phase when NRK lysates were partitioned in TX-114 according to the procedure of

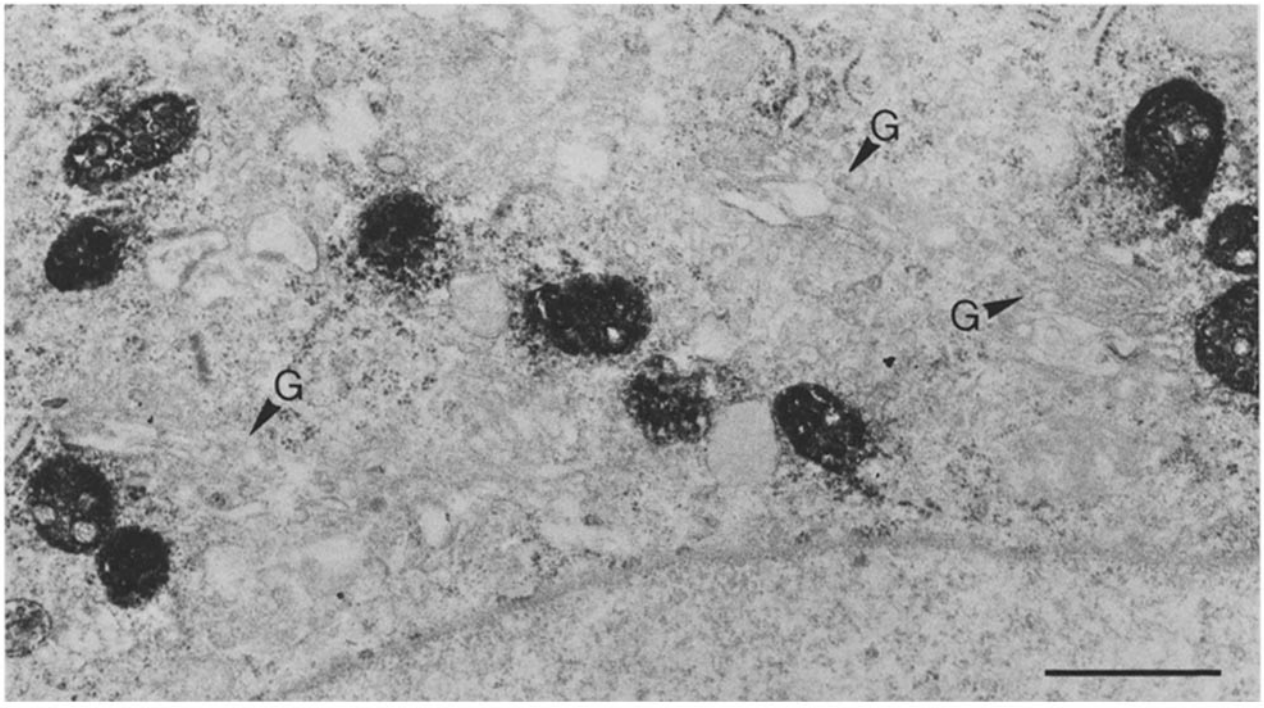

FIGURE 2 Immunoperoxidase staining of NRK cells using rabbit anti-lysosome antibody. The diaminobenzidine reaction produce was concentrated within vacuoles most often localized near the nucleus. Many of these vacuoles had multivesicular inclusions and, in general, had the morphological appearance of lysosomes. There was little or no staining of Golgi elements (G), the plasma membrane (not shown), or peripheral tubules and vesicles. Bar, 1.0 μm. × 24,000.

Bordier (23). This is illustrated in Fig. 4, where immunoprecipitations are shown using the monoclonal antibody Ly1C6 (lanes *1* and *4*), the rabbit anti-lysosome antibody (lanes *2* and *5*), and control rabbit antiserum (lanes *3* and *6*). The 120-, 100-, and 80-kD proteins were only detected in the detergent phase (lanes *1–3*). Although these antigens were not precipitated from the aqueous phase (lanes *4–6*), the rabbit anti-lysosome antiserum did recognize an 85-kD species (lane *5*) that was electrophoretically distinct from either the 100- or 80-kD protein. Conceivably, the 85-kD protein represents a soluble, proteolytic fragment of the 100-kD protein.

