Antibodies against Lysosomal Membranes Reveal a 100,000-mol-wt Protein That Cross-reacts with Purified H$^+$,K$^+$ ATPase from Gastric Mucosa

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ABSTRACT   Specific antibodies against lysosomal membranes were prepared by using techniques previously described (Louvard, D., H. Reggio, and G. Warren, 1982, J. Cell Biol., 92:92–107) for obtaining organelle-specific antibodies. The purified antibodies stained an acidic vacuolar compartment as shown by double-labeling experiments with acridine orange and indirect immunofluorescence. Characterization of the antibodies by immunoreplica methods revealed one major protein of ~100,000 mol wt. The antibodies cross-reacted with purified H$^+$,K$^+$ ATPase from pig gastric mucosa, the enzyme responsible for HCl secretion, but not with ATPases transporting other ions. They may therefore recognize a component of the proton pump involved in the acidification of lysosomes. As was expected, secondary lysosomes contained immunoreactive antigen, as determined by the fine-structural localization of reaction product for peroxidase or immunogold probes in several cell types. The antigen was also found in vacuoles containing phagocytosed bacteria in macrophages so it is present in at least some of the compartments of an endocytic pathway. In liver, the antigen was present in small amounts on the plasma membrane and in large amounts in some coated vesicles (near the sinusoidal surface of hepatocytes), putative endosomes, two cisternae on the cis side of the Golgi complex, adjacent vesicles and vacuoles, and pericanalicular dense bodies. In summary, the antigen seems to be present in those compartments that have recently been demonstrated to be acidified by an ATP-driven pump.

Secondary lysosomes are the major intracellular compartment where proteins, as well as many other molecules of biological significance, are processed and degraded through the action of various acid hydrolases. Recent morphological and biochemical studies have elucidated the pathway of some lysosomal hydrolases from their site of synthesis, the rough endoplasmic reticulum (RER),[1] to their final location within digestive vacuoles (11, 13, 36, 49–51, 61). Other studies have led to the identification of a phosphorylated mannose, covalently bound to several lysosomal hydrolases, which serves as a signal for directing these enzymes to their destination (7,

10, 14, 44, 52, 57). Although several of the proteins stored in lysosomes have been extensively characterized (see review, reference 61), to our knowledge, no specific integral membrane protein associated with the lysosome has yet been identified.

It has long been known that secondary lysosomes have an acid content, but the molecular mechanisms responsible for the acidification were until very recently a matter of speculation (38). Recently, studies on the pathway and fate of ligand-receptor complexes internalized by adsorptive endocytosis have produced some unexpected findings (1, 6, 27). The membrane vesicles involved in adsorptive endocytosis have an acidic content (6, 17, 31, 33, 46, 70), but do not contain

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[1] Abbreviations used in this paper: NRK, normal rat kidney; RER, rough endoplasmic reticulum.

typical lysosomal hydrolases. Two types of acidic prelysosomal vesicles have been identified: the coated vesicle (15) and a second compartment variously referred to as the endosome (16, 31, 32, 65, 71) the receptosome (70), the intermediate compartment (33, 67), or CURL (18). In both types of vesicle, an ATPase seems to be the driving force for H^+ accumulation (15, 16, 71). This ATPase, by pharmacological criteria, differs from the plasma-membrane- and mitochondrial-membrane-bound ATPases (reviewed in reference 46).

We have recently described an immunological technique for preparing and characterizing polyclonal antibodies specific for antigens associated with the membranes of the RER and the Golgi complex (29). By using a similar approach, we have now characterized an antibody that allows morphological and biochemical identification of an antigen associated with the membranes of lysosomes and other organelles of the lysosomal pathway. This antibody recognizes a previously unidentified protein (100,000 mol wt), which is immunologically related to the H^+,K^+-simulated ATPase found in parietal cells of stomach mucosa, but not to the ouabain-sensitive Na^+,K^+ ATPase of dog kidney, to the Ca^{2+} ATPase from muscle sarcoplasmic reticulum, or to mitochondrial ATPase. Thus, we have identified an integral lysosomal membrane protein, which may function in the acidification of certain intracellular organelles, such as coated vesicles, endosomes, secondary lysosomes, and certain cisternae of the Golgi complex.

MATERIALS AND METHODS

Preparation of Crude Antiserum to Purified Lysosome Membranes

CELL FRACTIONATION: We isolated rat liver lysosomes by a combination of isopycnic centrifugation in colloidal silica gel (41) and free-flow electrophoresis (58) (Fig. 1). A similar technique has been used to isolate lysosomes from human fibroblasts or lymphoblasts (23, 24). We used the same isotonic buffer system throughout the preparation (0.25 M sucrose, 10 mM triethanolamine, 1 mM EDTA, pH 7.4). The colloidal silica gel gradient was formed by centrifugation of the suspension (starting density 1.09 g/ml) at 40,000 g for 45 min in a Sorvall RC-5 centrifuge (DuPont Instruments, Sorvall Biomedical Div., Newton, CT) using an SS34-rotor. After centrifugation, the dense brownish fraction at the bottom of the tubes, containing most of the lysosomes, was collected, and the colloidal silica gel was removed by centrifugation and resuspension in buffer four times. We then performed electrophoresis using a free-flow electrophoresis model VAP 11 (Bender & Hobein GmbH, München, Federal Republic Germany) under the following conditions: 140 V/cm, buffer flow 4.5 ml/h in a fraction, at 5°C. We analyzed fractions for protein and marker enzymes as previously described (34). The fractions showing the greatest activity of lysosomal enzymes were pooled to form a final lysosomal fraction; the fractions showing the greatest activity of nonlysosomal enzymes were pooled to form a crude mitochondrial fraction, which contained mainly mitochondrial, but also some Golgi vesicles, endoplasmic reticulum vesicles, and peroxisomes. The recovery and relative specific activity of N-acetyl-β-D-glucosaminidase in the fractions used for further experiments are given in Table I. Lysosomes were lysed by freezing and thawing three times in distilled water. Sodium chloride was added to a final concentration of 0.9%. We centrifuged the membranes at 12,000 g for 10 min (Eppendorf centrifuge [Migge GmbH, Heidelberg, Federal Republic of Germany]) and washed three times with 0.9% sodium chloride. This fraction will be referred to hereafter as "purified lysosome membranes."

IMMUNIZATION: Rabbits were immunized by injection of the purified lysosome membranes into the popliteal lymph node, essentially as described by Louvard et al. (29). We used 100 μg of proteins for the first injection, and 50 μg for each booster injection. The antiserum was collected as described in detail in reference 29.

Purification of Crude Antiserum by Absorption Steps

The antiserum was then tested by indirect immunofluorescence on tissue culture cells or frozen thin sections of tissue. The unwanted antibodies to cell
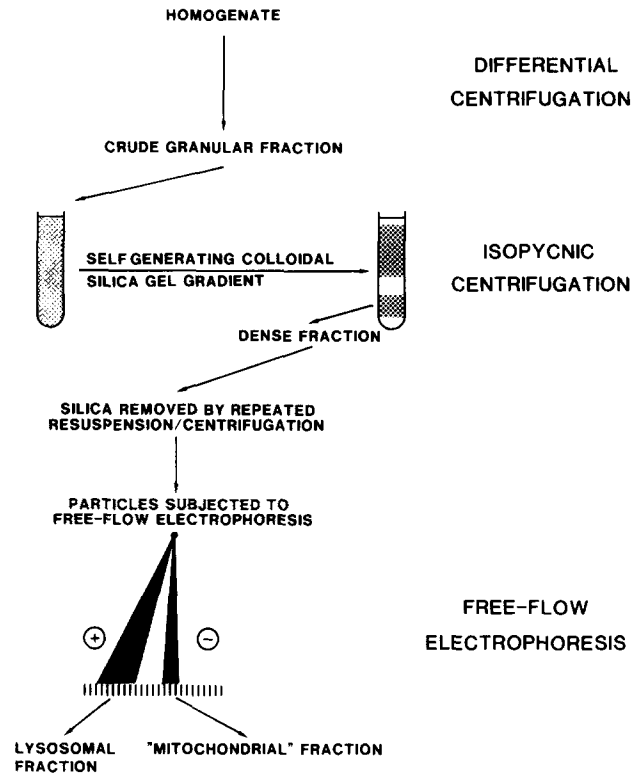

FIGURE 1   Steps in the preparation of rat liver lysosomes.

TABLE I
Purification of Rat Liver Lysosomes

Fraction	N-Acetyl-β-D-glucosaminidase	
	Relative specific activity	Recovery
		%
Crude granular fraction	5.0 ± 0.3	58.0 ± 9.1
Dense fraction from silica gradient (silica removed)	50.0 ± 10.9	15.1 ± 3.2
Pooled lysosomal fraction after free-flow electrophoresis	72.9 ± 9.1	5.2 ± 1.1

± indicates standard deviation. Data are the results of eight experiments. Results are expressed relative to the original homogenate.

components other than lysosomes were removed by suitable absorption steps. These steps were carried out until the pattern of fluorescent labeling was that expected for lysosomes.

CELLS: Normal rabbit kidney (NRK) cells, provided by Dr. S. J. Singer, University of California, San Diego, at La Jolla, CA, were grown in minimum essential medium containing 10% fetal calf serum (vol/vol). Several other fibroblast and epithelial-like cell lines were tested as previously described (29).

DETECTION OF UNWANTED ANTIBODIES BY IMMUNOFLUORESCENCE MICROSCOPY: NRK cells were fixed and labeled with the antiserum using the general procedure described by Wang et al. (68). Semithin frozen sections of rat liver, kidney, and intestine were prepared and stained by the method of Tokuyasu (63), using only paraformaldehyde as fixative. For permeabilization, Triton X-100 was used. The second antibody was an affinity-purified (62) sheep anti-rabbit IgG conjugated to rhodamine (5). The preparations were observed with a Zeiss Photomicroscope III.

ABSORPTION STEPS: Absorptions with the mitochondrial fraction from rat liver and the microvilli fraction from rat intestine were necessary. Freshly isolated mitochondria were obtained from the light band of the silica gel gradient (see Fig. 1) by centrifugation in a discontinuous sucrose gradient in an SW27-rotor of a Beckman L50 (Beckman Instruments, Inc., Fullerton, CA); they were then centrifuged at 130,000 g for 2 h (27,000 rpm). The gradient contained three sucrose layers: 54.1% (d-1.21), 46% (d-1.18), 35.7% (d-1.14).

