

Identification of Two Lysosomal Membrane Glycoproteins

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ABSTRACT Two murine lysosome-associated membrane proteins, LAMP-1 of 105,000–115,000 D and LAMP-2 of 100,000–110,000 D, have been identified by monoclonal antibodies that bind specifically to lysosomal membranes. Both glycoproteins were distinguished as integral membrane components solubilized by detergent solutions but not by various chaotropic agents. The lysosome localization was demonstrated by indirect immunofluorescent staining, co-localization of the antigen to sites of acridine orange uptake, and immunoelectron microscopy. Antibody binding was predominantly located at the limiting lysosomal membrane, distinctly separated from colloidal gold-labeled alpha-2-macroglobulin accumulated in the lumen during prolonged incubation. LAMP-1 and LAMP-2 also appeared to be present in low concentrations on Golgi *trans*-elements but were not detected in receptosomes marked by the presence of newly endocytosed^malpha-2-macroglobulin, or in other cellular structures. LAMP-1 and LAMP-2 were distinguished as different molecules by two-dimensional gel analysis, ¹²⁵I-tryptic peptide mapping, and sequential immunoprecipitations of ¹²⁵I-labeled cell extracts. Both glycoproteins were synthesized as a precursor protein of ~90,000 D, and showed a marked heterogeneity of apparent molecular weight expression in different cell lines. LAMP-2 was closely related or identical to the macrophage antigen, MAC-3, as indicated by antibody adsorption and tryptic peptide mapping. It is postulated that these glycoproteins, as major protein constituents of the lysosomal membrane, have important roles in lysosomal structure and function.

Lysosomes are membrane-bound vacuoles rich in hydrolytic enzymes that act as sites for the digestion of foreign materials and for specialized autolytic phenomena (1). The importance of lysosomal function in cellular metabolism is reflected by the large number of genetic abnormalities attributed to lysosomal defects (2). Extensive progress has been made in understanding some aspects of lysosomal function, in particular, the synthesis and assembly of the acid hydrolases, pathways of endocytosis, and the role of compartment acidification (for reviews, see references 1, 3, 4, and 5).

Membrane glycoproteins presumably have important roles in lysosomal structure and function, and lysosomes have been examined for their content of unique components. Using a method involving intravacuolar iodination in living cells, Muller et al. (6) studied the composition of phagolysosomes in cultured macrophages; these experiments did not reveal any major differences between the membrane proteins of

phagolysosomes and those of the plasma membrane. On the other hand, Burnside and Schneider (7) and Ohsumi et al. (8) partially purified lysosomes from rat liver and found that the lysosomal membranes differed in polypeptide composition from the plasma membrane, with characteristic glycoproteins in the 60,000 and 90,000–110,000-D range. Reggio et al. (9) have recently reported a 100,000-D rat glycoprotein that is antigenically cross-reactive with H⁺, K⁺-ATPase of porcine gastric mucosa and is present in lysosomes and a variety of other cell compartments that are acidified by an ATP-driven pump.

We here report two lysosome-associated membrane proteins (LAMP-1 and LAMP-2).¹ The two molecules were lo-

¹ *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; EGS, ethyldimethylaminopropyl carbodi-imide, glutaraldehyde, saponin; LAMP-1 and LAMP-2, lysosome-associated membrane proteins; Nonidet P-40, NP-40.

calized primarily on the periphery of the lysosome and appear to be constituents of the lysosomal membrane. The molecules were first recognized in studies designed to identify and characterize major membrane glycoproteins of mouse embryo 3T3 tissue culture cells (10). LAMP-1 was previously purified and was found to comprise ~0.1% of total cell protein, as measured by the yield of purified protein, with more than 10⁶ antibody binding sites per cell (11). The lysosomal membrane localization of the molecules was revealed by studies using indirect immunofluorescence, co-localization with acridine orange, and immunoelectron microscopy. The two glycoproteins were similar in several ways, but were distinguished as different molecules by their molecular and antigenic properties. In addition, we find that the macrophage differentiation antigen MAC-3 (12) is highly homologous to LAMP-2.

MATERIALS AND METHODS

Production of Monoclonal Antibodies: Monoclonal antibody ABL-93 (anti-LAMP-2) was prepared against glycoproteins purified by lectin affinity chromatography. Approximately 5 × 10⁸ Balb/c 3T3 cells were collected by centrifugation and lysed in 5.0 ml extraction buffer (5 mM Tris-HCl, 1 mM EDTA, 400 mM KCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pH 7.2), as previously described (10). The extract, containing 46.5 mg of protein, was dialyzed against 20 mM Tris-HCl, 100 mM NaCl, and 0.2% (wt/vol) Triton X-100, pH 7.6, and centrifuged for 1 h at 100,000 g. The supernatant was adjusted to 1 mM Mg⁺⁺, Ca⁺⁺, and Mn⁺⁺, and applied to an affinity column containing 2 ml of lentil-lectin Sepharose (Pharmacia Inc., Piscataway, NJ) that had been cross-linked with glutaraldehyde (13). Bound proteins were eluted with 0.3 M α -methyl-D-mannoside (Sigma Chemical Co., St. Louis, MO) in the same buffer, and were dialyzed against 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 100 mM NaCl, 0.2% Triton X-100, and a female Sprague-Dawley rat was injected subcutaneously and intraperitoneally with 50 μ g of the purified glycoproteins in complete Freund's adjuvant. The animal was boosted on days 14, 28, and 42 with subcutaneous and intraperitoneal injections of 50 μ g glycoprotein in incomplete Freund's adjuvant. The rat was injected intraperitoneally with 50 μ g glycoproteins in incomplete adjuvant on day 61, and cell fusion was performed three days later with P3x63-Ag8-653 mouse myeloma cells (14) as previously described (10). Hybridomas were selected by antibody precipitation of a specific protein from the lentil-lectin fraction labeled with ¹²⁵I. The isotype of ABL-93 is IgG2a as found by Ouchterlony double diffusion analysis using class specific antisera.

Monoclonal antibody 1D4B (anti-LAMP-1), IgG2a, was prepared as previously described (10). Monoclonal antibody M3/84 (anti-MAC-3) (15) was obtained from American Tissue Type Culture (Rockville, MD).

Light Microscopic Immunocytochemistry: NIH 3T3 or HaNIH cells were grown to 50% confluency on marked coverslips or in 35-mm culture dishes in Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum at 37°C and fixed *in situ* with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. The cells were incubated in hybridoma supernatant medium with 0.1% saponin (Sigma Chemical Co.) for 15 min at room temperature, washed, and further incubated with affinity-purified goat anti-rat IgG conjugated to rhodamine (Jackson ImmunoResearch, Avondale, PA) for an additional 15 min.

Double-label experiments with rhodamine alpha-2-macroglobulin were performed as follows: Cells were incubated with 50 μ g/ml rhodamine-labeled alpha-2-macroglobulin (16) at 4°C for 30 min, washed, and warmed to 37°C for 5 min. The cells were then fixed in paraformaldehyde for 10 min and treated with 0.1% Triton X-100 (Sigma Chemical Co.) for 3 min. This was followed by incubation in hybridoma culture supernatant with saponin and affinity-purified goat anti-rat IgG conjugated to fluorescein (Jackson ImmunoResearch), as described above. Cells were examined and photographed using a Zeiss RA microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epiillumination and a 63 \times , numerical aperture 1.4, oil planapochromat objective. Photographs were made with Kodak Tri-X film and Diafine development.

Co-localization of Acridine Orange with LAMP-1 and LAMP-2: Cells were propagated on marked coverslips as described above, and acridine orange (Sigma Chemical Co.) was added at 5–10 μ g/ml from a stock solution (1 mg/ml in H₂O). Cells were incubated for 10 min at 37°C, washed with medium, and viewed and photographed immediately by epifluorescence using a Zeiss RA microscope with rhodamine filters. When dishes

were used, the cells were mounted in medium under a coverslip and viewed with a 40 \times , numerical aperture 1.0, planapochromat objective. The cells were marked in the plastic dish by scratching a 0.5-mm square on the bottom of the dish using a needle. The coverslips or dishes were then washed in PBS and fixed either in 100% methanol (10 min at 23°C) or in 3.7% paraformaldehyde in PBS (10 min at 23°C). This was followed by incubation with monoclonal antibodies 1D4B, ABL-93, or control antibody P3x63Ag8 (30 min, 23°C). When cells were fixed with formaldehyde, the antibody incubations included 0.1% saponin. The cells were then washed and incubated in affinity-purified rabbit or goat anti-rat IgG conjugated to rhodamine (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD; Jackson ImmunoResearch) (50 μ g/ml, 30 min, 23°C). The cells were then mounted in glycerol and the same fields photographed previously with acridine orange were viewed and photographed again by rhodamine epifluorescence. Photography was performed using either 3M 640-T film or Kodak Tri-X film with Diafine development.