In addition to being integral membrane proteins, each of these antigens was found to be heavily glycosylated (see below). Accordingly, they were designated lysosomal membrane glycoproteins lgp120, lgp100, and lgp80.

Purification of lgp120

To better characterize the Ly1C6 antigen, and to obtain antibodies with a wider species reactivity (Ly1C6 reacted only with rat cells), lgp120 was purified by immunoaffinity chromatography. NRK cells, trace labeled with [^{35}S]methionine, were lysed in TX-114, and a high speed post-nuclear supernatant was prepared. The lysate was then phase separated and the detergent phase, which contained 27% of the solubilized protein (Table I), was incubated with Ly1C6 coupled to Sepharose 4B. Specifically adsorbed proteins were eluted with 0.5 M propionic acid and analyzed by SDS PAGE. As shown in Fig. 5, only a single polypeptide corresponding to lgp120 was obtained. The amount of lgp120 recovered accounted for ~0.05% of the solubilized protein (Table I).

Rabbits were immunized with the purified protein, and the antibody obtained exhibited an immunofluorescent staining pattern identical to that of Ly1C6. It was also judged to be monospecific for lgp120 by immunoprecipitation (see below) and by Western blots. Significantly, rabbit anti-lgp120 recognized not only the rat antigen, but also the homologous 120-kD protein in cultured mouse cells, e.g., J774 and NIH 3T3 cells. Being in general more efficient at precipitation than Ly1C6, the anti-lgp120 rabbit antiserum was used for all immunoprecipitation in subsequent experiments.

Glycosylation of lgp120, lgp100, and lgp80

To determine whether the lysosomal antigens were glycoproteins, digestion with the endoglycosidases Endo H and Endo F was performed. Endo F, which removes both mature (complex) and immature (high mannose) N-linked oligosaccharides, greatly reduced the apparent molecular weights of all [^{35}S]methionine-labeled proteins, (Fig. 6, lanes *3* and *6*). lgp120 gave a diffuse band in the 50–90-kD region that almost certainly still contained considerable carbohydrate. lgp100 and lgp80, on the other hand, yielded a single sharp band at 60 kD. These results suggested that each polypeptide contained multiple asparagine-linked oligosaccharide chains. Digestion with Endo H, which removes only immature N-linked oligosaccharide chains, decreased the apparent molecular weights of the three proteins only slightly (lanes *2* and *4*), indicating that most of their oligosaccharide chains were of the mature, or complex, type.

Further characterization of the glycosylation and processing of lgp120 was performed using J774 cells labeled with [^{3}H]-mannose or [^{35}S]methionine. A short pulse of [^{3}H]mannose (10 min) revealed that lgp120 was synthesized as a 90-kD precursor that was sensitive to both Endo H and Endo F (Fig. 7). After a 2 h chase in [^{3}H]mannose-free medium, most of the antigen was converted to the mature 120-kD form that was, as expected, insensitive to Endo H but fully sensitive to