The load deposited on the top was adjusted to 8.6% sucrose. The fraction collected was the upper part of the 46%/54.1% layer and contained only mitochondria as indicated by marker enzymatic measurements (not shown). The microvilli fraction from rat intestine was prepared as previously described (30). Unwanted antibodies were removed from the antiserum by absorption with these two fractions as described by Louvard et al. (29). The resulting antiserum will hereafter be referred to as the "purified antiserum." One other absorption step, with a freshly prepared plasma membrane fraction from rat liver (45), was tested but was found to be unnecessary.

Double Labeling of NRK Cells with Acridine Orange and the Purified Antiserum

NRK cells, grown on coverslips engraved with circles, were incubated for 5 min at 37°C in normal serum-free minimum essential medium containing 1 μM acridine orange. The cells were then rinsed three times with serum-free minimum essential medium. Photographs were immediately taken at room temperature with a ×63 water-immersion Zeiss lens. They were observed using a fluorescence setup equipped with filter combinations for specimens labeled with rhodamine-conjugated probes. The cells were then rapidly fixed with formaldehyde and labeled by indirect immunofluorescence techniques with the purified antiserum to lysosome membranes. Another set of pictures was taken of the same cells. These cells were easily found by returning to the same circle on the coverslip. It should be noted that acridine orange is washed away during the processing for immunolabeling.

Immunochemical Experiments

IMMUNORADIOMETRIC ASSAY: Binding of the purified antiserum was assayed on different subcellular fractions as described by Green et al. (20).

IMMUNOREPLICA EXPERIMENTS: SDS-PAGE was performed as described by Laemmli (28), in 6.5-17% linear acrylamide gradient slab gels. Electrotransfer of the proteins to nitrocellulose and immunolabeling were performed according to Burnette's technique (8), as modified by Coudrier et al. (9).

TRITON X-114 EXTRACTION: Membrane proteins were extracted with Triton X-114 according to the method of Bordier (4) for phase separation of integral membrane proteins.

AFFINITY PURIFICATION ON NITROCELLULOSE: Proteins from the lysosomal fraction were separated on a preparative SDS PAGE and transferred to nitrocellulose. The sheet was stained with Ponceau S, and the 100,000-mol-wt band was cut out. This strip was used for affinity purification of antibodies to the 100,000-mol-wt proteins according to the technique of Olmsted (40), as modified by Coudrier et al. (9).

ATPASE PREPARATIONS: Ca^{++} ATPase was prepared as described by Warren et al. from rabbit muscle (69). Preparations of purified Na$^+$,K$^+$ ATPase (dog kidney) and K$^+$,H$^+$ ATPase (pig stomach) were generously provided by Dr. Ponzio and Dr. B. Rossi (University of Nice, France) and Dr. S. L. Bonting and associates (Nijmegen University, Holland), respectively. Homogeneity of these preparations was assessed by PAGE in SDS.

Immunocytochemistry and Enzyme Cytochemistry

IMMUNOPEROXIDASE LABELING: Immunolabeling of NRK cells was performed as described by Ohtsuki et al. (39) and Tougard et al. (64), with the modifications described by Louvard et al. (29). Because lysosome membranes were very sensitive to saponin treatment, the conditions of fixation were varied in order to observe well-preserved lysosomes or to ascertain whether the antigens were present on other membranes that are more difficult to permeabilize (i.e., Golgi membrane, endoplasmic reticulum, nuclear membrane, etc.) (47). The cells were fixed with 2% paraformaldehyde in 100 mM phosphate buffer, pH 7.2, containing 0.1%, 0.05%, 0.03%, or 0% glutaraldehyde (Ladd Research Industries, Inc., Burlington, VT). The primary antibody was used in a dilution of 1:50 for 90 min at 22°C. The secondary antibody was a sheep anti-rabbit Fab conjugated to horseradish peroxidase (Institut Pasteur Production, Paris). To assess permeability, we performed control experiments using purified antibodies to RER and to Golgi membranes (29) as the primary antibody.

Rat macrophages from peritoneal exudate were fixed and labeled as described for NRK cells. The exudates were induced in rats by the intraperitoneal injection of 20 ml of endotoxin (2 μg of *Salmonella typhosa* lipopolysaccharide dissolved in 100 ml of saline). 4 d later, 10 ml of heparinized Hanks' balanced salt solution were injected into the peritoneal cavity to wash out the macrophages. In some experiments, heat-killed *Escherichia coli* were allowed to be phagocytized 10 min before fixation.

For immunolabeling of rat liver, the tissue was perfused with saline and fixed for 30 min at room temperature with 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The tissue was then chopped into 40-μm sections and given three 5-min washes in PBS containing 50 mM of ammonium chloride (NH$_4$Cl). The sections were then processed as described above, except that they were incubated for 16-18 h with the primary antibody and for 6 h with the secondary antibody.

FROZEN THIN SECTIONS AND IMMUNOGOLD PROBES: For preparation of frozen thin sections, rat macrophages were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The techniques for ultracryotomy described by Tokuyasu (63) were used. Thin sections were incubated with the primary antibody in 1:50 dilution for 90 min, and then with goat anti-rabbit IgG linked to 20-nm particles of colloidal gold (Janssen Pharmaceutica, Beerse, Belgium) in 1:100 dilution for 90 min (56). Embedding and staining were performed as described by Griffiths et al. (21, 22). In control procedures for both experiments, nonimmune, purified rabbit IgG or buffer was used in place of the primary antiserum.

ENZYME CYTOCHEMISTRY: Acid phosphatase cytochemistry was performed as described by Bainton and Farquhar (2) using β-glycerolphosphate as substrate.

Preparation of Coated Vesicles

Clathrin-coated vesicles were prepared from calf brain as described (15). This preparation was kindly provided by Dr. Daniel Branton (Harvard University).

RESULTS

Sequential Absorption Steps to Obtain Purified Antibodies against Lysosome Membranes

Highly purified preparations of lysosomes can be obtained from rat liver by free-flow electrophoresis as outlined in Fig. 1. The specific activity of the purified fraction was at least 70 times that of the original homogenate (Table I). These membranes were used for immunization and the resulting antiserum stained a number of cell structures including perinuclear vacuoles, which closely resembled the vacuoles stained by acid phosphatase, a lysosomal marker. Unwanted antibodies to organelles other than lysosomes were then removed by the following two steps.

STEP 1—ABSORPTION WITH MITOCHONDRIAL FRACTION: The crude antiserum from rabbits immunized with highly purified lysosome membranes was first assayed for antigen localization by immunofluorescence techniques on NRK cells, as described in similar studies on antibodies to RER and Golgi membranes (29). Fixed, permeabilized NRK cells showed weak internal labeling, typical of mitochondria (25), and strong punctate labeling throughout the interior of the cell, concentrated near the nucleus (not shown). The latter pattern was interpreted as the staining of lysosomes in that it resembled the distribution of acid phosphatase (to be seen later in Fig. 9). The unwanted antibodies to mitochondria were absorbed out of the antiserum with a fraction enriched in liver mitochondria.

On permeabilized NRK cells (Fig. 2*A*), the resulting antiserum labeled numerous small intracellular vesicles, concentrated near the Golgi region, which were presumably lysosomal. On fixed but nonpermeabilized NRK cells, this antiserum produced weak, but significant, punctate images typical of surface labeling (Fig. 2*B*). It is also worth noting that under these fixation conditions, some intracellular labeling also occurs. When the antiserum had been subjected to absorption with a very large excess of a fraction enriched in liver plasma membranes, both surface and intracellular labeling were lost. We concluded that the antigens on the plasma membrane were probably recognized by the antibodies that labeled the intracellular structures, a conclusion supported by electron
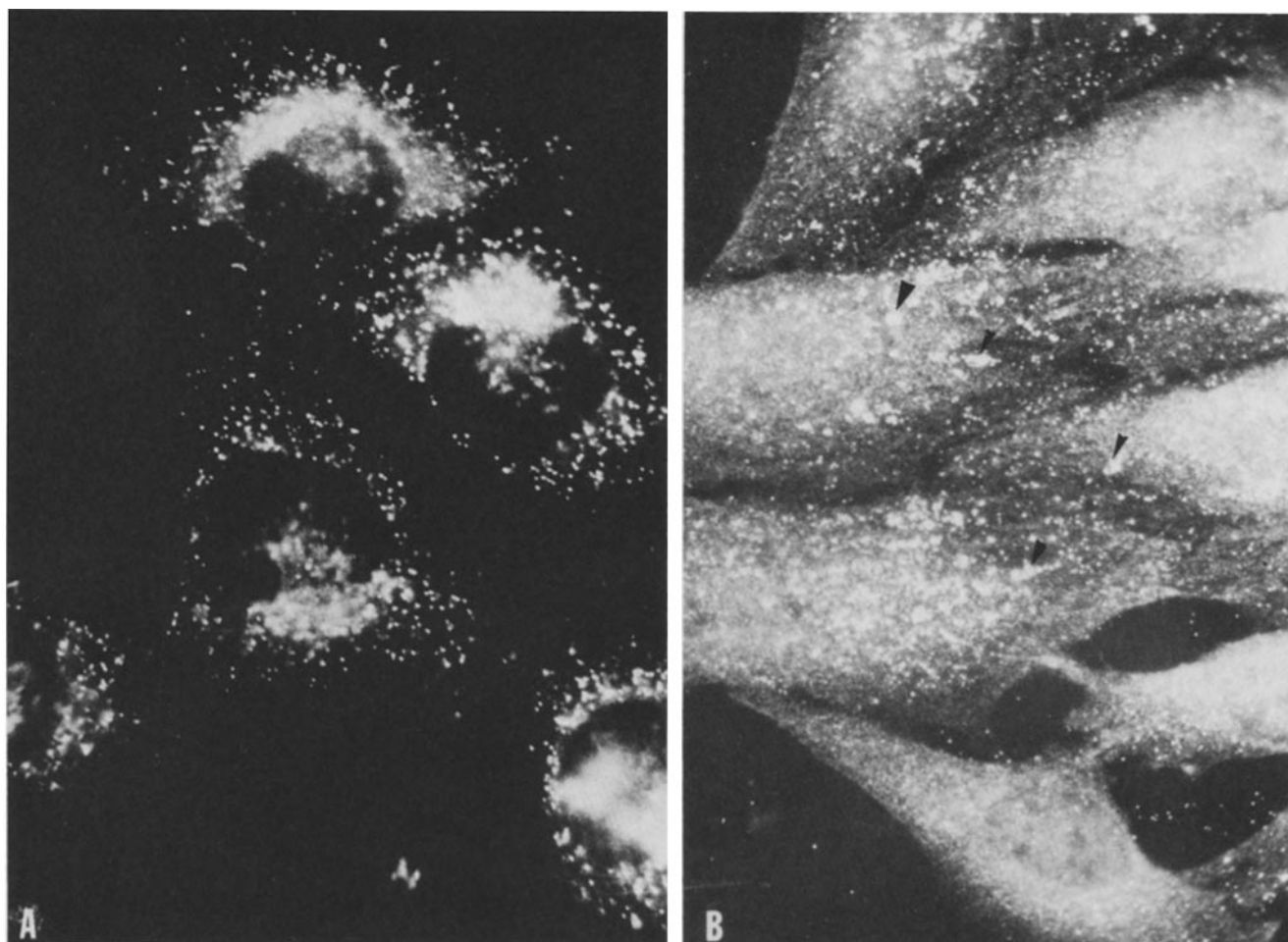
FIGURE 2 Immunofluorescent labeling of NRK cells with antiserum to lysosome membranes after absorption with the mitochondrial fraction. (A) the antibody-labeled intracellular vesicles present throughout the cytoplasm of permeabilized cells are concentrated in a perinuclear region (probably the Golgi area). (B) In a field similar to the one shown in A, nonpermeabilized cells show labeling of the plasma membrane, which appears as a fine punctate uniformly distributed pattern. In addition, under these fixation conditions, the cells frequently show some intracellular labeling, particularly in lysosomes (arrows). × 1,045.

microscopic study detailed below.