Electron Microscopic Immunocytochemistry: Cells propagated in 35-mm plastic dishes were fixed with 0.1% glutaraldehyde and processed as previously described (17) for the ethyldimethylaminopropyl carbodiimide, glutaraldehyde, saponin (EGS) procedure. In some experiments, the cells were preincubated with colloidal gold-labeled alpha-2-macroglobulin for 60 min at 4°C and then warmed to 37°C for 5 or 60 min before being fixed and processed. Preparation of the gold-labeled protein was previously described (18). Antibody bound to antigen was localized by the ferritin bridge method: The cells were incubated with tissue culture supernatant media of 1D4B, ABL-93, or a rat monoclonal antibody that did not react with these cells (30 min, 23°C) in media containing 10% fetal bovine serum and 0.1% saponin. Rabbit anti-rat IgG (Cappel Laboratories, West Chester, PA), affinity-purified rat anti-ferritin (Jackson ImmunoResearch), and horse spleen ferritin were then added. Each incubation was performed in the continuous presence of 0.1% saponin and 4 mg/ml normal rabbit globulin. The cells were secondarily fixed, dehydrated, and embedded as described (17). Thin sections were counterstained using lead citrate and bismuth subnitrate, and examined using a Philips 400T electron microscope at 40 kV.

Cell Extraction and Protein Immunoprecipitation: Metabolically labeled cells were extracted with a lysis buffer containing 10 mM Tris-HCl, pH 7.5, 0.5% Nonidet-P40 (NP-40, Particle Data Inc., Elmhurst, IL), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.15 M NaCl (10). After 30 min on ice, the lysate was freeze-thawed three times, and the detergent-insoluble material was removed by centrifugation at 100,000 g for 60 min at 4°C. The soluble extract containing 1–5 × 10⁶ acid precipitable cpm was incubated with 200 μ l monoclonal antibody from tissue culture supernatants for 1 h at 4°C followed by addition of goat anti-rat second antibody and incubation on ice for 5 h. The antibody-antigen complexes were washed twice with 20 mM Tris-HCl, pH 7.6 containing 2.5 M KCl, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, and once with 20 mM Tris HCl, pH 7.6.

Extraction of LAMP-1 and LAMP-2: HaNIH cells grown to 90% confluence in T-150 flasks were preincubated with 6 ml of methionine-free media (Gibco Laboratories, Grand Island, NY) for 2 h at 37°C. The cells were incubated for 4 h with 6 ml of methionine-free media supplemented with 150 μ Ci/ml [³⁵S]methionine (Amersham Corp., Arlington Heights, IL), washed with warm Hank's buffered saline, and incubated an additional 2 h with DME containing 10% fetal bovine serum. After washing with Hank's buffered saline, cells were collected by scraping into a conical centrifuge tube, suspended in Hank's buffered saline, and aliquoted into seven preweighed conical tubes. After re-pelleting the cells, various extraction agents were added to the pellet at a ratio of extraction agent/cell pellet = 10:1 (wt/wt), as follows: A, Hank's buffered saline; B, 1.0% Triton X-100; C, 5 mM EDTA with three cycles of freeze-thaw; D, 1.0 M KI; E, 0.5 M guanidine HCl; F, 1.0 M urea; and G, 0.5% NP-40. To all of these, phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM. After incubation at room temperature for 30 min, the cells were centrifuged at 100,000 g for 1 h at 4°C. The supernatants were dialyzed exhaustively against lysis buffer (0.5% NP-40, 5 mM EDTA, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) and centrifuged at 100,000 g for 1 h at 4°C. The pellets from the first 100,000 g centrifugation were extracted a second time with lysis buffer, and the suspension was centrifuged at 100,000 g. The supernatant and the pellet extracts were incubated with the 1D4B or ABL-93 monoclonal antibodies, and the immune complexes were processed and analyzed by SDS PAGE as described (10).

Cells: NIH 3T3 cells (19) were obtained from Dr. Don Blair of the Frederick Cancer Institute, MD. HaNIH cells (20) and P388D1 (21) were obtained as previously described (10).

Other Procedures: The following procedures have been described: two-dimensional gel electrophoresis (22), biosynthetic labeling with [³⁵S]methionine (11), iodination of proteins with chloramine-T (23), SDS PAGE (24), and fluorography (25).

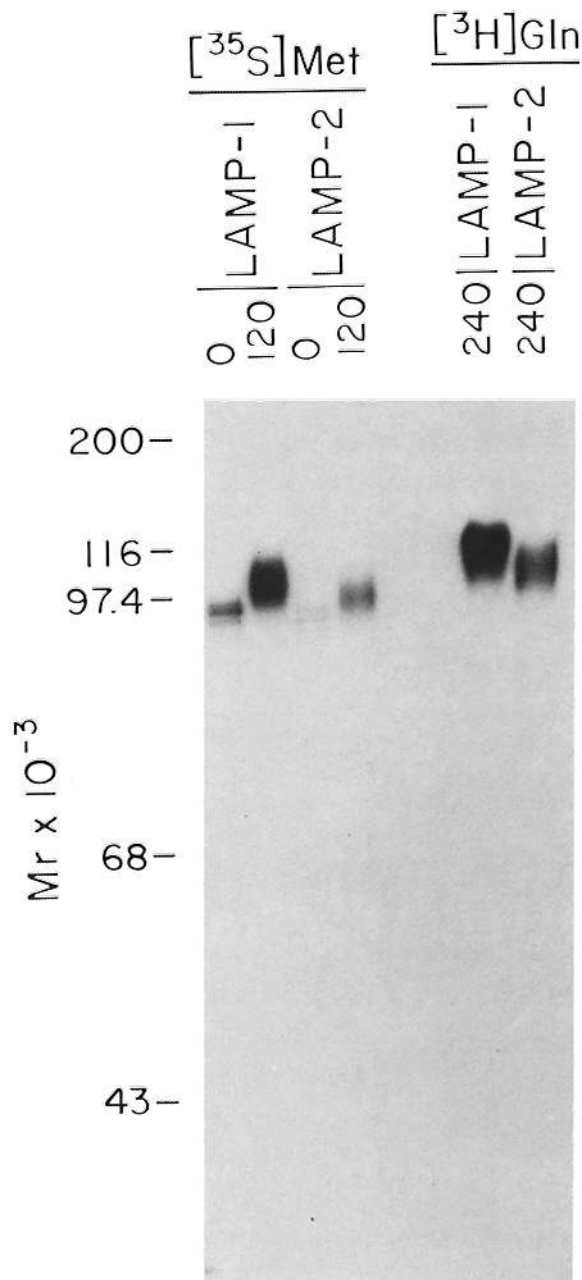


FIGURE 1 Immunoprecipitation of LAMP-1 and LAMP-2. HaNIH cells grown in T-75 cm² flasks to 80% confluence were washed with 10 ml of warm Earle's salt solution (Gibco Laboratories) and incubated for 2 h at 37°C with 10 ml of DME without methionine (Gibco Laboratories). Cells were then incubated for 5 min at 37°C with 4 ml of methionine-free media containing 125 μ Ci/ml of [³⁵S]-methionine (Amersham Corp.). Cells were collected immediately (T = 0) and extracted as described in Materials and Methods, or they were washed with 10 ml of warm Earle's salt solution and incubated for 2 h in DME with 10% fetal bovine serum. These cells were then collected and extracted. [³H]glucosamine labeling was performed in the same way except that the cells were incubated for 4 h at 37°C with 4 ml of glutamine-free DME (Gibco Laboratories) containing 50 μ Ci/ml of [³H]glucosamine (New England Nuclear, Boston, MA). The radiolabeled proteins were immunoprecipitated with the 1D4B and ABL-93 antibodies and analyzed by discontinuous SDS PAGE under nonreducing conditions. The labeled proteins were visualized by fluorography with Kodak XAR film. Molecular weight standards were: myosin, *M_r* 200,000; B-galactosidase, *M_r* 116,000; phosphorylase B, *M_r* 97,400; BSA, *M_r*

RESULTS

Biosynthetic Labeling and Immunoprecipitation of LAMP-1 and LAMP-2

The antigens recognized by anti-LAMP-1 and anti-LAMP-2 monoclonal antibodies were identified by immunoprecipitation of biosynthetically labeled proteins. Antigens were labeled in two ways: (a) cells were pulsed with [³⁵S]methionine for 5 min and collected immediately or incubated for 120 min in the presence of unlabeled methionine; (b) cells were incubated for 4 h with [³H]glucosamine and chased for 120 min in the presence of unlabeled glucosamine.

Polypeptides labeled during a 5-min incubation with [³⁵S]-methionine and immunoprecipitated with the 1D4B or ABL-93 antibodies both showed an apparent *M_r* of ~90,000 (Fig. 1). Mature LAMP-1 labeled with [³⁵S]methionine for 5 min and immunoprecipitated after a 2-h chase was 105,000–115,000 in apparent molecular weight, and LAMP-2, 100,000–110,000; the results were the same under reducing and nonreducing conditions. These findings were confirmed by a kinetic analysis of protein synthesis (data not shown). The amount of [³⁵S]methionine immunoprecipitated as LAMP-1 was consistently greater than that immunoprecipitated as LAMP-2.