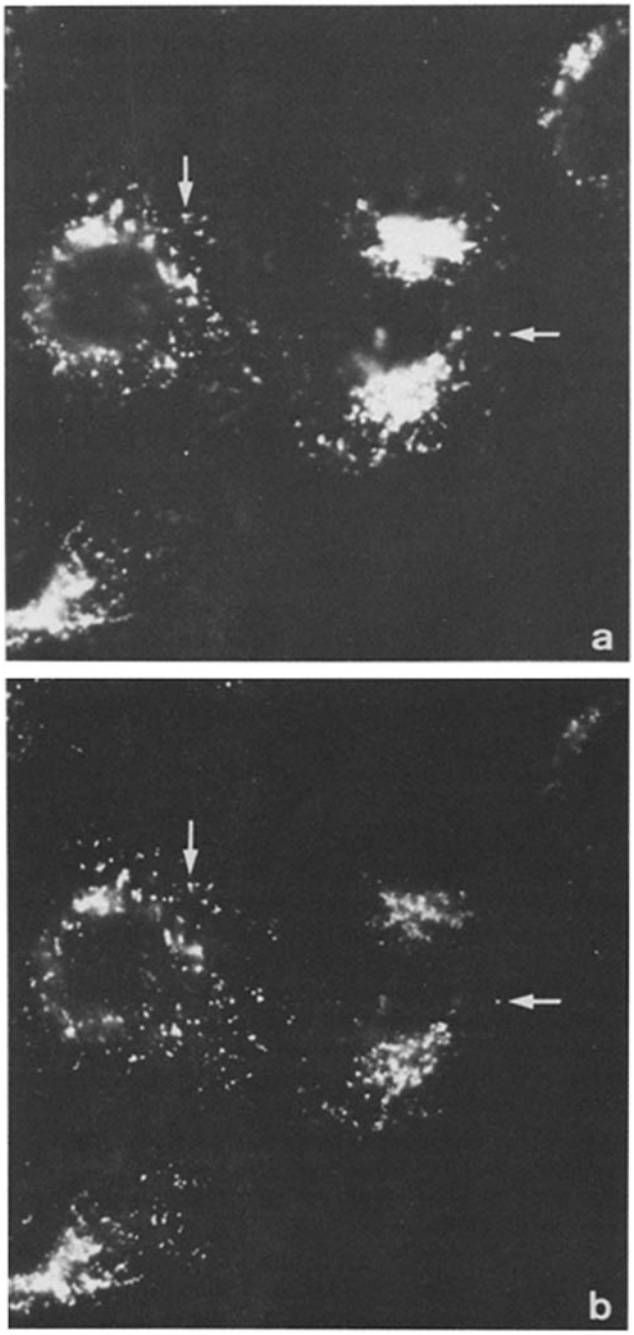

FIGURE 3 Co-localization of immunofluorescent staining using Ly1C6 and antibody to lysosomal acid phosphatase. NRK cells were analyzed by double immunofluorescent staining by using an affinity purified rabbit antibody to human lysosomal acid phosphatase (1:400 dilution) (*a*) and the mouse monoclonal antibody Ly1C6 (*b*). Rabbit and mouse IgG's were visualized using rhodamine- and fluorescein-labeled, affinity purified second antibodies, respectively. Strong co-localization of acid phosphatase-positive lysosomes and L1C6-positive structures was readily apparent (arrows). × 2,900.

Endo F. A minor 90-kD band, corresponding to unprocessed precursor, was completely sensitive to both glycosidases.

To determine the actual polypeptide size of lgp120, J774 cells were labeled in the presence of 10 μg/ml tunicamycin, an inhibitor that prevents the transfer of high-mannose oligosaccharides to nascent glycoproteins. After pulse labeling (5 min) with [^{35}S]methionine, immunoprecipitation with the