STEP 2—ABSORPTION WITH INTESTINAL MICRO-VILLI FRACTION: With NRK cells, as well as with several other fibroblast-like and epithelial-like cell lines, the antiserum purified by absorption with mitochondria labeled only the intracellular and surface structures shown in Fig. 2, even when a large amount was used. However, in frozen sections of tissues and particularly of absorptive epithelia, this antiserum produced a strong apical labeling of brush borders (proximal tubule cells of kidney or intestine) or bile canaliculi (liver). Labeling was absent from other domains of the cell surface, such as the basolateral membranes (kidney, intestine) and sinusoidal front (liver). Highly purified preparations of intestinal microvilli were therefore used to absorb out the

unwanted antibodies to brush borders and bile canaliculi in the antiserum.

In liver sections stained with the resulting purified antiserum (Fig. 3, A and B), the label within parenchymal cells was distributed near the bile canaliculi in a pattern very similar to that for acid phosphatase in liver (not shown). As expected, Kupffer cells were labeled; but, more surprising, the cell membrane of erythrocytes was also labeled. Kidney sections (Fig. 3, C and D) showed labeling in intracellular vacuoles strongly resembling those usually labeled with acid phosphatase. In proximal tubule cells, the labeling was mainly found in large structures at the apex of the cell, probably corresponding to the apical ultralucent vesicles (endosomes) described by others in kidney (60).

FIGURE 3 Immunofluorescent labeling of thin sections of rat tissues with purified antiserum to lysosome membranes (A and C) with corresponding phase-contrast view (B and D). (A) In liver, Kupffer cells (KC) showed extremely strong labeling, and labeled structures were scattered throughout the cytoplasm of hepatocytes. The plasma membranes of the bile canaliculi (BC) were not labeled, but erythrocyte membranes were labeled (arrowhead). (B) Phase-contrast view of the field illustrated in A. (C) In kidney, the labeling was prominent in the cells of the proximal tubule (PT), which were identified by their brush border (bb). Distal tubules (DT) were less extensively labeled. (D) Phase-contrast view of the field illustrated in C. (A–D) × 1,250.
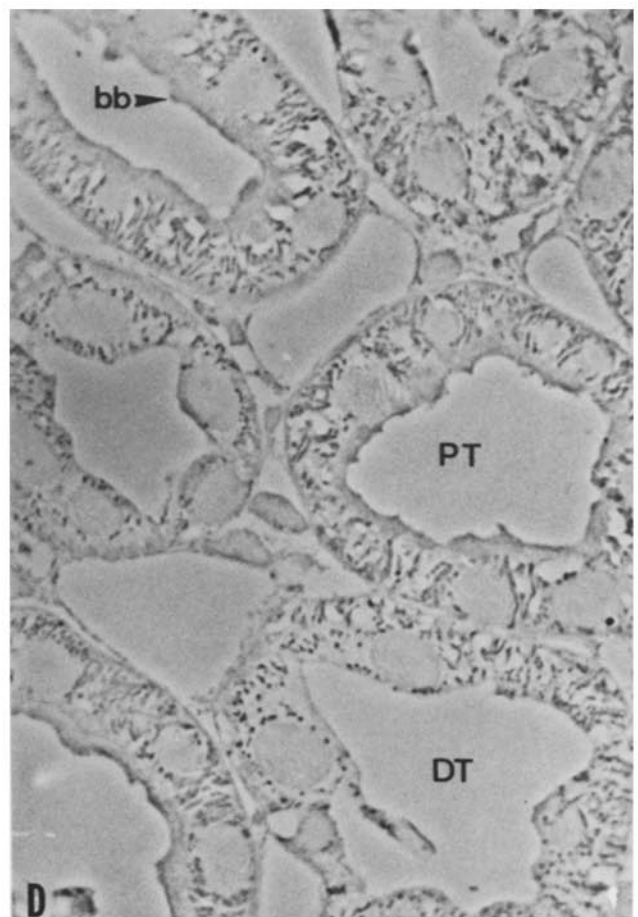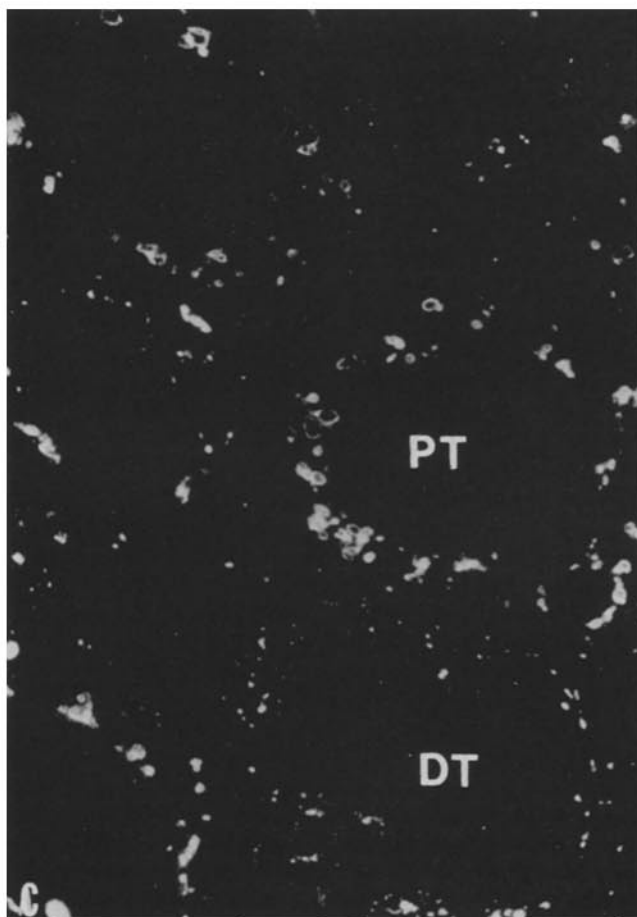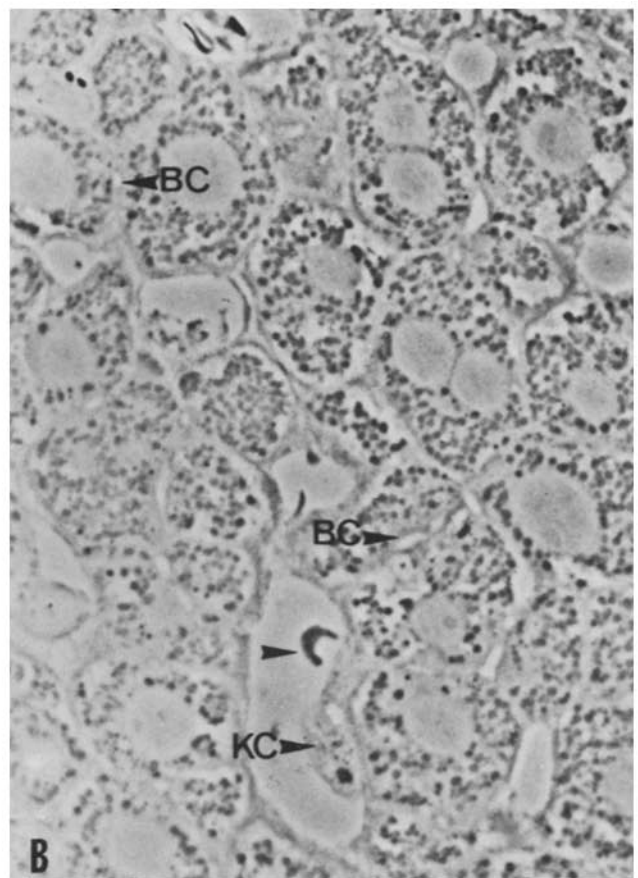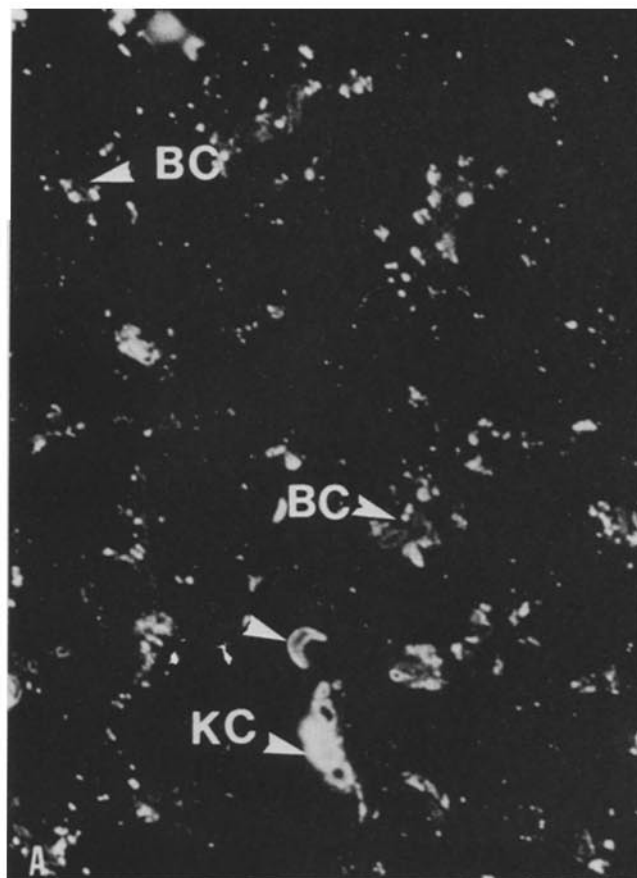
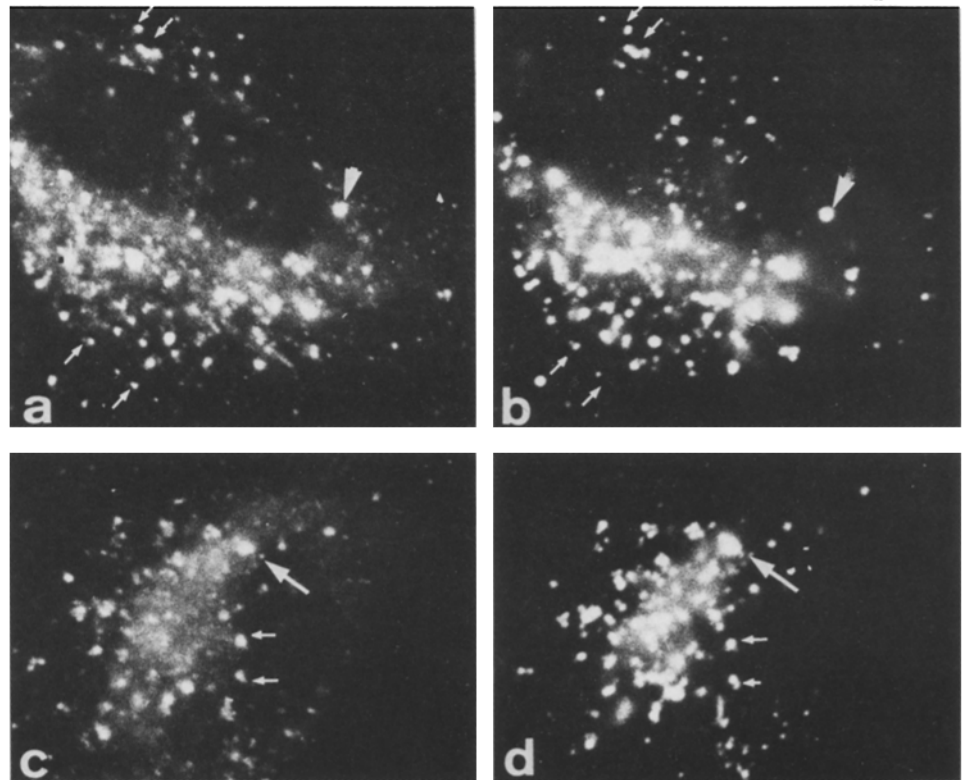# Antibodies