The mature glycoproteins were readily labeled with [³H]-glucosamine, with the intensity of labeling similar for both glycoproteins. A remarkable finding repeatedly observed was that the apparent molecular weight of the sugar-labeled glycoprotein appeared greater than that of the [³⁵S]methionine-labeled specimen (Fig. 1). In other studies, we found that the heterogeneity in apparent molecular weight of the molecules results from posttranslational processing with marked differences in either the composition or number of oligosaccharide chains of different molecules (26). It appeared that a small fraction of the protein labeled with [³⁵S]methionine contained a majority of the sugar residues labeled by [³H]glucosamine; this heavily glycosylated fraction correspondingly migrated at higher apparent molecular weights. In accord with this explanation, the purified glycoproteins detected by staining with silver or Coomassie Brilliant Blue coincided with the [³⁵S]-methionine-labeled glycoprotein bands (data not shown). These results attest to an extreme heterogeneity of both LAMP-1 and LAMP-2 in oligosaccharide composition.

Two-Dimensional Gel Electrophoresis

LAMP-1 and LAMP-2 were compared by two-dimensional gel electrophoresis of proteins isolated by immunoprecipitation from cells labeled with [³⁵S]methionine. The two glycoproteins differed in their isoelectric point. LAMP-1 demonstrated marked isoelectric heterogeneity with more than 16 distinct fractions between pH 4.1 and 7.0 (Fig. 2). In contrast, LAMP-2 contained many fewer fractions focusing between pH 4.1 and 5.2.

LAMP-1 and LAMP-2 As Integral Membrane Glycoproteins

HaNIH cells radiolabeled with [³⁵S]methionine were extracted by a variety of chaotropic agents and nonionic detergents as described in Materials and Methods. Both the super-

68,000; RNA polymerase subunit, *M_r* 43,000; and chymotrypsin, *M_r* 23,000.

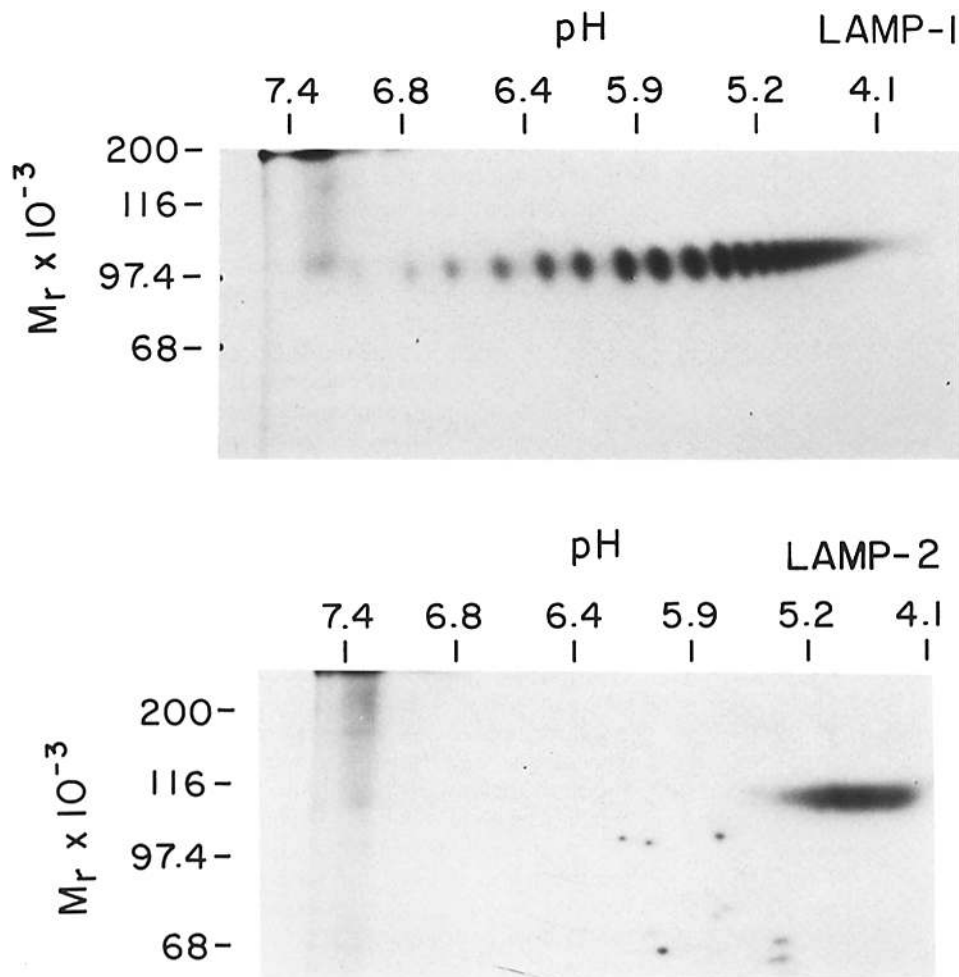


FIGURE 2 Two-dimensional electrophoresis of immunoprecipitated LAMP-1 and LAMP-2 antigens metabolically labeled with [^{35}S]methionine. HaNIH cells were labeled with [^{35}S]methionine and for 120 min as described in Fig. 1. LAMP-1 and LAMP-2 were immunoprecipitated from 5×10^6 dpm of acid-precipitable protein. The immunoprecipitates were analyzed by two-dimensional gel electrophoresis. The gels were visualized by fluorography with Kodak XAR-5 film.

natant and pellet fractions were analyzed for the presence of LAMP-1 and LAMP-2 by immunoprecipitation and SDS PAGE (data not shown). Both glycoproteins were completely solubilized by the detergents Triton X-100 or NP-40. In contrast, freeze-thawing in low ionic strength buffer containing EDTA, or treatment with chaotropic agents such as KI, guanidine HCl, or urea, failed to extract LAMP-1 or LAMP-2, with the majority of the antigen remaining in the pellet.

Lysosomal Localization of LAMP-1 and LAMP-2

Antibodies bound to LAMP-1 and LAMP-2 and visualized by indirect immunofluorescence light microscopy were present in a perinuclear "ring" pattern that suggested localization of the glycoproteins at the perimeters of lysosomes (Fig. 3, *A* and *B*). The patterns with LAMP-1 and LAMP-2 were indistinguishable.

The site of antibody binding was compared with the localization of acridine orange, a supravital dye known to accumulate in lysosomes (27). Cells treated with acridine orange were photographed and then fixed and analyzed by indirect immunofluorescence for binding of the monoclonal antibodies. The localizations of acridine orange and the antibodies to LAMP-1 and LAMP-2 were very similar (Fig. 3, *C*, *D*, and *E*). There was no localization of the control antibody (P3x63Ag8) indicating that the fluorescence seen with 1D4B or ABL-93 was not due to residual acridine orange or non-specific binding of antibodies. The same results were obtained when the experiment was repeated with HaNIH cells.

Electron Microscopy

Electron microscopy with ferritin bridge labeling showed extensive localization of the two glycoproteins just beneath the limiting lysosomal membrane of large "dense body" lysosomes and smaller multivesicular lysosomes (Fig. 4*A*). Small lysosomes on the *trans*-face of the Golgi stacks and some Golgi *trans*-elements also showed labeling (Fig. 4, *B* and *C*). Control samples with normal rat globulin showed only low background levels of labeling (Fig. 4*D*).

Other cellular structures, including the plasma membrane, coated pits, and structures with the morphological appearance of receptosomes, showed no significant labeling of both LAMP-1 and LAMP-2.

Localization Compared with Alpha-2-macroglobulin

Many ligands, such as alpha-2-macroglobulin, enter cells through coated pits and receptosomes, and are then delivered to lysosomes. The antibodies to LAMP-1 and LAMP-2 provided an opportunity to investigate whether newly formed receptosomes contained these lysosomal membrane markers. Previous studies had shown that receptosomes lack detectable lysosomal enzyme activities (28, 29). Thus, the absence of these LAMP markers in receptosomes would be another criterion for the nonlysosomal nature of receptosomes.