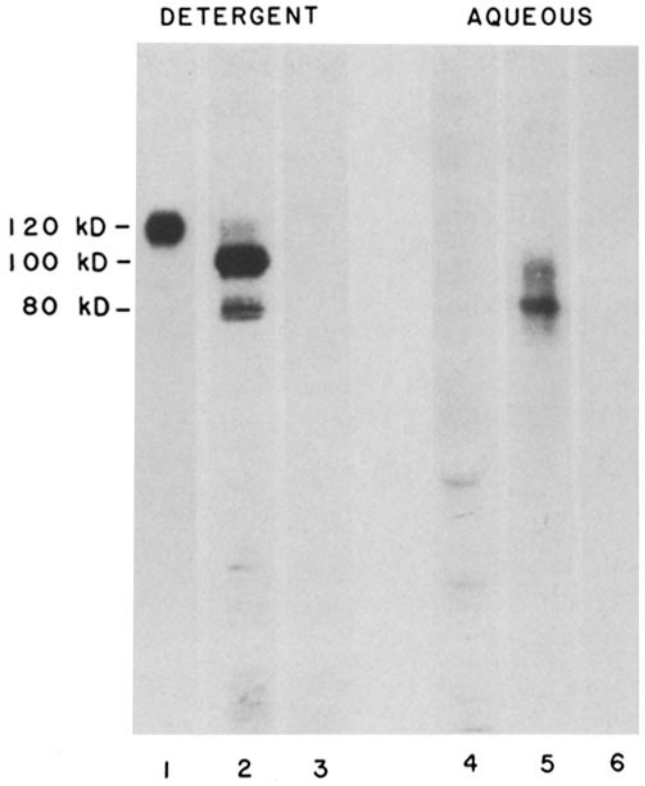

FIGURE 4 Phase separation of lgp120, lgp100, and lgp80 in Triton X-114. NRK cells were labeled overnight with [^{35}S]methionine, lysed in TX-114, and separated into detergent and aqueous phases, as described in Materials and Methods. Immunoprecipitations from the detergent phase (lanes *1–3*) and the aqueous phase (lanes *4–6*) were performed using the monoclonal antibody Ly1C6 (lanes *1* and *4*), the polyclonal anti-lysosomal membrane antibody (lanes *2* and *5*), and control rabbit serum (lanes *3* and *6*).

TABLE I

Affinity Purification of lgp120

Purification stage	Protein	[^{35}S]methionine	Solubilized protein
	mg	*cpm*	*%*
TX-114 lysate* (post-particulate)	158.2	1.57×10^8	100
TX-114 aqueous phase	114.6	1.15×10^8	72.4
TX-114 detergent phase	43.4	0.42×10^8	27.4
Purified protein	0.082‡	8.02×10^4	0.05

* Lysate was prepared from 12 confluent roller bottles of NRK cells, as described in Materials and Methods. One 100-mm dish of cells, labeled overnight with 0.5 mCi [^{35}S]methionine, was added as tracer. Lysate refers to post-particulate (45,000 *g*) supernatant after TX-114 solubilization.

‡ Calculated from [^{35}S]methionine cpm, assuming proteins in the detergent phase were at a specific activity of 9.76×10^5 cpm/mg protein. All other protein values given were measured using the Fluorescamine procedure.

rabbit anti-lgp120 antiserum detected a 42-kD polypeptide (Fig. 8, lane *T*). A control experiment using [^{3}H]mannose-labeled cells showed that mannose was not present in the 42-kD band (Fig. 7, lanes *4* and *8*). To estimate the actual number of oligosaccharide chains present, a time course of Endo H digestion was performed using as substrate the 90-kD precursor from [^{35}S]methionine-labeled J774 cells. As shown in Fig. 8, at least 18 digestion intermediates could be counted, indicating that lgp120 contains at least this number of asparagine-linked chains.

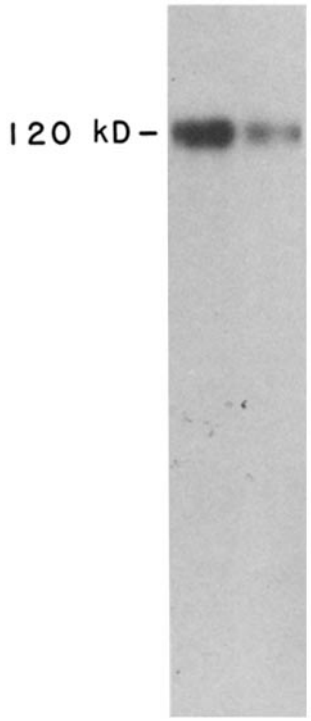

FIGURE 5 Purification of lgp120 by immunoaffinity chromatography. lgp120 was isolated from the detergent phase of TX-114 lysates of NRK cells and trace-labeled with [^{35}S]methionine, using the monoclonal antibody LY1C6 coupled to cyanogen bromide-activated Sepharose 4B. Aliquots of the affinity column eluate were precipitated with 10% trichloroacetic acid washed with ethanol/ether (1:1) and analyzed by electrophoresis and fluorography. The amount of sample added to the left lane was three times that added to the right lane.