Acridine Orange

FIGURE 4 Double labeling of NRK cells with acridine orange (b and d) and purified antiserum to lysosome membranes (a and c). Most of the labeled structures were coincident (arrows), but some may have moved during processing. × 3,550.

Double Labeling of NRK Cells with Acridine Orange and the Purified Antiserum

To further determine whether our antiserum labeled lysosomal structures, we used a fluorescent weak base as a marker for lysosomal localization (35). Living NRK cells were incubated with acridine orange, which penetrates them and fluoresces brightly in an acidic environment. We photographed the living cells, then quickly fixed and processed them for immunofluorescent labeling. Careful comparison of identical fields revealed that the acridine orange staining could be almost completely superimposed on the immunolabeling (Fig. 4). The small differences between the two patterns are probably due to changes in cell shape and in the distribution of lysosomes that occurred during the time between the initial observation and fixation, or to differences in the sensitivity of the two staining methods.

Binding of the Purified Antiserum to Different Cellular Fractions

We used our purified antiserum to follow the fate of lysosome membranes as they were purified from rat-liver homogenates by cell fractionation as described in Fig. 1. For this purpose, we used immunoradiometry to assay the binding of the antiserum to immobilized fractions on polyvinyl chloride plates (Fig. 5). Significant binding occurred only in the dense fraction of the silica gel gradient and purified lysosomal fractions. Particularly noteworthy is the complete lack of binding with the crude mitochondrial fraction obtained during the last step of purification (free-flow electrophoresis).
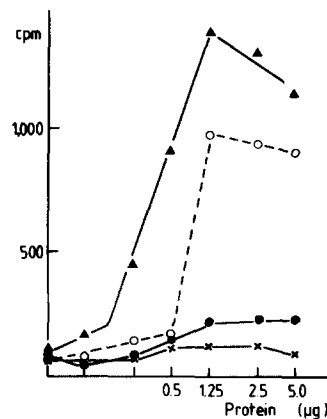

FIGURE 5 Radioimmunometric measurement of the binding of purified antiserum to lysosome membranes in different subcellular fractions of rat liver. ●, crude granular fraction; O, dense fraction of silica gel gradient; ▲, lysosomal fraction after free-flow electrophoresis; ×, mitochondrial fraction after free-flow electrophoresis.

Identification of the Polypeptides Recognized by the Purified Antiserum

We performed immunoreplica experiments to identify the polypeptide(s) recognized by the purified antiserum. Two polypeptides, mol wt 33,000 and 100,000, reacted strongly with the analyzed fractions (Fig. 6). The 100,000-mol-wt antigen was particularly abundant in the purified lysosomal fraction (Fig. 6, lane C). It was also detected in the enriched dense fraction of the silica gel gradient (Fig. 6, lane B), but was barely detectable in the crude granular fraction (Fig. 6, lane A). It was undetectable in the crude mitochondrial fraction (free-flow electrophoresis Fig. 6, lane D). Clearly, the 100,000-mol-wt polypeptide recognized by our antiserum co-purifies with the lysosomal fraction (Fig. 6, lane C). This was not the case with the other antigen of 33,000-mol-wt, which

A B C D
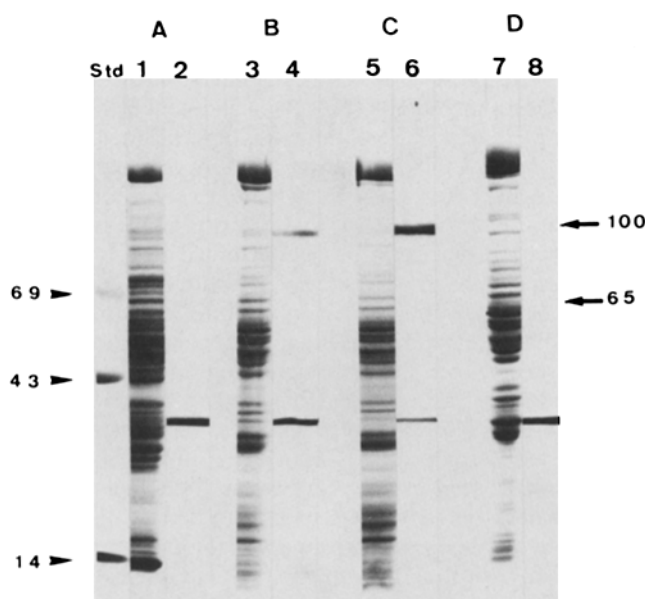Std 1 2 3 4 5 6 7 8

←100
69 ►
←65
43 ►

14 ►

FIGURE 6 Identification of the antigens recognized by the purified antiserum to lysosome membranes in different subcellular fractions of rat liver by immunoreplica experiments. Membrane fractions as defined in Fig. 5 were separated on SDS PAGE and stained using Coomassie Blue (lanes 1, 3, 5, and 7) or by immunoperoxidase labeling of a replica with the purified antiserum (lanes 2, 4, 6, and 8). (A) crude granular fraction; (B) dense fraction from the silica gel gradient; (C) lysosomal fraction; (D) crude mitochondrial fraction.

was reduced in the lysosomal fraction as evidenced by immunoreplica techniques, but was very abundant in the crude mitochondrial fraction (Fig. 6, compare lanes 6 and 8). However, radioimmunometric measurements, performed under nondenaturing conditions, indicated that our purified antibodies did not bind to this mitochondrial fraction (Fig. 5). One explanation for this discrepancy is that one or more antigenic determinants of the 33,000-mol-wt polypeptide are accessible for binding to the antibodies only after SDS treatment. Alternatively, this binding may be an artifact of our experimental procedures. In support of the latter interpretation, one should recall that Riezman et al. (48) recently reported nonspecific binding to a 29,000-mol-wt polypeptide membrane protein (perhaps porin) of purified mitochondria membranes during immunoreplica analysis.

By use of a microtechnique (9), antibodies specific for the 100,000-mol-wt polypeptide were affinity purified on isolated 100,000 mol wt bound to nitrocellulose strips. These affinity-purified antibodies were then used in immunofluorescence studies with NRK cells. The pattern of surface and internal labeling was identical to that produced by the purified antiserum (Fig. 2). We concluded, therefore, that the 33,000-mol-wt polypeptide probably was not recognized in situ by our antibodies and that the immunolocalization pattern observed can be attributed to the 100,000-mol-wt polypeptide.

Evidence for the Amphipathic Character of the 100,000-mol-wt Lysosomal Antigen

A simple and reliable assay to discriminate between integral and peripheral membrane proteins was carried out according to Bordier (4). The lysosomal fraction was extracted with Triton X-114, and the detergent and aqueous phases were analyzed by immunoreplica in a parallel experiment. The

33,000- and 100,000-mol-wt polypeptides were highly enriched in the detergent phase but depleted in the aqueous phase (Fig. 7). This indicates that the two polypeptides have an amphipathic character and therefore are probably integral membrane proteins (4).