In one set of experiments, the intracellular localization of fluorescein-labeled anti-LAMP-1 was compared with that of

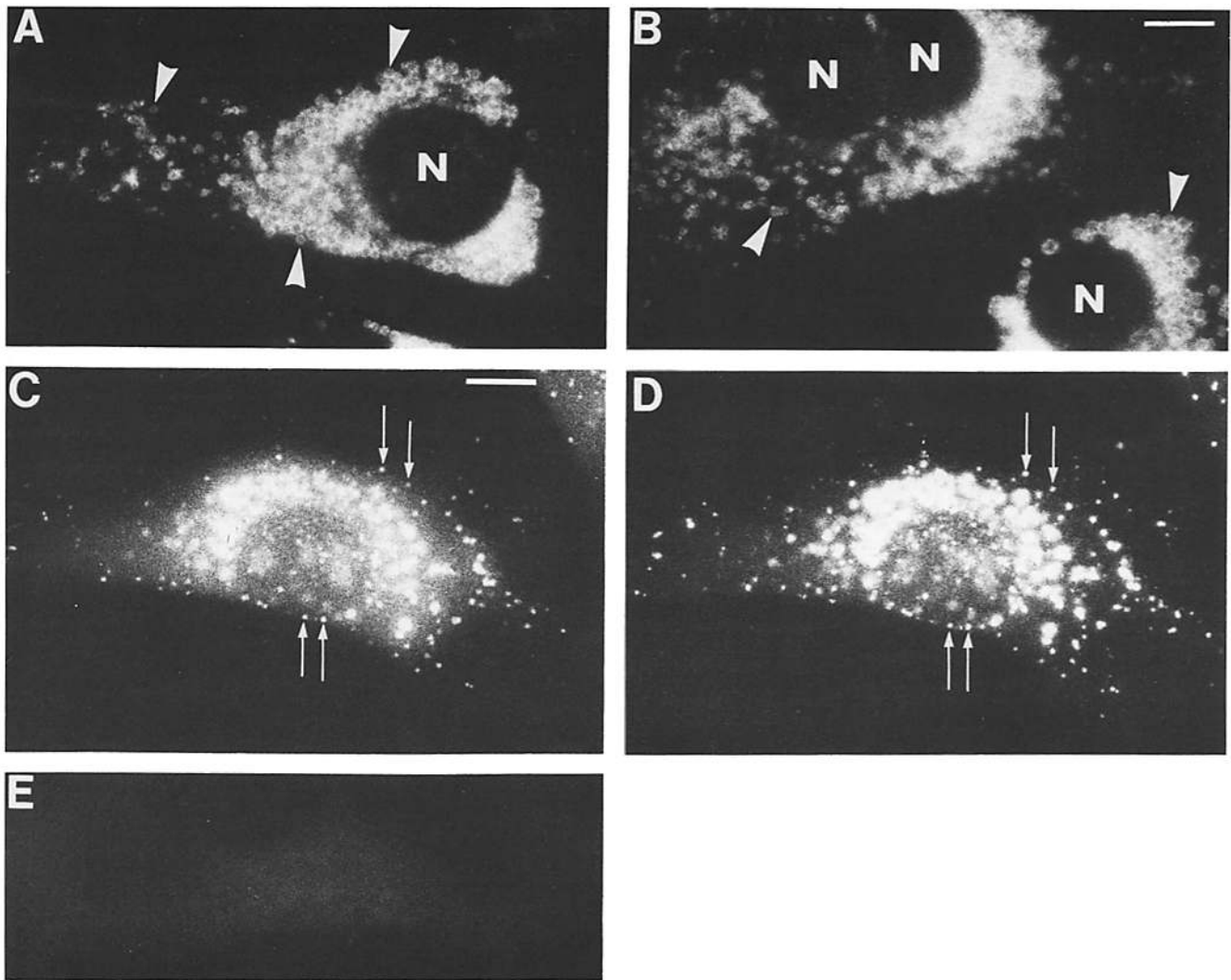


FIGURE 3 Immunocytochemical localization of LAMP-1 and LAMP-2 and co-localization with acridine orange. NIH 3T3 cells were fixed and processed using formaldehyde-saponin with rhodamine epifluorescence (A and B) as described in Materials and Methods. The fluorescence patterns of ABL-93 (A) and 1D4B (B) show a "ring" pattern at the perimeters of lysosomes, some of which are labeled in this figure with arrowheads. Co-localization with acridine orange (C and D) was as described in Materials and Methods. The cells were photographed using epifluorescence (C). They were then fixed in formaldehyde, incubated with 1D4B followed by anti-rat rhodamine in the presence of saponin, and then rephotographed using rhodamine epifluorescence (D). Examples of the almost exact coincidence of acridine orange and 1D4B are shown by arrows. There was no fluorescence localization with a control nonreactive monoclonal antibody, P3X63Ag8 (E). A, ABL-93. B, 1D4B. Bar in B, 5 μm . Bar in C, 10 μm . (A and B) $\times 2,050$. (C, D and E) $\times 1,000$.

recently endocytosed rhodamine-labeled alpha-2-macroglobulin. There was no correspondence in the localization of LAMP-1 and receptosomes containing the rhodamine-alpha-2-macroglobulin (Fig. 5, A and B). The localization was also examined by electron microscopy, with colloidal gold-labeled alpha-2-macroglobulin and ferritin bridge-labeled 1D4B antibody. Gold-labeled receptosomes marked by the recently endocytosed alpha-2-macroglobulin (5-min incubation) contained little or no antibody, whereas adjacent lysosomes (*Ly*) were heavily labeled with ferritin-conjugated antibody (Fig. 5C). When the cells were incubated for 60 min at 37°C to allow the colloidal gold-labeled probe to reach lysosomes, all of the lysosomes containing colloidal gold in the lumen were also labeled at the peripheral membrane with the 1D4B antibody (Fig. 5D). Thus, LAMP-1, while detected at high levels in lysosomes, was not found in newly formed endocytic vesicles (receptosomes).

Antigen Expression in Different Cell Lines

LAMP-1 and LAMP-2 were further analyzed by examining the expression and apparent mass of [³⁵S]methionine-labeled glycoproteins in NIH 3T3, HaNIH, and P388 cells (Fig. 6). Both pulse-labeled unprocessed and mature glycoproteins were examined. The unprocessed forms of LAMP-1 and LAMP-2 were very similar in the different cell lines, with a large amount of both glycoproteins in the macrophage cell line P388. In contrast, the apparent molecular weights of both mature glycoproteins differed markedly between the different cell lines. LAMP-1 was 105,000–115,000 D in NIH 3T3 and HaNIH cells, and 115,000–160,000 D in P388 cells. LAMP-2 was 100,000–110,000 D in 3T3 cells, and 115,000–130,000 D in P388. In other studies, we have found that LAMP-1 from HaNIH and P388 cells were highly homologous, if not identical, by tryptic peptide mapping (26) and NH₂-terminal