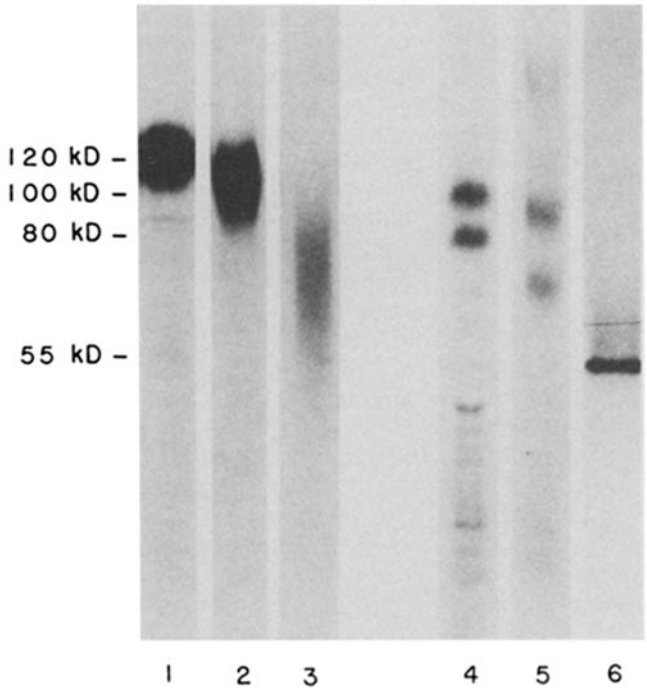

FIGURE 6 Glycosidase digestion of lgp120, lgp100, and lgp80. lgp120 (lanes *1–3*) and lgp100 and lgp80 (lanes *4–6*) were immunoprecipitated from [^{35}S]methionine-labeled NRK cells. Precipitates were incubated with Endo H (lanes *2* and *5*), with Endo F (lanes *3* and *6*), or without added glycosidase (lanes *1* and *4*) for 2 h at 37°C before electrophoresis. See Materials and Methods for details.

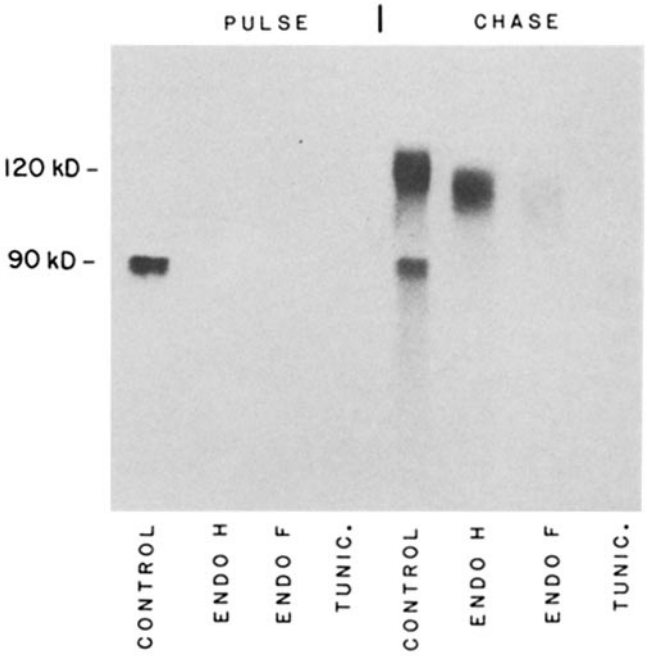

FIGURE 7 Glycosidase digestion of [^{3}H]mannose-labeled lgp120. lgp120 was immunoprecipitated from J774 cells labeled with [^{3}H]-mánnose in the presence or absence of tunicamycin (*TUNIC.*). Samples on left were from cells pulsed for 10 min and then chased in complete medium for 2 h before lysis. Precipitated proteins were incubated with Endo H, Endo F, or without glycosidase as indicated. Tunicamycin (10 μg/ml) was added 90 min before [^{3}H]mannose and was present throughout pulse and chase periods.

Neuraminidase Digestion of lgp120

The extent of sialylation of lgp120 was determined by neuraminidase digestion of antigen immunoprecipitated from [^{35}S]methionine-labeled J774 cells. Digested samples and controls were analyzed by two-dimensional isoelectric focusing and SDS PAGE (31). Undigested lgp120 was found to have a pI of <4.0, as it migrated at the very extreme of the acidic end of the focusing gel (Fig. 9*a*). After treatment with neuraminidase, however, the pI increased dramatically to 8.0–8.5 (Fig. 9*b*), whereas the apparent molecular weight of the digested antigen was reduced only slightly. These results demonstrated that lgp120 is highly anionic. Moreover, its net negative charge is derived mainly from numerous sialic acid residues, since its core polypeptide appears to be rather basic.