Homologies between the 100,000-mol-wt Polypeptide and Membrane-bound ATPases

Recent pharmacological evidence suggests that the acidity of the lysosomal content is attributable to an ATPase that transports H^+ to the interior of the lysosome (reviewed in reference 46). We have been intrigued by the fact that several well-characterized ATPases have a catalytic subunit of 100,000-mol-wt (as measured by SDS PAGE): e.g., Na^+,K^+ ATPase (ouabain-sensitive) (43), Ca^{2+} ATPase (69), and stomach H^+,K^+ ATPase (42). We therefore investigated possible homologies between the 100,000-mol-wt polypeptide of the lysosome membrane and several membrane-bound ATPases. As can be seen in Fig. 8, Ca^{++} ATPase preparation shows a unique band of 100,000-mol-wt. Na^+,K^+ ATPase display a major band at 100,000-mol-wt polypeptide (catalytic α-subunit) and a diffuse band around 55,000-mol-wt (glycosylated β-subunit); a few contaminating low-molecular-weight polypeptides were also seen. H^+,K^+ ATPase preparations were nearly homogeneous and display a major polypeptide at 100,000-mol-wt as well as few poorly visible polypeptides below 50,000-mol-wt. Purified H^+,K^+ ATPase from pig stomach, the enzyme responsible for the secretion of HCl, exhibited a clear cross-reactivity with the 100,000 mol wt as shown by immunoreplica techniques (Fig. 8, lanes c and d). In contrast, neither the ouabain-sensitive ATPase of the cell surface (Fig. 8, lanes e and f) nor the Ca^{++} ATPase from sarcoplasmic reticulum (lanes g and h) reacted under the same conditions.
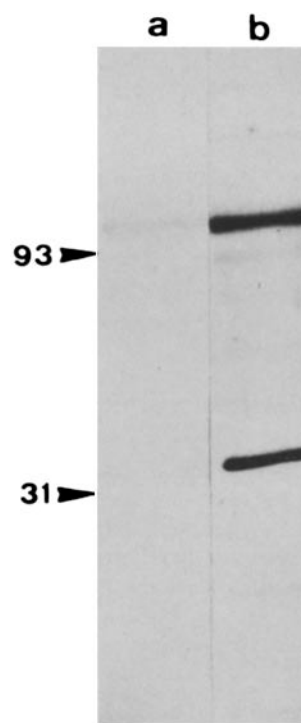

a b

93 ►

31 ►

FIGURE 7 Evidence for amphipathic character of the 100,000-mol-wt lysosomal antigen. The lysosomal fraction, 100 μg protein (see Fig. 6, lane C) was extracted with Triton X-114 and analyzed by using an immunoreplica procedure. The aqueous phase and detergent phase were recovered and were rerun on an 8% polyacrylamide gel in SDS. Note that the 100,000-mol-wt polypeptide was mostly recovered in the detergent phase (lanes B), whereas a weak co-migrating band was found in the aqueous phase (lane A). Also notice the complete recovery in the detergent phase of the 33,000-mol-wt polypeptide, which is thought to correspond to a mitochondrial integral membrane protein (see Results). Molecular weight markers DNAse (31,000). Phosphorylase a (93,000).
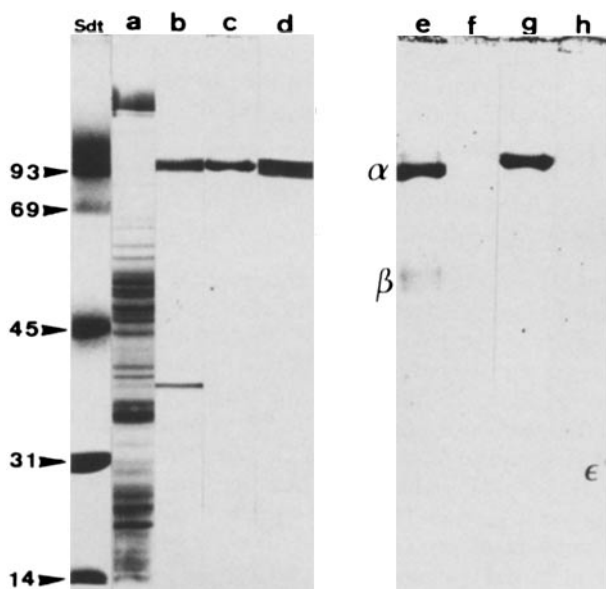
FIGURE 8 Immunological cross-reaction between different ATPase preparations and purified antibodies to the lysosomal membrane antigen (100,000-mol-wt). Immunoreplica on nitrocellulose strips (lanes b, d, f, and h) and corresponding protein pattern (Coomassie Blue staining) analyzed by 10% polyacrylamide gel electrophoresis in SDS (lanes a, c, e, and g). (Lanes a and b) Purified lysosome fraction; total protein 100 μg. (Lanes c and d) K⁺H⁺-ATPase from pig stomach (10 μg). (Lanes e and f) Na⁺ + K⁺-ATPase ouabain-sensitive (10 μg). (Lanes g and h) Ca⁺⁺-ATPase (10 μg). Molecular weight standards (Sdt) are phosphorylase a (93,000 mol wt). Bovine serum albumin (69,000 mol wt). Ovalbumin (45,000 mol wt). Pancreatic DNAse (31,000 mol wt). Egg lysozyme (14,000 mol wt). Notice in lane b the reaction with a 33,000-mol-wt polypeptide thought to be an artifact due to a mitochondrial antigen (see Results). Under our conditions Ca⁺⁺-ATPase migrates slightly slower than the other ATPases, Na⁺ + K⁺-ATPase catalytic subunit α and glycosylated subunit β.

Localization of the Antigen by Immunoperoxidase and Immunogold Techniques

NRK CELLS: NRK cells permeabilized with saponin were labeled by the immunoperoxidase technique. At low magnification, rounded structures of various sizes could be seen scattered throughout the cytoplasm (Fig. 9a). At higher magnification, typical secondary lysosomes (Fig. 9b) and small vesicles could be easily recognized. In rare instances, parts of a few Golgi cisternae were also labeled (Fig. 9b). The cell surface exhibited a weak reaction, similar to that observed in immunofluorescence studies (Fig. 2b). Acid phosphatase was distributed mainly in secondary lysosomes (Fig. 9c), as anticipated.

To assess differential permeability of the various organelle membranes when treated with 0.05% saponin, we used purified antibodies to RER or to Golgi membranes as the primary antibody (29). The appropriate organelles were stained (not shown). For example, many Golgi cisternae were stained with the anti-Golgi antibody, so we believe that the limited Golgi cisternae reaction seen with this purified antiserum to lysosomes is real and not artifactual.

MACROPHAGES: Because macrophages have many secondary lysosomes and are active in endocytosis, we attempted to determine the location of the antigen in this cell type. We used both the immunoperoxidase procedure that we had used

with NRK cells and the frozen thin-section method with an immunogold probe. On frozen thin sections, immunogold particles were most consistently present in small quantities on the plasma membrane (Fig. 10B) and concentrated near the inner membrane face of numerous large or small vacuoles (Fig. 10, A and B). Note that the immunogold particles are adjacent to the membrane and not homogeneously distributed within the content of the vacuoles. In order to test whether or not this antigen could be demonstrated in a phagocytic vacuole, macrophages were allowed to endocytize bacteria for 10 min before fixation. Using the immunoperoxidase-saponin method, peroxidase reaction product appeared consistently in vacuoles containing bacteria (Fig. 11A) as well as in small and large vacuoles, and one or two cisternae of the Golgi complex. No reaction product was seen when normal IgG was used as a control. This clearly illustrates that this antigen can be seen in organelles of the lysosomal pathway. Unfortunately, we have not been able to determine whether or not our antibody stains primary lysosomes, but we are currently investigating this possibility. One interesting technical variable was encountered. If saponin was omitted, distinct plasma membrane antigen could then be seen (Fig. 11B). This observation is consistent with the clear plasma membrane staining seen with the immunogold method (Fig. 10), which contains no saponin.

LIVER: Finally, because we used lysosome membranes from rat liver to produce antiserum, we determined the localization of the antigen in that organ by the immunoperoxidase procedure. Reaction product could be seen in some of the coated vesicles near the sinusoidal surface and in adjacent, smooth-surfaced, irregularly shaped vacuoles of varying sizes, presumed to be endosomes (Fig. 12) and occasionally multivesicular bodies (Fig. 12A). The secondary lysosomes and plasma membranes of Kupffer cells were heavily labeled (not shown), as well as the large coated pits and vesicles in endothelial cells (Fig. 12, inset). Often, two of the cisternae on the cis side of the Golgi complex were reactive (Fig. 13, A–C), as well as adjacent vesicles and vacuoles. As we expected because of our findings by immunofluorescence in rat liver (Fig. 3A), reaction product was present in large vacuoles, presumed to be the typical secondary lysosomes (or pericanalicular dense bodies) of liver. Under our conditions, these organelles were frequently broken, and the reaction product had bled into the adjacent cytoplasm (Fig. 13A). When well-preserved, their appearance can be seen in Fig. 14 (and inset). Near the bile canaliculi, multivesicular bodies containing the antigen were frequently present (Fig. 14).

Do Coated Vesicles Have the 100,000-mol-wt Antigen?

The presence of a 100,000-mol-wt polypeptide antigen in coated vesicles from calf brain was also confirmed. The results of such an experiment are shown in Fig. 15.

DISCUSSION

Using rat liver lysosomal fractions, purified by free-flow electrophoresis, we produced an antiserum that we screened for specific organelle reactivity by rapid immunofluorescence methods on NRK cells and sections of rat liver, kidney, and intestine. The crude antiserum contained antibodies that reacted with lysosomes, as well as with a few other intracellular structures, which we have identified morphologically. The
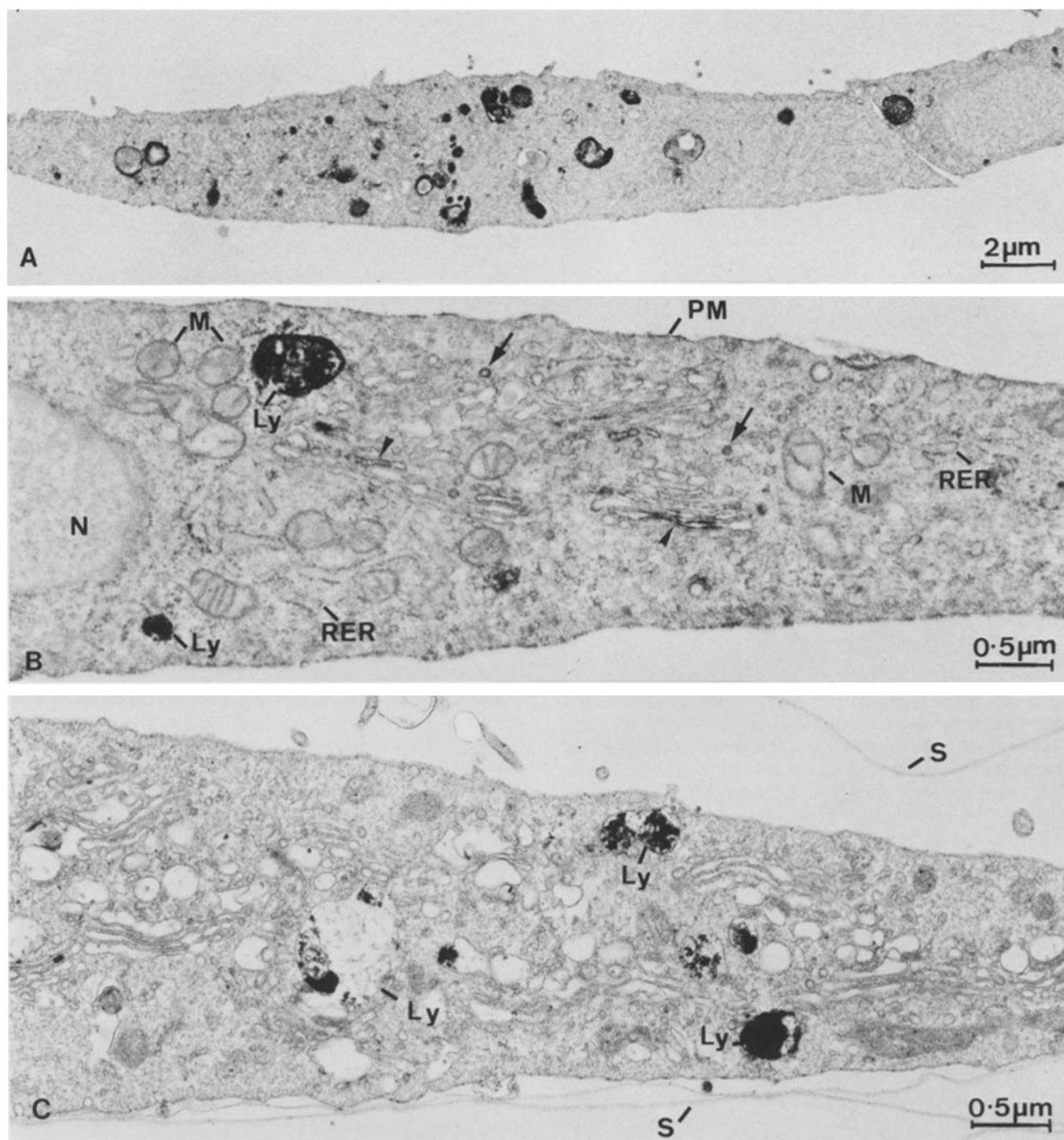
FIGURE 9   Immunoperoxidase (*a* and *b*) and acid phosphatase (*c*) labeling of NRK cells. (*a*) At low magnification, labeled structures of different shapes and sizes can be seen throughout the cytoplasm. (*b*) At higher magnification, labeled secondary lysosomes (*Ly*) can be seen. The plasma membrane (*PM*) was often slightly labeled. The membranes of the Golgi stacks (arrowheads) and of some small vesicles (arrows) within the Golgi region were occasionally labeled. *M*, mitochondria; *N*, nucleus; *RER*, rough endoplasmic reticulum. (*c*) Reaction product for acid phosphatase was found primarily within secondary lysosomes (*Ly*). The Golgi stacks were usually free of reaction product. *S*, surface of dish. (*a*) × 6,000; (*b* and *c*) × 25,000.