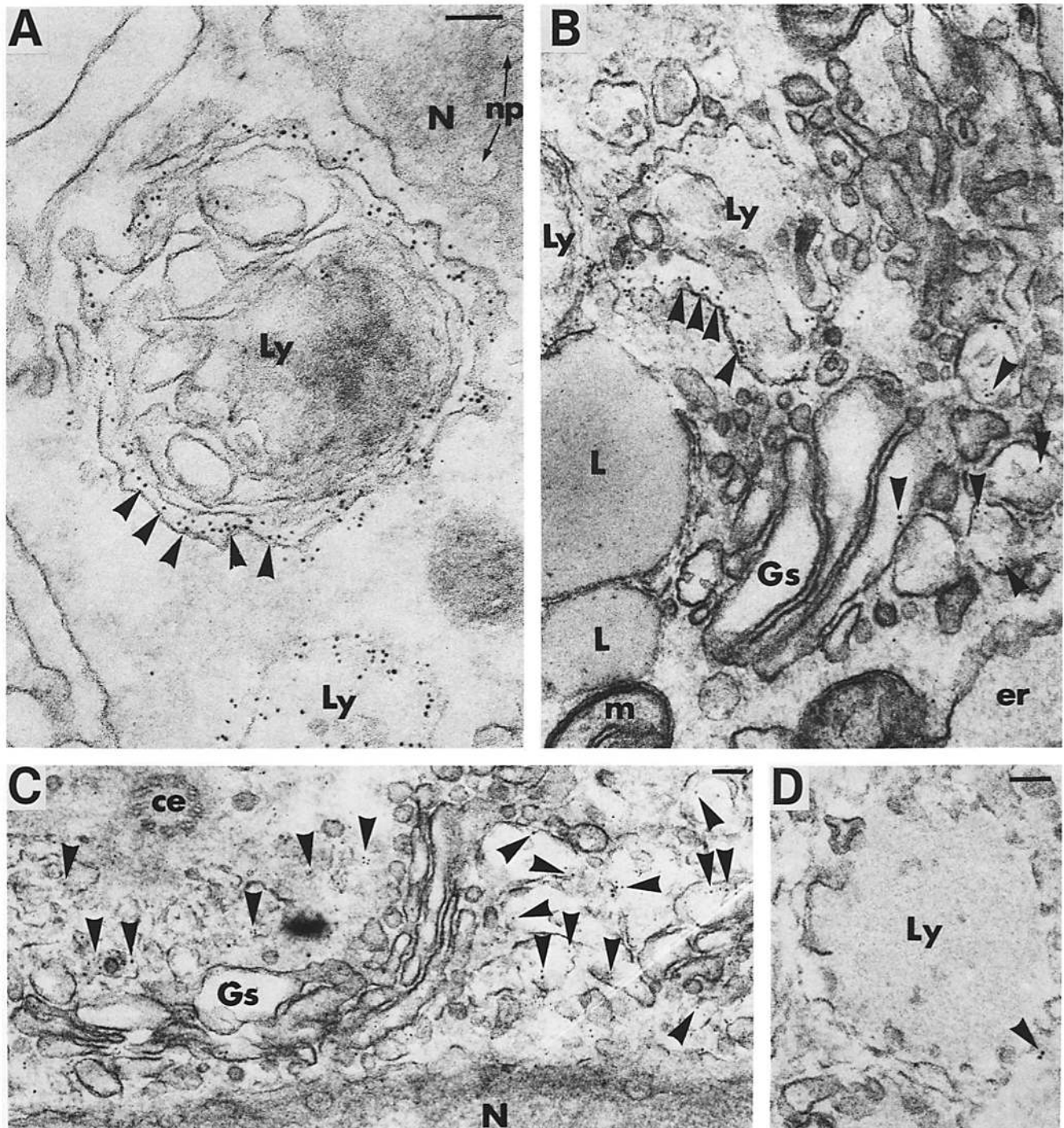


FIGURE 4 Electron microscopy of NIH 3T3 cells. Cells were processed by EGS fixation and ferritin bridge labeling (C-F) as described in Materials and Methods. Large "dense body" lysosomes and smaller multivesicular lysosomes show extensive antigen localization just beneath the limiting lysosomal membrane using 1D4B (A) (arrowheads). Note that the internal myelin-figure membranes within the "dense body" lysosome do not contain large amounts of label. Small lysosomes (Ly) on the *trans*-face of the Golgi stacks also show extensive labeling (B), with lower concentrations of label in *trans*-elements (B). In cross-sections of some Golgi stacks, a large number of these swollen *trans*-elements show low concentrations of label (C). These *trans*-elements are more readily identified because of the interruptions in their membranes due to the extraction of their high cholesterol content by saponin (C). A normal rat globulin control sample shows only low background levels of labeling (D). A and C, 1D4B. B, ABL-93. D, normal rat globulin. N, nucleus. L, lipid droplet. er, endoplasmic reticulum. Ly, lysosome. np, nuclear pore. m, mitochondrion. Gs, Golgi stack. ce, centriole. Arrowheads in A-D, ferritin cores. Bars, 0.1 μ m. (A) \times 94,000. (B and C) \times 73,000. (D) \times 57,000.

amino acid sequence (30 residues) (unpublished observations). We attributed the differences in apparent molecular weight of the glycoproteins in different cells to heterogeneity in number or composition of oligosaccharides. It was notable that both LAMP-1 and LAMP-2 shared this property.

Tryptic Peptide Maps of LAMP-1, LAMP-2, and MAC-3

An important question was whether LAMP-1 and LAMP-2 were the same or different since the cellular localizations of

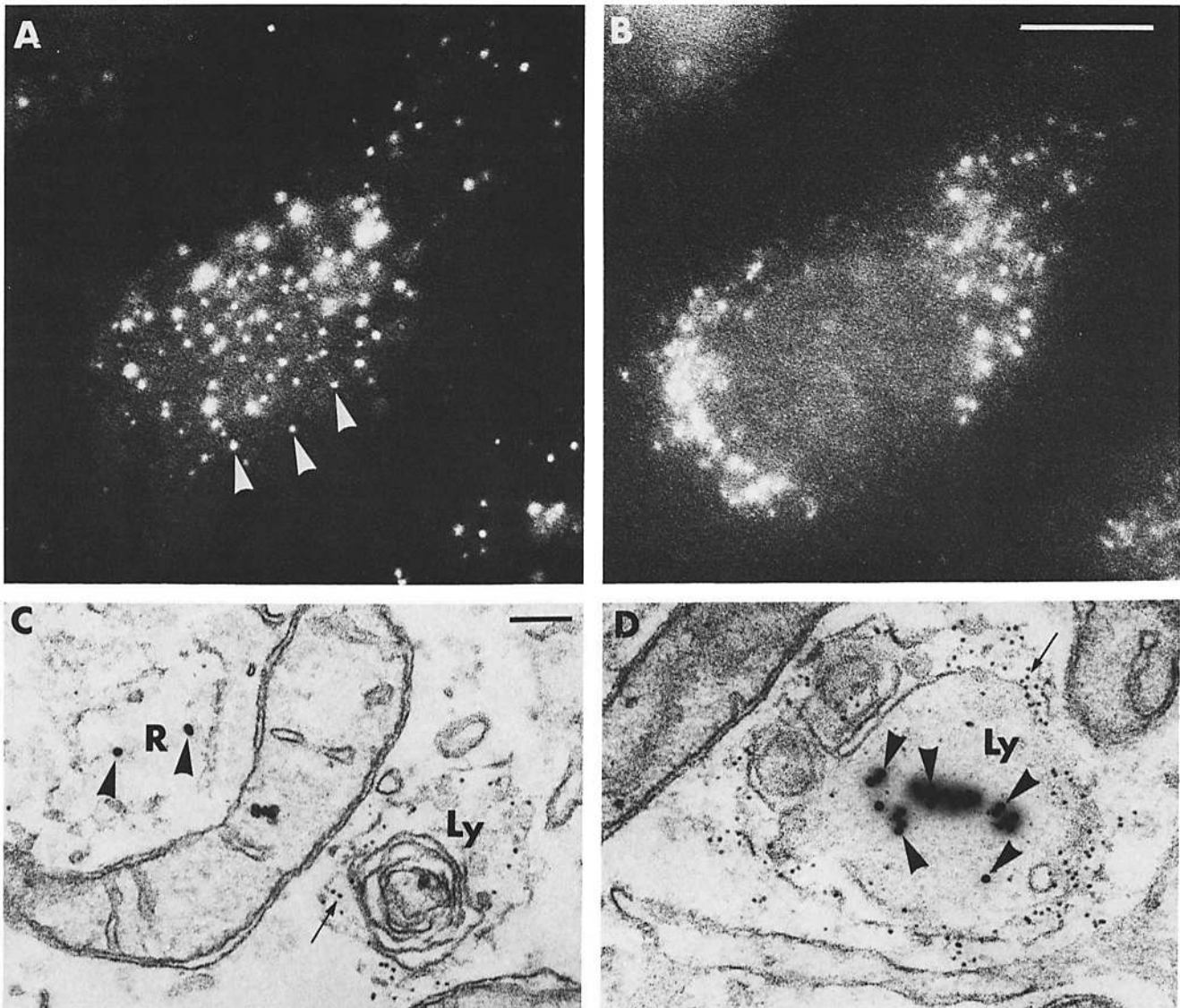


FIGURE 5 Localization of LAMP-1 and alpha-2-macroglobulin. NIH 3T3 cells were incubated with rhodamine-labeled alpha-2-macroglobulin at 4°C for 30 min. The cells were warmed to 37°C for 5 min, fixed in formaldehyde, and treated with Triton X-100, then incubated with 1D4B (anti-LAMP-1) followed by fluorescein-labeled anti-rat IgG. An image of such a cell on the rhodamine channel is shown in *A* (alpha-2-macroglobulin) and on the fluorescein channel in *B* (LAMP-1). Arrowheads in *A* show examples of endocytic vesicles (receptosomes) labeled with rhodamine that show no detectable label with 1D4B (*B*). For electron microscopy (*C* and *D*), cells were incubated with colloidal gold-labeled alpha-2-macroglobulin for 2 h, warmed to 37°C for 5 (*C*) or 60 (*D*) min, then fixed and processed using the EGS procedure. 1D4B was localized using the ferritin bridge method as described in the Results section. Antibody was not present on the receptosome (*R*) containing recently endocytosed colloidal gold-alpha-2-macroglobulin (large arrowheads), and an adjacent lysosome (*Ly*) was strongly labeled with 1D4B-ferritin (small arrowheads). After a 60-min incubation at 37°C, all of the endocytosed colloidal gold-alpha-2-macroglobulin was found in lysosomes which were also heavily labeled with 1D4B (*D*). *Ly*, lysosome. *R*, receptosome. Bar in *B*, 10 μm. Bar in *C*, 0.1 μm. (*A* and *B*) × 2,050. (*C* and *D*) × 90,000.

the two molecules, as described above, were indistinguishable. The two glycoproteins were therefore compared in a number of ways, including tryptic peptide mapping. MAC-3, a macrophage differentiation antigen (12, 15, 30), was included in these experiments as the reported properties of MAC-3 were very similar to the LAMP molecules in molecular weight, isoelectric point, molecular weight heterogeneity between different cell types, and histological staining patterns of mouse tissues.

Glycoproteins of NIH 3T3 cells were purified by lentil-lectin affinity chromatography, labeled with ¹²⁵I, and immunoprecipitated with each of the three antibodies. The immu-

noprecipitates were analyzed by SDS PAGE, and the bands corresponding to LAMP-1, LAMP-2, and MAC-3 were excised and analyzed by two-dimensional tryptic peptide mapping (Fig. 7). LAMP-1 was clearly different from LAMP-2 and MAC-3, whereas LAMP-2 and MAC-3 were very similar.