DISCUSSION

We have identified three highly glycosylated proteins of the lysosomal membrane—lgp120, lgp100, and lgp80—by producing monoclonal and polyclonal antibodies against highly purified preparations of lysosomal membranes. lgp120 was purified to homogeneity and found to be rich in sialic acid. Although we have yet to define the functions of any of the antigens, their analysis has already provided some insight into the structural properties of the lysosomal membrane.

Carbohydrate Content and Glycosylation

Thus far, the most distinguishing characteristic of these proteins is their high oligosaccharide content. Endo F digestion decreased their apparent molecular weights by 20–50 kD. Since the contribution of a single N-linked chain is ~2–4 kD, shifts of 20 kD suggest the removal of at least 5–10 chains per polypeptide. On the basis of the number of detectable digestion intermediates generated during glycosidase treatment, we estimated that lgp120 contains at least 18 asparagine-linked oligosaccharides. The actual polypeptide portion of this antigen was only 42 kD. Thus, at least one out of every 7–8 amino acids must be involved in generating a canonical three

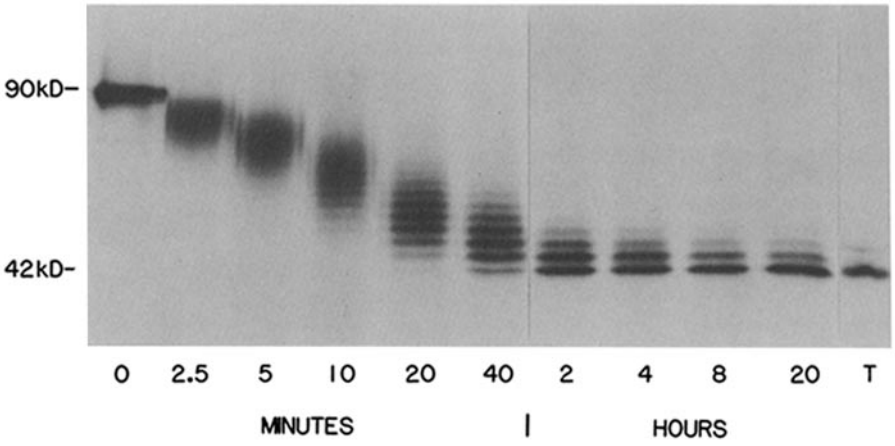

FIGURE 8 Timecourse of Endo H digestion of the lgp120 precursor. The 90-kD precursor of lgp120, immunoprecipitated from J774 cells labeled for 5 min with [^{35}S]methionine, was incubated with Endo H for the times indicated at 37°C. Up to 18 digestion intermediates could be counted as individual "ladder steps" at each timepoint. Lane *T* shows the 42-kD protein devoid of all N-linked oligosaccharides that is immunoprecipitated from tunicamycin-treated cells by anti-lgp120 antibody.