unwanted antibodies were removed from the antiserum by absorption with two subcellular fractions: mitochondria and intestinal microvilli. These were sufficient to give a labeling pattern characteristic of lysosomes. We then found by immunoreplica techniques that our purified antibodies bound almost entirely to one major integral membrane protein, with a molecular weight on SDS PAGE of 100,000. Binding to

another polypeptide (33,000 mol wt) was also observed but this binding may have been artifactual (see comments in Results). We cannot exclude the possibility that our absorbed antiserum contains antibodies against other polypeptides that escaped our analysis either because they were present in very low amounts or because they fail to react under our experimental conditions. However, the similarity between the im-
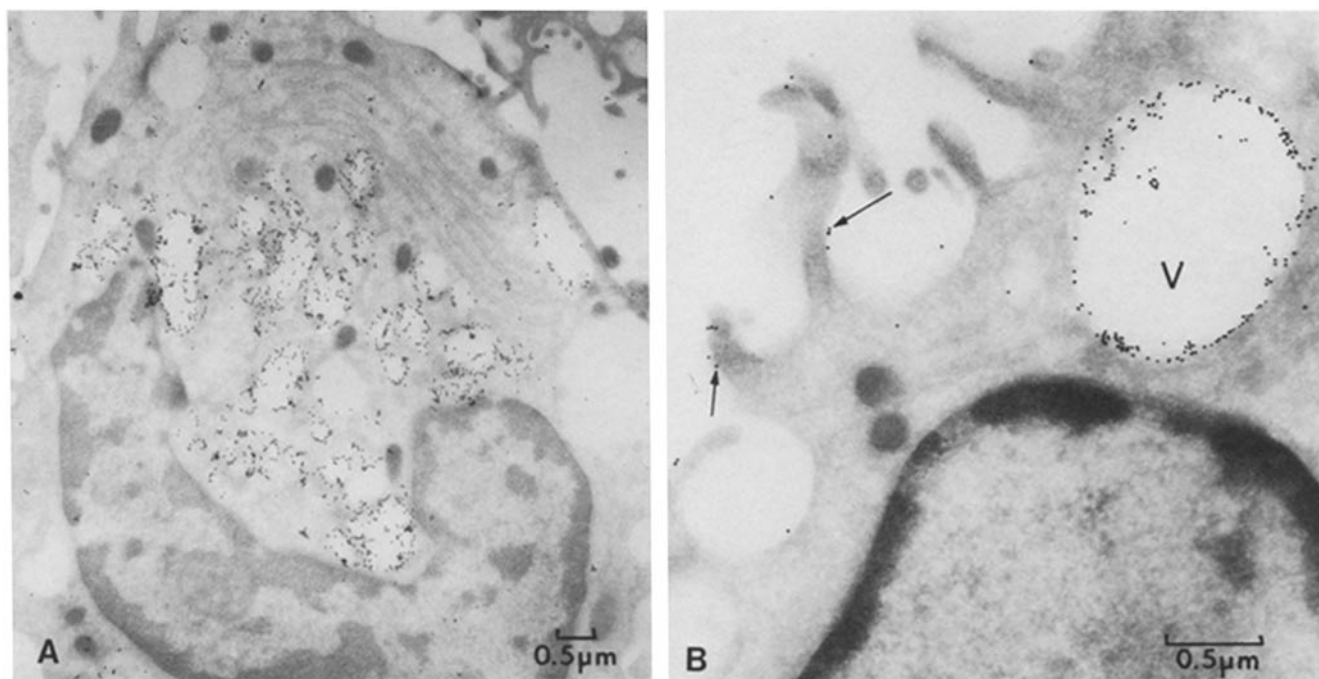
FIGURE 10   The localization of antigen in elicited rat peritoneal macrophages on frozen thin sections in which immunogold was used as a probe for the primary antigen. (A) A low-magnification view shows numerous labeled cytoplasmic vacuoles; (B) a higher-magnification view shows heavy label near the membrane of a vacuole (V) and scantier label on the plasma membrane (arrows). (A) × 10,000; (B) × 26,000.

munofluorescent labeling with the purified antiserum and that with affinity-purified antibodies against the 100,000-mol-wt antigen strongly suggests that this protein is found in the structures that were labeled by the antiserum. To our knowledge, this is the first antibody produced that recognizes an antigen on the membranes of lysosomes.

Our electron-microscopic immunolocalization of the antigen, which was performed with the purified antiserum, clearly showed that it is present on the membranes of secondary lysosomes. It was also detected in rare instances in RER and more frequently in a few cisternae on the *cis* side of the Golgi complex, as well as in adjacent vesicles. These organelles may be the sites of synthesis and transport of the antigen. It was also weakly present on the plasma membrane, and found in greater amounts in some nearby coated pits and vesicles, and in putative vacuolar "endocytic" structures. We have also observed that not all coated vesicles nor multivesicular bodies appear to have demonstrable antigen. In addition, in endothelial cells of rat liver, adjacent coated pits and vesicles display even more heterogeneity in that the large coated pits and vesicles contain this antigen whereas the smaller coated pits and vesicles do not (see Fig. 12, *inset*). This indicates that there are differences in coated pits and vesicles other than size, and that one may therefore anticipate differences in transport activities of these plasma membrane microdomains. We have also demonstrated that permeabilizing agents, such as saponin, may markedly diminish labeling of antigen on the plasma membrane. This artifact has been recently investigated in depth by Stenberg et al. (59).

What is the nature of this 100,000-mol-wt lysosomal membrane protein present in many structures recognized as organelles of the endocytic pathway? It may be significant that the antibody cross-reacted with a purified H^+,K^+ ATPase from pig stomach, but not with other membrane-bound ATPases

of similar molecular weight. This specific cross-reactivity indicates that the two proteins have common antigenic determinants. The proteins may therefore participate in a common function, such as the transfer of protons across the plasma membrane, the membranes of endocytic vesicles, and the lysosome membrane. In this respect, it is worth mentioning that pharmacological studies have shown that stomach H^+ ATPase is vanadate sensitive (12), whereas the lysosome H^+ ATPase is not (16). Therefore, our antibodies may bind to a family of membrane-bound H^+ ATPases, cross-reactive because they are structurally related, but with different catalytically active sites. In this regard, the labeling of red blood cells seen in Fig. 3A may reflect a cross-reactivity with the anion-sensitive Mg^{++} ATPase of erythrocytes (66).