Sequential Immunoprecipitation

A further comparison of LAMP-1, LAMP-2, and MAC-3 was carried out with a series of sequential immunoprecipitations of the ¹²⁵I-labeled lentil-lectin purified glycoproteins by the different antibodies. Equal aliquots of the labeled extract

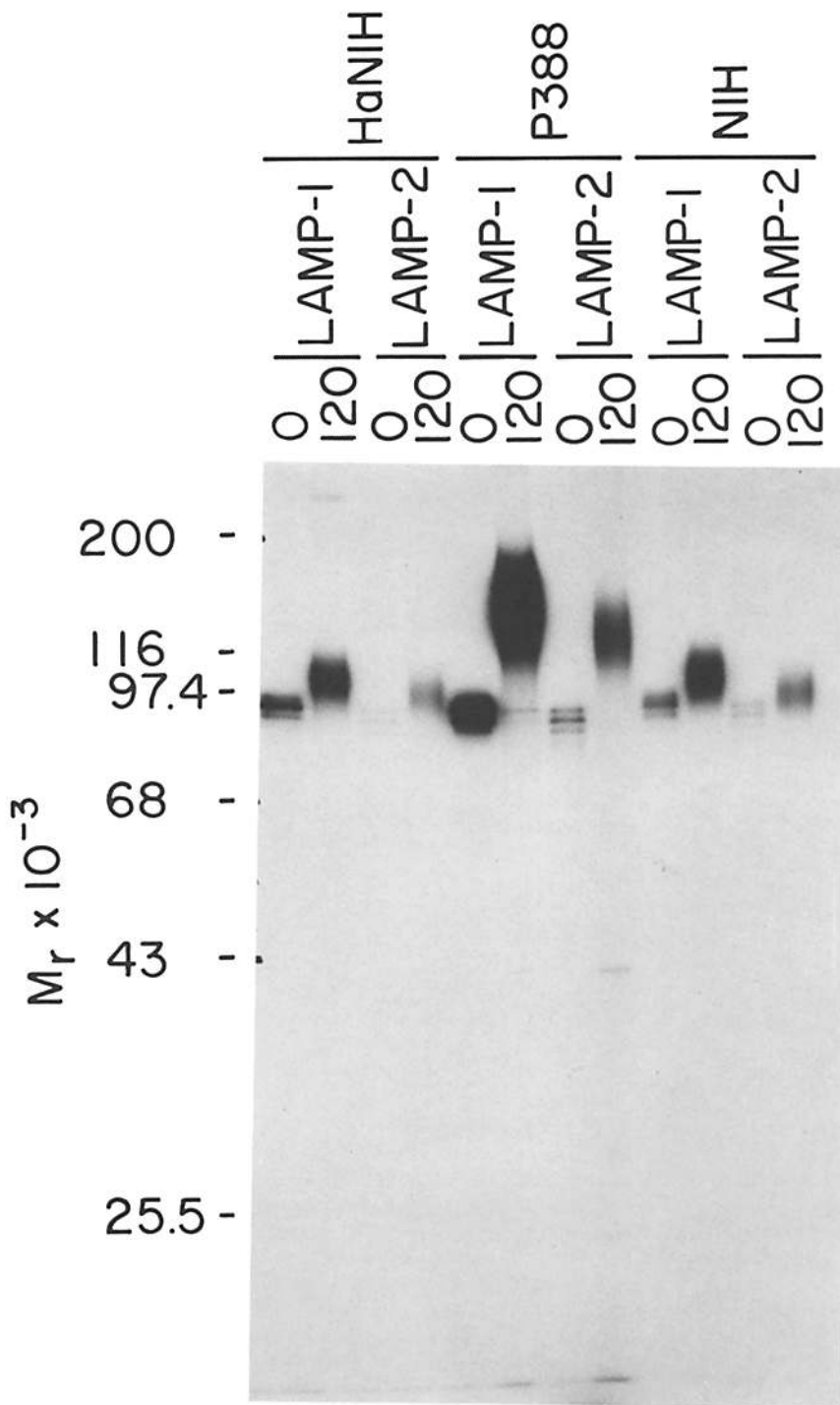


FIGURE 6 Antigen expression in different cell lines. HaNIH, P388D1, and NIH 3T3 cells were grown to 80% confluence in T-75 flasks. The cells were pulse-labeled with [³⁵S]methionine as described in Fig. 1. An aliquot was collected immediately (0) or incubated with unlabeled methionine for 120 min (120). The cells were extracted and 10⁶ dpm of acid-precipitable radioactivity of each sample were immunoprecipitated with 1D4B or ABL-93 monoclonal antibodies for the LAMP-1 or LAMP-2 glycoproteins, respectively. The immunoprecipitates were analyzed by discontinuous SDS PAGE under reducing conditions.

were depleted of an individual antigen by two cycles of immunoprecipitation with an excess of either anti-LAMP-1, anti-LAMP-2, or anti-MAC-3 antibodies. Antibody complexes were removed by centrifugation, and the supernatant fraction was divided into four equal aliquots that were reacted individually with anti-LAMP-1, anti-LAMP-2, anti-MAC-3, or control antibodies (P3).

Adsorption of the extract with a given antibody completely removed the corresponding antigen (Fig. 8). After removal of LAMP-1, 84–90% of the total radioactivity of LAMP-2 and MAC-3 glycoproteins remained and could be immunoprecipitated by the corresponding antibodies. After removal of LAMP-2 or MAC-3, 90% of LAMP-1 remained in the extract.

However, immunoprecipitation of LAMP-2 after prior absorption of MAC-3 was markedly reduced. Correspondingly, preabsorption of LAMP-2 markedly reduced immunoprecipitation of MAC-3. These results indicated that LAMP-1 was antigenically distinct from LAMP-2 and MAC-3, whereas LAMP-2 and MAC-3 were antigenically similar, if not identical.

DISCUSSION

These studies provide evidence that two glycoproteins, LAMP-1 of 105,000–115,000 D and LAMP-2 of 100,000–110,000 D, reside predominantly in the lysosomal membranes of NIH 3T3 and HaNIH cells. The distribution of the antigens