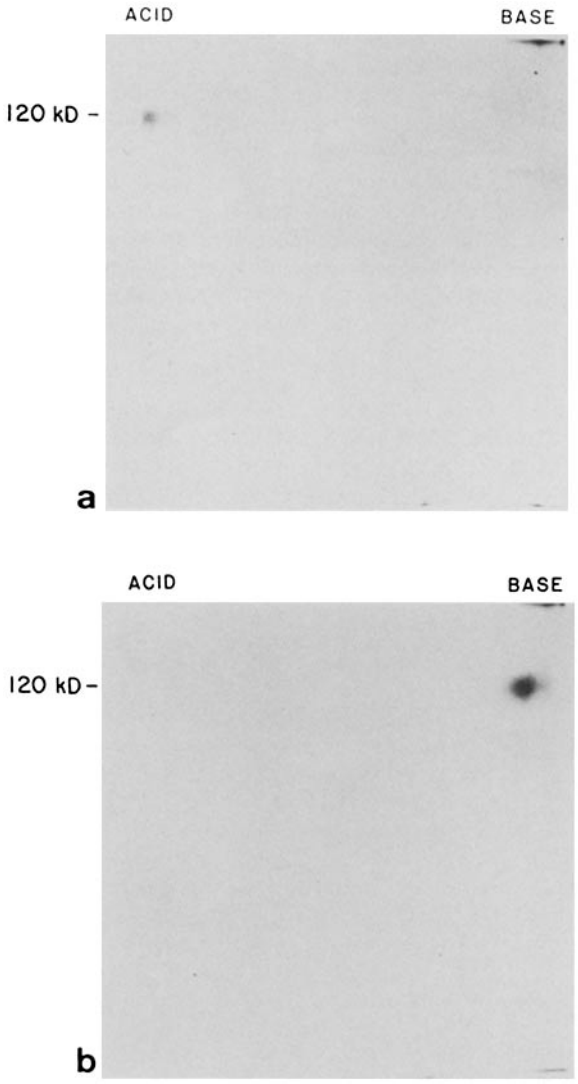

FIGURE 9 Neuraminidase digestion of lgp120. lgp120 immunoprecipitated from J774 cells labeled overnight with [^{35}S]methionine was eluted from *S. aureus* and incubated for 4 h at 37°C with or without neuraminidase. Samples were then analyzed by two-dimensional isoelectric focusing SDS PAGE (34). (*a*) Untreated lgp120 migrated at the extreme acidic end of the isoelectric focusing gel, indicating a pI of <4.0. A faint band corresponding to the 90-kD lgp120 precursor is visible at the basic end of the gel. (*b*) Neuraminidase-treated lpg120 migrated at the basic end of the isoelectric focusing gel, indicating a pI of 8.0–8.5.

amino acid N-linked glycosylation signal. In addition, since exhaustive Endo F digestion of mature [^{35}S]methionine-labeled lgp120 generated a broad 50–90-kD band rather than a sharp band at 42 kD, it is conceivable that lgp120 also contains O-linked oligosaccharides. This possibility was supported by the finding that Endo F could completely remove [^{3}H]mannose from mature lgp120 (Fig. 7). Since N-linked but not O-linked oligosaccharides contain mannose residues, the removal of all mannose label suggests that all N-linked chains were cleaved. Thus, the failure of Endo F to convert lgp120 to its 42-kD polypeptide core is probably not due to incomplete digestion of N-linked chains, but due to the presence of Endo F–resistant, e.g., O-linked, glycosides.

In addition to the 18 or more N-linked oligosaccharide chains (as well as possible O-linked sugars), lgp120 was found to contain a remarkable amount of sialic acid. Digestion of the mature glycoprotein with neuraminidase increased its pI from <4 to >8. The Endo H–sensitive 90-kD precursor form of lgp120 (barely visible in Fig. 9) had a pI of ~8 and was not affected by neuraminidase treatment. Although we have not examined the neuraminidase sensitivity of lgp100 and lgp80, it is possible that they too will be rich in sialic acid. Kato (34) has purified by chromatography on lectin and anion exchange columns two glycoproteins (115 kD and 105 kD) associated with rat liver lysosome membranes; both were found to have acidic pI's (<4) and were rich in sialic acid. The relationship between these two glycoproteins and the antigens described here is not yet known. Endo F treatment of lgp100 and lgp80 yielded a single, apparently nonglycosylated band at 60 kD. This result indicates that lgp80 cannot be a proteolytic fragment of lgp100, but further studies are needed to determine whether both glycoproteins share a common polypeptide moiety.

Lysosomal Biogenesis

During the past few years, there has been considerable interest in the problem of lysosomal biogenesis. Thus far, most work has concentrated on the synthesis and targeting of lysosomal hydrolases. It is now well established that most if not all lysosomal enzymes are, like secretory proteins, synthesized and core glycosylated in the rough endoplasmic reticulum (35, 36) and transported to the Golgi where mannose residues are phosphorylated in the 6 position (37). The modified sugars then serve as recognition markers for a specific receptor for mannose-6-phosphate in the Golgi that, in turn, mediates enzyme transport to lysosomes (1). At least in some

cell types, the enzyme-receptor complex is thought to leave the Golgi via *cis* cisternae (22, 38).