The mechanisms responsible for the acidification of lysosomes have not been extensively studied until recently (46). In 1978, in a very important paper, Ohkuma and Poole (38) described a sensitive fluorescence method for measuring internal pH in lysosomes. They showed that the intralysosomal pH of macrophages that had ingested fluorescent dextran was ~4.7–4.8 and that the pH became more neutral within 1 min after the cells were exposed to a weak base such as NH_4Cl. They also demonstrated that the pH increased to ~5.4 shortly after the cells were exposed to both NaN_3 and 2-deoxyglucose, metabolic inhibitors that block ATP production by oxidative phosphorylation and glycolysis, respectively. They postulated that these substances affected the pH by interfering with the supply of energy from an active proton pump in the lysosome membrane. These findings, confirmed and extended by Ohkuma et al. (37) and other investigators (53–55), suggest that lysosomes contain a proton pump in which the hydrolysis of ATP by a membrane-bound ATPase is coupled to the translocation of protons from the external medium to the interior of the lysosome. Until recently, little has been known about
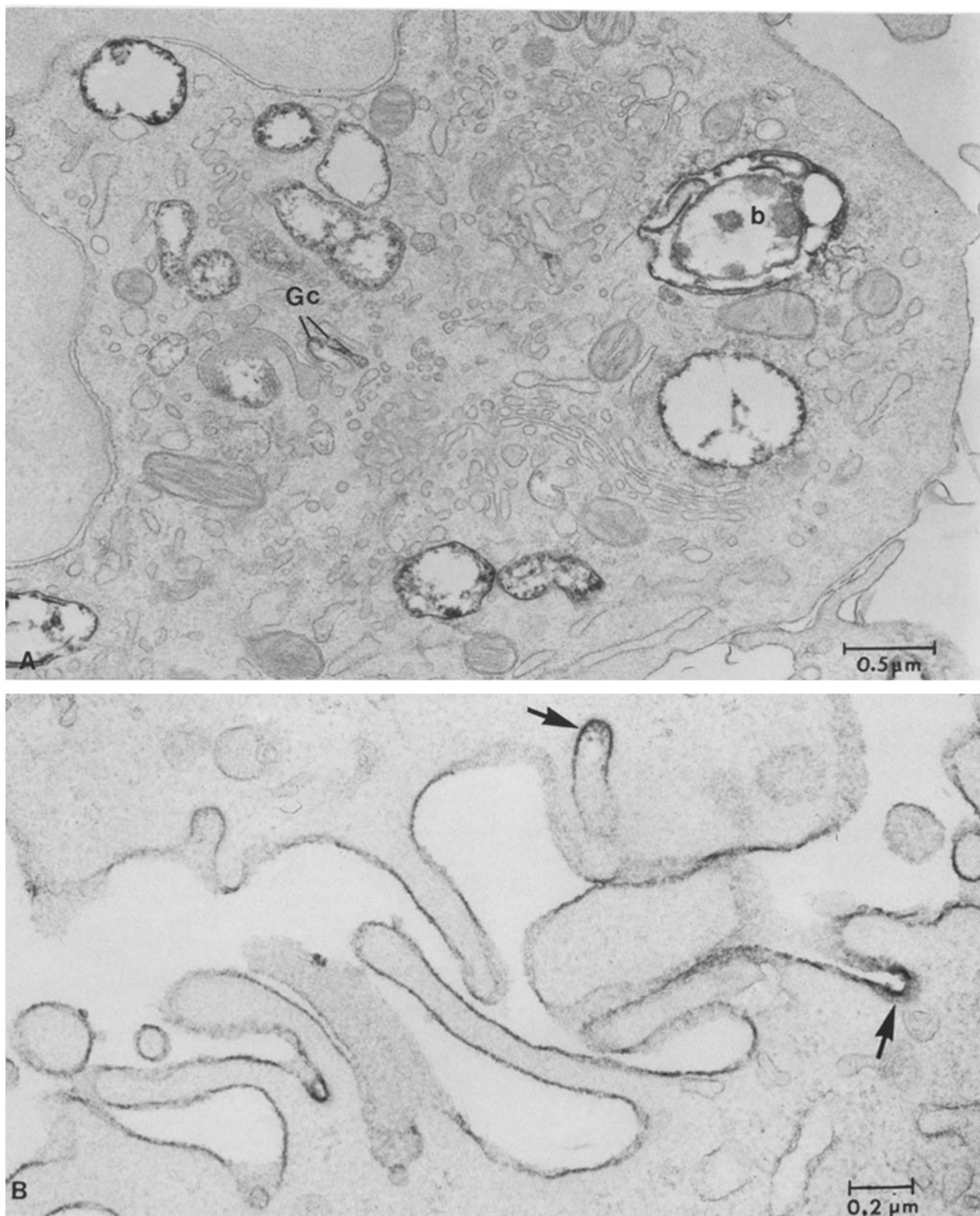
FIGURE 11 (A) The localization of antigen in macrophages 10 min after phagocytosis of E. coli using the immunoperoxidase-saponin procedure. Note the reaction product in the endocytic vacuoles that contain the bacterium (b) as well as in a few Golgi cisternae (Gc) and numerous vacuoles. (B) Same experimental circumstances as A, but the permeabilizing agent saponin was omitted. Note the presence of reaction product on the plasma membrane as well as possible coated pit areas (arrows). (A) × 33,000; (B) × 56,000.

the mechanism of action or the exact nature of the lysosomal proton pump (17). Various studies (53–55) have demonstrated ATPase activity on the lysosome membrane, but it has not been conclusively shown that this activity is related to a proton pump. Galloway et al. (16) have now demonstrated that both isolated endosomes and isolated lysosomes
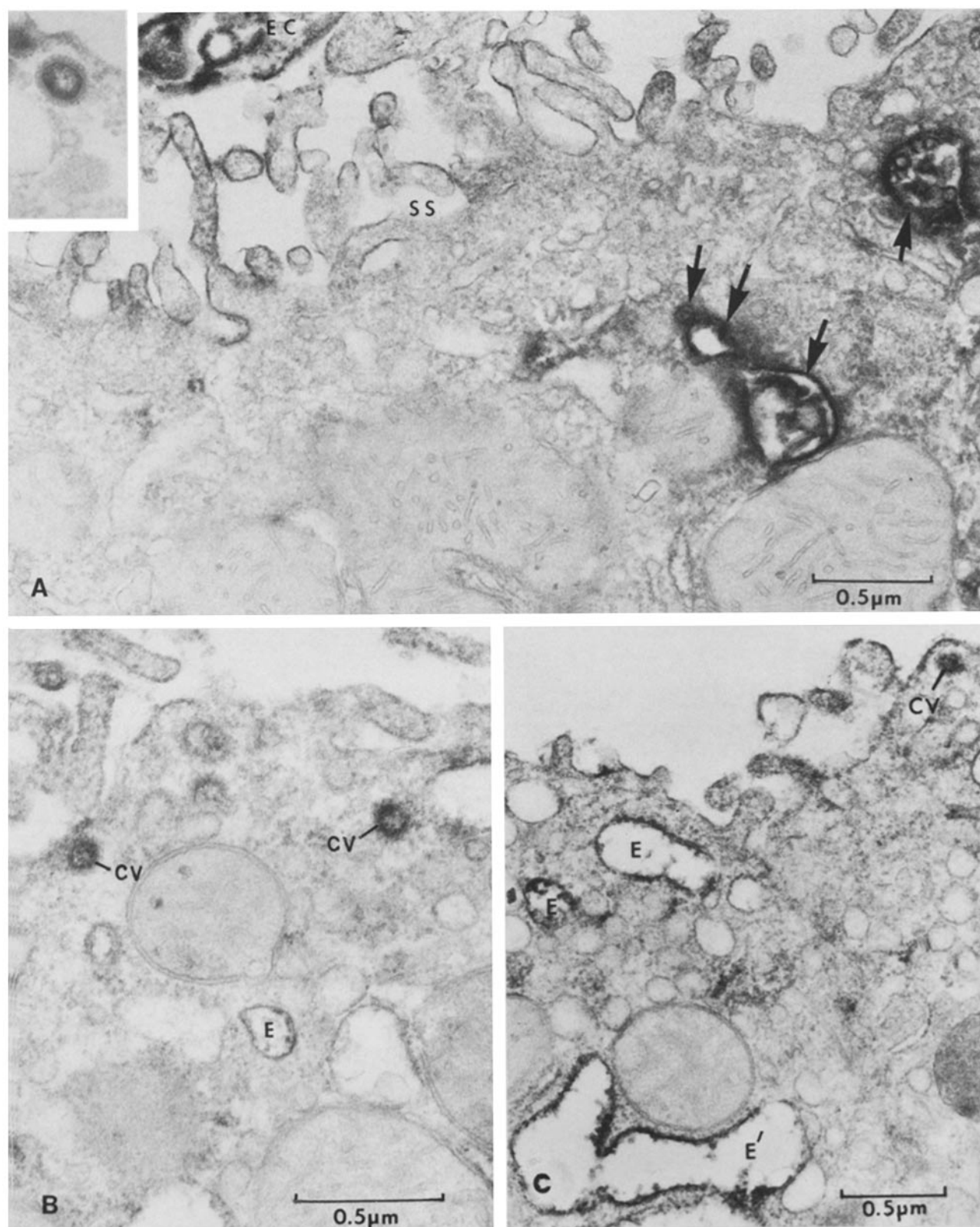
FIGURE 12 The localization of antigen in rat liver by the immunoperoxidase technique showing the sinusoidal face (SS) of the parenchymal cell and an adjacent endothelial cell (EC). It is difficult to say with certainty whether or not the sinusoidal plasma membrane contains antigen because the cells have been treated with saponin for permeabilization. (A) It is clear, however, that the endothelial cell (EC) contains large amounts of antigen and, as can be seen in the inset, the antigen is frequently found in the large coated vesicles but not the small coated vesicles. Close beneath the sinusoidal surface (SS) are reactive vacuoles of varying sizes and content (arrows). (B and C) Reaction product is also present in some coated vesicles (CV) and in small and large irregularly shaped vacuoles, possibly endosomes (E), near the sinusoidal surface of parenchymal cells. These vacuoles were sometimes swollen, possibly as an artifact of the mild fixation and the immunoperoxidase procedure (E'). (A) × 42,000; (inset) × 47,000. (B) × 53,000. (C) × 44,000.
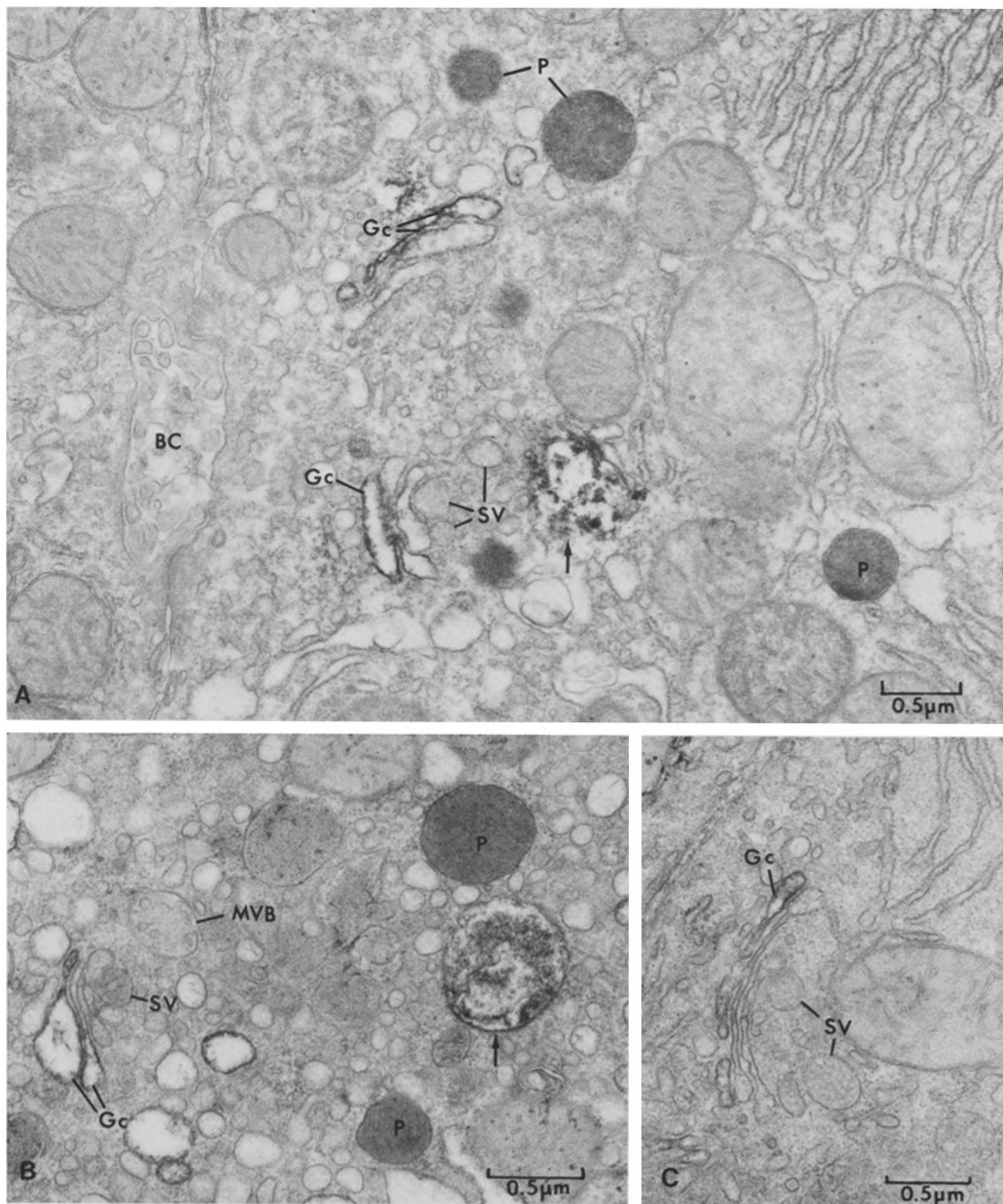
FIGURE 13   The Golgi regions near the bile canaliculi (BC) in rat liver parenchymal cells processed for the localization of antigen by the immunoperoxidase technique. Antigen can be found in several Golgi cisternae (Gc) on the cis side of the Golgi complex. Peroxisomes (P) are scattered throughout this area. Note that the secretory vacuoles (SV) and multivesicular bodies (MVB) contain no antigen. The arrows in A and B point to large reactive vacuoles, probably secondary lysosomes. Note that the tissue preservation in C is better than in A or B (i.e., fewer vacuoles, and broken membranes, less bleeding of reaction product), but the amount of reaction product is less. (A) × 30,000; (B) × 34,000; (C) × 30,000.