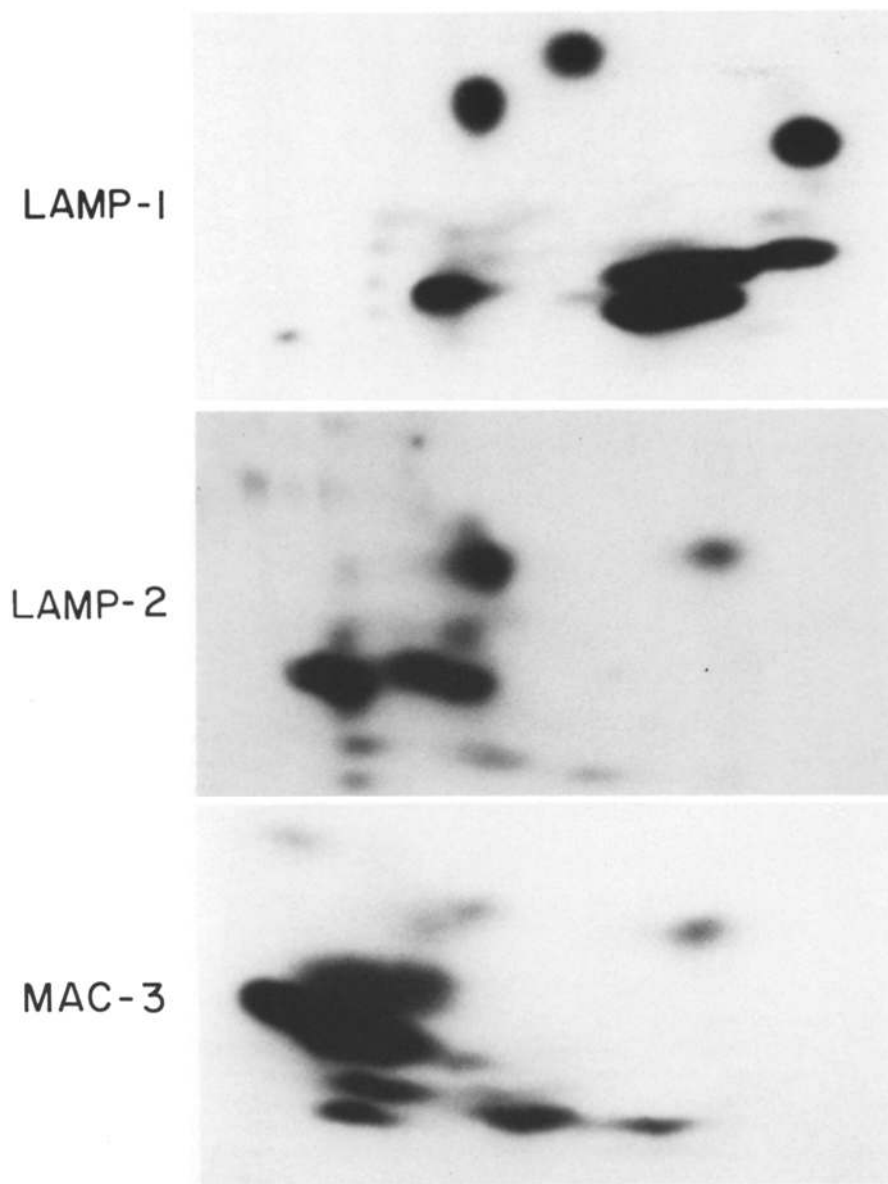


FIGURE 7 ^{125}I -tryptic peptide maps. Glycoproteins of Balb/c 3T3 cells purified by lentil-lectin affinity chromatography used for the production of monoclonal antibodies were labeled with ^{125}I by the chloramine T procedure and immunoprecipitated with 1D4B, ABL-93, or M3/84 monoclonal antibodies. The immunoprecipitates were analyzed by SDS PAGE, as described in Fig. 1, and exposed to Kodak XAR-5 film. Tryptic peptide analysis was performed as described by Krantz et al. (35). The bands were excised and incubated at 37°C for 24 h with $80\ \mu\text{g}/\text{ml}$ of TPCK trypsin in $50\ \text{mM}\ \text{NH}_4\text{HCO}_3$. The entire reaction was spotted onto cellulose TLC plates (EM reagents, Dramstadt, Germany) and initially chromatographed for 2 cm to remove contaminating electrolytes. First dimension electrophoresis was followed by second dimension chromatography. The labeled peptides were visualized by autoradiography of Kodak XAR-5 film.

was perinuclear, readily distinguished from antigens of the Golgi stacks (31, 32), rough endoplasmic reticulum (32), or mitochondria (33). Co-localization of the antigens with sites of acridine orange uptake confirmed the presence of the glycoproteins in acidic vesicles. Electron microscopy showed that antibodies bound to the glycoprotein were present on the luminal side of the lysosomal membrane of both large and small lysosomes. Verification of the structures as lysosomes rests on their content of endocytosed alpha-2-macroglobulin in extended (60 min) incubations, their uptake of acridine orange, and their morphological appearance. All structures with the appearance of lysosomes in these cells labeled heavily with antibodies to LAMP-1 and LAMP-2. The antigens were also detected in low concentration in the *trans*-elements of the Golgi stacks, possibly representing molecules undergoing terminal processing in the Golgi before movement to the primary lysosomes. Antigens were not detected in other organelles, coated pits, endocytic vesicles, or the plasma membrane. Moreover, LAMP-1 and LAMP-2 were not labeled by vectorial iodination of NIH 3T3 or HaNIH cells (Chen, J., and T. August, unpublished experiments).

These assays measured the relative concentration of the glycoproteins at different sites, and did not address movement of the glycoproteins in the cell. Possibly, there was a flow of the molecule through the plasma membrane at concentrations below that detected by short-term lactoperoxidase iodination or by the experiments described herein. Previously it was observed that NIH 3T3 cells lightly fixed with 0.12% glutaraldehyde and incubated for 120 min with 1D4B antibody showed a high level of antibody bound to the cell (11). A trivial explanation is that the lightly fixed cells were fractured by centrifugation in the antibody binding assay, allowing access of the antibody to the lysosome-associated antigen. Alternatively, if there is a flow of these glycoproteins through the plasma membrane, the molecules may continue to move in lightly fixed cells and become trapped on the surface during prolonged incubation with antibody. J. Lippincott-Schwartz and D. M. Fambrough (personal communication) find that a chicken lysosomal membrane glycoprotein shuttles continuously between the cell surface and an intracellular pool with ~2% of the antigen on the cell surface.

Similarities in LAMP-1 and LAMP-2 suggest possible re-

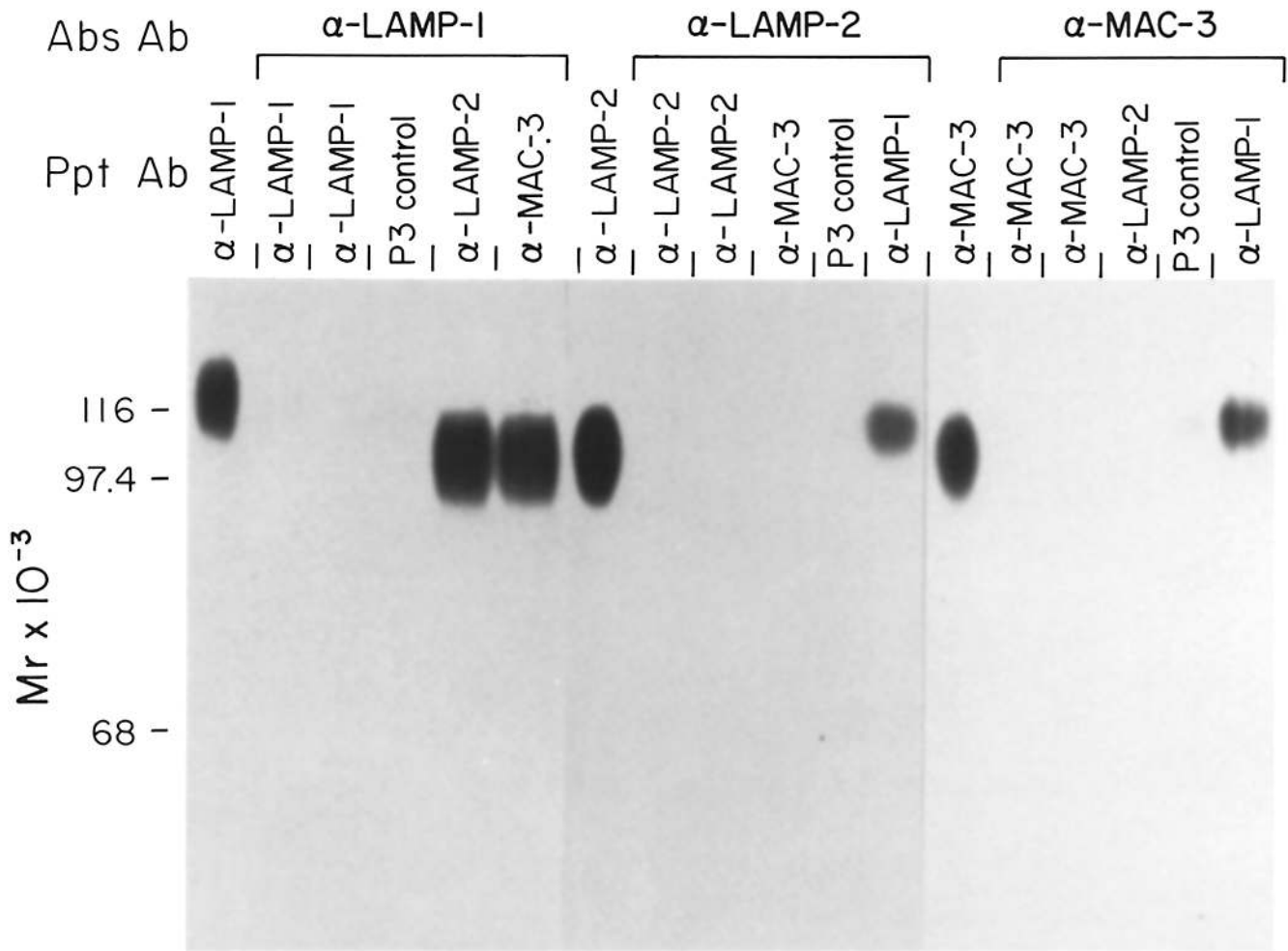


FIGURE 8 Antigen adsorption by sequential immunoprecipitation. Glycoproteins of Balb/c 3T3 cell were purified by lentil-lectin affinity chromatography and labeled with ^{125}I by the chloramine T procedure. Aliquots containing 2×10^6 cpm of acid-precipitable radioactivity were subjected to two cycles of immunoprecipitation with the anti-LAMP-1, anti-LAMP-2, anti-MAC-3 or P3x63Ag8 antibodies and goat anti-rat immunoglobulin secondary antibody. All antibody complexes were collected and washed as described in the Results section. The supernatants from the final immunoprecipitation were divided into four equal aliquots and immunoprecipitated with each of the four monoclonal antibodies as indicated. These precipitates and one-fourth of the antibody complexes collected in the first two absorptions were analyzed by SDS PAGE and visualized by exposure to Kodak XAR-5.

relationships between the molecules. They were both integral membrane proteins with indistinguishable lysosomal localization and tissue distribution; both labeled intensely with ^3H -glucosamine and showed similar patterns of posttranslational glycosylation resulting in a marked heterogeneity of apparent molecular weight. However, they were identified as unique molecules by tryptic peptide mapping and by the distinct antigenicity of the two glycoproteins. The cellular concentrations of the two glycoproteins also differed. Purified LAMP-1 constituted $\sim 0.1\%$ of the total detergent-extracted protein of the cell, with over 10^6 molecules/cell (11), and immunoprecipitation of ^{35}S methionine-labeled proteins yielded 0.08% of the total acid precipitable-labeled proteins as LAMP-1. LAMP-2, in contrast, comprised only 0.02% of total ^{35}S methionine-labeled protein. In addition, we consistently noted more intense immunohistochemical staining of tissues for LAMP-1 in comparison to LAMP-2 (Chen, J., and T. August, unpublished experiments). Finally, initial results from protein sequencing indicated that the amino-terminal sequences of the two molecules were different and there was no significant homology between cloned cDNA of LAMP-1

and LAMP-2 (Chen, J., and T. August, unpublished experiments).