Our finding that lysosomal membrane proteins contain complex (Endo H–resistant) asparagine-linked oligosaccharides and sialic acid has important implications for the biogenesis of the lysosomal membrane. Like glycoproteins of the plasma membrane, lysosomal membrane glycoproteins are apparently synthesized and core glycosylated in the rough endoplasmic reticulum, transported to the Golgi, and terminally glycosylated. Since lgp120 was also heavily sialylated and may contain O-linked glycosides, the processing of at least some lysosomal membrane proteins must involve transport to a "late" Golgi compartment represented by cisternae at the *trans* side of the Golgi stack (39–42).

The molecular signals that control the targeting of lysosomal membrane proteins remain to be identified. Although we do not yet have evidence for their phosphorylation, it is conceivable that lysosomal membrane glycoproteins, like lysosomal enzymes, rely on the mannose-6-phosphate recognition system. However, as discussed above, this mechanism is in conflict with the concept that the mannose-6-phosphate receptor and proteins such as lgp120 may exit from opposite sides of the Golgi stack. A second possibility is that lysosomal membrane glycoproteins use the alternative but as yet uncharacterized receptor system that mediates the targeting of acid hydrolases in cells that lack the mannose-6-phosphate receptor (43). Alternatively, lysosomal membrane proteins may be encoded with the appropriate structural information to signal their own transport to lysosomes, and indeed may comprise the mannose-6-phosphate receptor-independent pathway.

Functions of Lysosomal Membrane Proteins

Lysosomes carry out a variety of important functions, a number of which are likely to be mediated by membrane proteins. Among the best characterized of these functions is acidification. Lysosomes lower their internal pH through the activity an an ATP-driven H^+ pump that is mechanistically related to H^+ pumps found in endosomes, coated vesicles, Golgi membranes, and acidic secretory organelles such as the chromaffin granule (5, 32, 33). Although the lysosomal H^+-ATPase remains to be identified, it is conceivable that one (or more) of the antigens described here might be related to lysosomal acidification. Of particular interest in this regard, we have begun to characterize a series of monoclonal antibodies that were generated by immunization with highly purified bovine adrenal chromaffin granule membranes and that recognize four additional N-glycosylated lysosomal membrane proteins (150, 55, 30, and 20 kD) (manuscript in preparation). Since lysosomes and secretory chromaffin granules would be expected to share few (if any) functional activities aside from acidification (33), the recognized glycoproteins may be involved in the generation of transmembrane pH gradients. Reggio et al. (17) recently identified a 100-kD antigen in rat liver lysosomal membrane an antibody to which reacted on Western blots with a putative H^+, K^+-ATPase from porcine gastric mucosa. Using antiserum kindly supplied by Daniel Louvard, we have found that their protein appears to be immunologically related lgp100. However, unlike antibodies to lgp100 and lgp80, Reggio et al.'s anti–100-kD antiserum displays detectable reactivity in Golgi cisternae by immunoperoxidase cytochemistry (17). It remains to be determined whether either of these antigens is related to the lysosomal H^+-ATPase, which is mechanistically distinct from the mucosal K^+, H^+-ATPase (5, 32, 33).

In addition to proton transport, the lysosomal membrane must be able to transport to the cytosol the products of intralysosomal digestion. Presumably, hydrophilic molecules such as amino acids, dipeptides, mono- and disaccharides (6, 7), and cobalamin (10) require transport systems to permit their egress.

Although the role of carbohydrate in general and sialic acid in particular on lysosomal membrane proteins is not clear, the extent of the glycosylation suggests strongly that it serves one or more crucial functions. For example, extensive glycosylation may protect lysosomal membrane components from degradation by hydrolytic enzymes. Preliminary results suggest that lgp120, like many plasma membrane proteins, is relatively long lived ($t_{1/2} > 15$ h in J774 cells). In addition, the extensive sialylation of glycoproteins such as lgp120 could help maintain the acidic internal pH of lysosomes by serving as impermeant polyanions that may act to establish a Donnan potential for protons (44).

Finally, a variety of functional activities must also be associated with the cytoplasmic surface of the lysosome. In particular, membrane components must exist that control the specific interaction and fusion of lysosomes with other organelles such as endosomes. Putative specific binding sites for actin and/or tubulin (45, 46) may be relevant to these activities. We expect that the immunological approach that has thus far been used only to define the structure of organelle membrane proteins will be adaptable to define their functions as well (47).

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