reduce their internal pH via an ATP-dependent process, and the studies of Yamashiro et al. (71) support this conclusion.

If the 100,000-mol-wt protein is a component of the proton pump (by analogy with the purified gastric H+,K+ ATPase), its presence in small amounts on the plasma membrane, as well as in putative prelysosomes (coated vesicles derived from
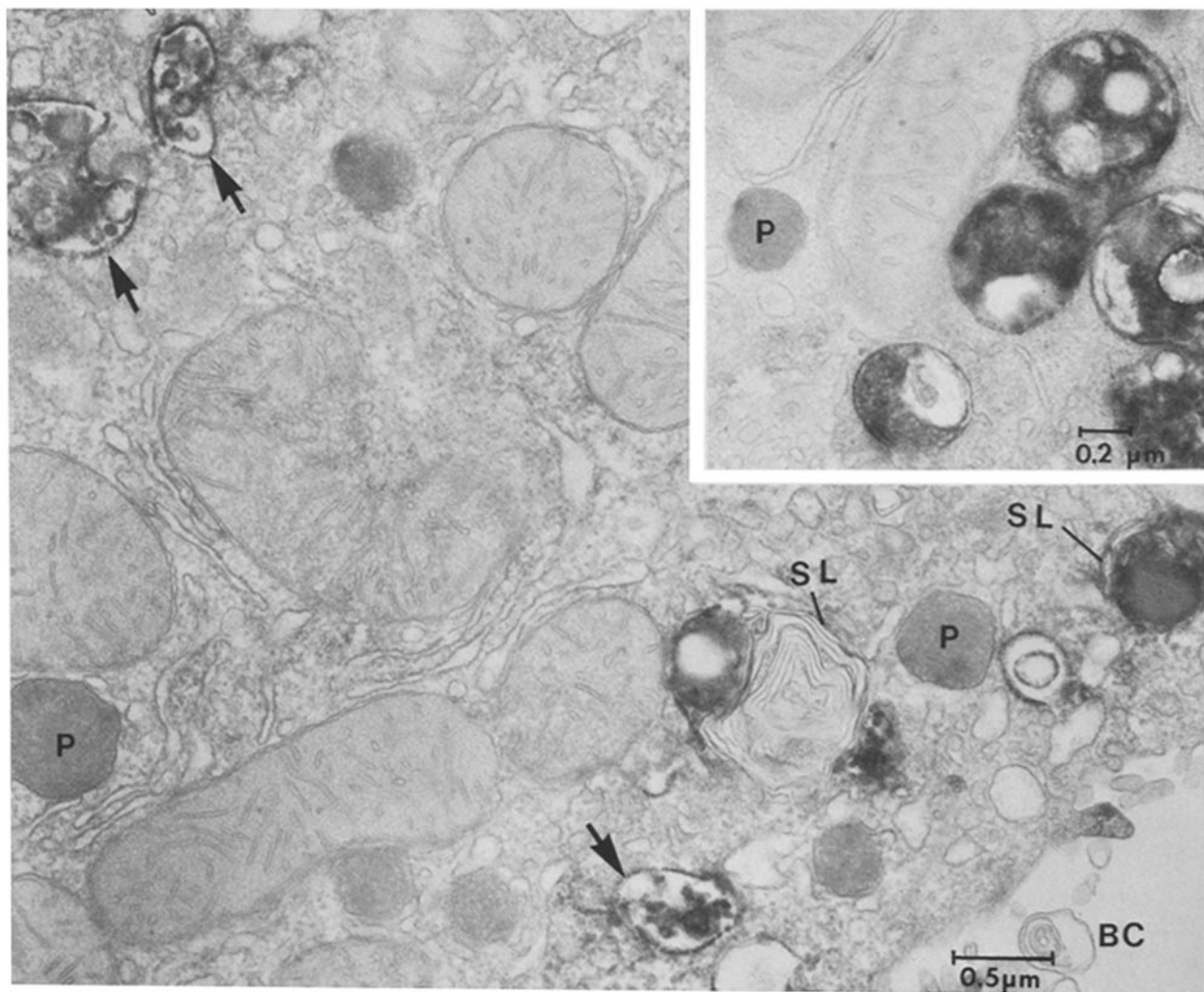
FIGURE 14 The localization of antigen near the bile canaliculi (BC) in rat liver using the immunoperoxidase technique. Near the bile canaliculi (BC) are several reactive obvious secondary lysosomes (SL) containing membranes, fat, and other materials as well as the antigen (arrows). Note that the peroxisomes (P) are not labeled. Note also that vacuoles are present resembling multivesicular bodies that contain antigen both on the membrane and within the matrix and vesicles (arrows). The inset demonstrates more variations in the morphology of obvious pericanalicular dense bodies (the secondary lysosomes) which contain the lysosomal antigen in their matrix as well as in their limiting membrane. × 31,000. (Inset) 36,000.

the cell surface and vacuoles that are possibly endosomes) and in secondary lysosomes, suggests that it participates in the acidification of the structures of the endocytic pathway. At the plasma membrane, H^+ would be pumped into the extracellular space. Upon invagination of the plasma membrane and formation of the coated vesicles, H^+ ions would be trapped within the small enclosed vesicles. Indeed, Forgac et al. (15) have now shown that some coated vesicles are acidic. We analyzed their preparation of coated vesicles with our purified antibody to lysosome membranes, and indeed a 100,000-mol-wt polypeptide was labeled (see Fig. 15). The next structures in the prelysosomal pathway of endocytosis are the endosomes, which are also acidic (16, 26, 31, 32, 65, 71). At this site, in an acid environment, many ligands become dissociated from their receptors, with the ligands going to the lysosome for degradation and the receptors being quickly recycled to the cell surface (reviewed in references 6 and 27). Although we have conclusively demonstrated that phagocytic vacuoles of macrophages contain the 100,000-mol-wt protein, we have no direct evidence that it is in endocytic coated

vesicles or endosomes. Future work with this antibody should be attempted with double-label methods to test this point. It is therefore of interest that Tougard, Louvard, Picart, and Tixier-Vidal (manuscript submitted for publication) have investigated the localization of our antibody during adsorptive endocytosis in rat prolactin cells in culture using cationized ferritin as tracer. Their results indicate that the 100,000-mol-wt antibody reacts with both prelysosomal and lysosomal compartments involved in the endocytic pathway of these cells. In addition, Baron et al. (3) have shown the presence of this antigen on the ruffled border of osteoclasts where lower pH conditions allow bone resorption and not on the plasma membrane facing away from the bone.

Finally, the recent paper of Glickman et al. (19) indicates that isolated Golgi membranes do contain an active ATP-dependent H^+ pump. This finding suggests a possible role for the antigen that we detected in cisternae on the cis side of the Golgi complex in rat liver parenchymal cells. In this position, the presumed "receiving face" of the Golgi complex, an active H^+ ATPase might create an acidic environment that could
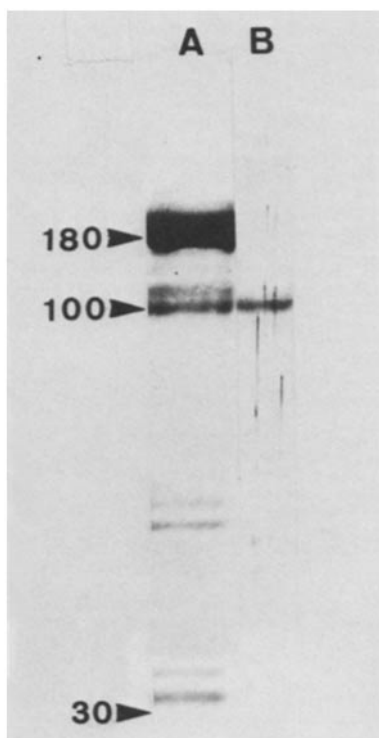
FIGURE 15 Presence of an antigen (100,000 mol wt) in a highly purified coated-vesicle preparation which cross-reacted with the purified antiserum raised against a lysosomal membrane preparation. (A) Polypeptide composition of calf brain coated vesicles (50 μg) analyzed by 10% PAGE in SDS (Coomassie Blue staining). (B) Immunoreplica on nitrocellulose strips showing antigen cross-reacting (100,000 mol wt) with lysosomal membrane antigen. Notice in A the clathrin heavy chain (180,000 mol wt) and its associated light chains (~36,000 and 39,000 mol wt).

play a role in receptor-ligand association or dissociation (6), in activation of certain enzymes involved in posttranslational modification of synthesized substances, or possibly even in fusion–fission membrane phenomena (31).

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