The results also indicated that LAMP-2 is the same molecule or is highly homologous to MAC-3, a macrophage glycoprotein reported by Ho and Springer (12). MAC-3 on intact peritoneal exudate cells was accessible to vectorial iodination and to antibody-binding, indicating that at least some of the antigen was present on the macrophage cell surface (12). LAMP-1 and LAMP-2 were also lightly labeled by lactoperoxidase-catalyzed iodination of the macrophage-like cell line P388 (Chen, J., and T. August, unpublished experiments). It thus appears that the topology of the glycoproteins is different in macrophage membranes as compared with NIH 3T3 or HaNIH cell membranes. This is possibly due to the fusion of intracellular membrane pools with the cell surfaces of macrophages (34).

Other reports of lysosome-specific membrane glycoproteins from rat liver lysosome membranes (7, 8; Mellman, I., and A. Helenius, personal communication), and chicken cells (Lippincott-Schwartz, J., and D. M. Fambrough, personal communication) all describe components of $\sim 100,000$ D. In

addition, Reggio et al. (9) describe adsorbed, polyclonal antibodies prepared against rat liver lysosomal membranes that react with a protein of 100,000 D and stain lysosomes, some coated vesicles, other vesicles and vacuoles, and (to a lesser extent) the plasma membrane of macrophages; these antibodies also reacted with purified H⁺, K⁺-ATPase from porcine gastric mucosa, the enzyme active in the HCl secretion, but not with other ATPase's. Despite the similarities in apparent molecular weight, properties of the Lippincott-Schwartz and Fambrough (personal communication) and Reggio et al. (9) glycoproteins suggest that they are different from LAMP-1 and LAMP-2.

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REFERENCES

- de Duve, C. 1983. Lysosomes revisited. *Eur. J. Biochem.* 137:391-397.
- McKusick, V. A., and E. F. Neufeld. 1983. The mucopolysaccharide storage diseases. In *The Metabolic Basis of Inherited Disease*. J. B. Stanbury, J. B. Wyngaarden, D. S. Frederickson, J. L. Goldstein, and M. S. Brown, editors. McGraw-Hill Book Co., New York. 751-777.
- Helenius, A., I. Mellman, D. Wall, and A. Hubbard. 1983. Endosomes. *Trends Biochem. Sci.* 7:245-250.
- Pastan, I. H., and M. C. Willingham. 1983. Receptor-mediated endocytosis: coated pits, receptosomes, and the Golgi. *Trends Biochem. Sci.* 8:250-257.
- Neufeld, E. F. 1981. Recognition and processing of lysosomal enzymes in cultured fibroblasts. In *Lysosomes and Lysosomal Storage Diseases*. J. W. Callahan and J. A. Lowden, editors. Raven Press, New York. 115-129.
- Muller, W. A., R. M. Steinman, and Z. A. Cohn. 1983. Membrane proteins of the vacuolar system. III. Further studies on the composition and recycling of endocytic vacuole membrane in cultured macrophages. *J. Cell Biol.* 96:29-36.
- Burnside, J., and D. L. Schneider. 1982. Characterization of rat liver lysosomes: composition, enzyme activities and turnover. *Biochem. J.* 204:525-534.
- Ohsumi, Y., T. Ishikawa, and K. Kato. 1983. A rapid and simplified method for the preparation of lysosomal membranes from rat liver. *J. Biochem. (Tokyo)*. 93:547-556.
- Reggio, H., D. Bainton, E. Harms, E. Coudrier, and D. Louvard. 1984. Antibodies against lysosomal membranes reveal a 100,000-mol-wt protein that cross-reacts with purified H⁺, K⁺ ATPase from gastric mucosa. *J. Cell. Biol.* 99:1511-1526.
- Hughes, E. N., and J. T. August. 1981. Characterization of plasma membrane proteins identified by monoclonal antibodies. *J. Biol. Chem.* 256:664-671.
- Hughes, E. N., and J. T. August. 1982. Murine cell surface glycoproteins. Identification, purification, and characterization of a major glycosylated component of 110,000 daltons by use of a monoclonal antibody. *J. Biol. Chem.* 257:3970-3977.
- Ho, M.-K., and T. A. Springer. 1983. Tissue distribution, structural characterization, and biosynthesis of MAC-3, a macrophage surface glycoprotein exhibiting molecular weight heterogeneity. *J. Biol. Chem.* 258:636-642.
- Kowal, R., and R. G. Parsons. 1980. Stabilization of proteins immobilized on sepharose from leakage by glutaraldehyde crosslinking. *Anal. Biochem.* 102:72-76.
- Kearney, J. F., A. Radbruch, B. Liesegang, and K. Rajewsky. 1979. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting cell lines. *J. Immunol.* 123:1548-1550.
- Springer, T. A. 1981. Monoclonal antibody analysis of complex biological systems. *J. Biol. Chem.* 256:3833-3839.
- Pastan, I. H., and M. C. Willingham, W. Anderson, and M. Gallo. 1977. Localization of serum-derived alpha-2-macroglobulin in cultured cells and decrease after Moloney sarcoma virus transformation. *Cell.* 12:609-617.
- Willingham, M. C. 1980. Electron microscopic immunocytochemical localization of intracellular antigens in cultured cells: the EGS and ferritin bridge procedures. *Histochem. J.* 12:419-434.
- Dickson, R. B., M. C. Willingham, and I. Pastan. 1981. alpha-2-Macroglobulin adsorbed to colloidal gold: a new probe in the study of receptor-mediated endocytosis. *J. Cell Biol.* 89:29-34.
- Jainchill, J. L., S. A. Aaronson, and G. J. Todaro. 1969. Murine sarcoma and leukemia viruses: assay using clonal lines of contact-inhibited mouse cells. *J. Virol.* 4:549-553.
- Scolnick, E. M., and W. P. Parks. 1974. Harvey sarcoma virus: a second murine type C sarcoma virus with rat genetic information. *J. Virol.* 13:1211-1219.
- Shevach, E. M., J. D. Stobo, and I. Green. 1972. Immunoglobulin and O-bearing murine leukemias and lymphomas. *J. Immunol.* 108:1146-1151.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
- Hunter, W. M. 1967. The preparation of radioiodinated proteins of high activity, their reaction with antibody *in vitro*: the radioimmunoassay. In *Handbook of Experimental Immunology*. D. M. Weir, editor. F. A. Davis Co., Philadelphia. 608-692.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83-88.
- Chen, J. W., W. Pan, M. P. D'Souza, and J. T. August. 1985. Lysosome associated membrane proteins: characterization of LAMP-1 of macrophage P388 and mouse embryo 3T3 cultured cells. *Arch Biochem Biophys.* In press.
- Zelenin, A. V. 1966. Fluorescence microscopy of lysosomes and related structures in living cells. *Nature (Lond.)*. 212:425-426.
- Willingham, M. C., and I. Pastan. 1980. The receptosome: an intermediate organelle of receptor-mediated endocytosis in cultured fibroblasts. *Cell.* 21:67-77.
- Dickson, R. B., L. Beguinot, J. A. Hanover, N. D. Richert, M. C. Willingham, and I. Pastan. 1983. Isolation and characterization of a highly enriched preparation of receptosomes (endosomes) from a human cell line. *Proc. Natl. Acad. Sci. USA.* 80:5335-5339.
- Flotte, T. J., T. A. Springer, and G. J. Thorbecke. 1983. Dendritic cell and macrophage staining by monoclonal antibodies in tissue sections and epidermal sheets. *Am. J. Pathol.* 111:112-124.
- Lin, J. J.-C., and S. A. Queally. 1982. A monoclonal antibody that recognizes Golgi-associated protein of cultured fibroblast cells. *J. Cell Biol.* 92:108-112.
- Louvard, D., H. Reggio, and G. Warren. 1982. Antibodies to the Golgi complex and the rough endoplasmic reticulum. *J. Cell Biol.* 92:92-107.
- Johnson, L. V., M. L. Walsh, and L. B. Chen. 1980. Localization of mitochondria in living cells with rhodamine 123. *Proc. Natl. Acad. Sci. USA.* 77:990-994.
- Buys, S. S., E. A. Keogh, and J. Kaplan. 1984. Fusion of intracellular membrane pools with cell surfaces of macrophages stimulated by phorbol esters and calcium ionophores. *Cell.* 38:569-576.
- Krantz, M. J., M. Strand, and J. T. August. 1977. Biochemical and immunological characterization of the major envelope glycoprotein gp69/71 and degradation fragments from Rauscher leukemia virus. *J. Virol.* 22:804-815.