Lysosomal Membrane Dynamics: Structure and Interorganellar Movement of a Major Lysosomal Membrane Glycoprotein

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Abstract. The biochemistry and intracellular transit of an integral membrane glycoprotein of chicken fibroblast lysosomes were studied with monoclonal antibody techniques. The glycoprotein had an apparent molecular weight of 95,000–105,000. Structural analysis involving metabolic labeling with [^{35}S]methionine and cleavage with glycosidases revealed the presence of numerous oligosaccharide chains N-linked to a core polypeptide of apparent molecular weight 48,000. A primary localization of the glycoprotein to lysosomes was demonstrated by the coincidence of antibody binding sites with regions of acridine orange uptake, electron immunocytochemical labeling on the inner surface of lysosome-like vacuolar membranes, and preferential association of the glycoprotein with lysosome-enriched subcellular fractions from Percoll gradients. In addition, small quantities of the glycoprotein were detected on endocytic vesicle and plasma membranes. To study the intracellular pathway of the glycoprotein, we used a monoclonal antibody whose binding to the glycoprotein at the cell surface had no effect on the number or subcellular distribution of antigen molecules. Incubation of chicken fibroblasts with monoclonal antibody at 37°C led to the rapid uptake and subsequent delivery of antibody to lysosomes, where antibody was degraded. This process continued undiminished for many hours on cells continuously exposed to the antibody and was not blocked by the addition of cycloheximide. The rate at which antigen sites were replenished in the plasma membrane of cells prelabeled with antibody ($t_{1/2} = 2$ min) was essentially equivalent to the rate of internalization of antibody bound to cell surfaces. These results suggest that there is a continuous and rapid exchange of this glycoprotein between plasma membrane and the membranes of endosomes and/or lysosomes.

OF vital importance to the proper functioning of the lysosome is its unique surrounding membrane that sequesters numerous acid hydrolases used for degradation and modification of substances passing through the vacuolar membrane system. In addition to playing this structural role, the lysosomal membrane is specialized for acidification of the intralysosomal environment (26, 27, 32); release of the final products of lysosomal digestion; and specific interactions with other membranous organelles, including endosomes and the plasma membrane (5, 6, 8, 10, 21, 25, 34, 35, 38, 41). Despite recent interest in studying the lysosomal membrane (7, 17, 28, 32), the chemical and structural characteristics that account for its properties are not known. Nor is it understood how the lysosomal membrane is formed and maintained amidst the rapid and extensive flow of membrane occurring throughout the vacuolar membrane system.

To learn more about the properties and dynamics of the lysosomal membrane, we have generated a monoclonal antibody to one of the major lysosomal membrane proteins in chicken cells. We have used the monoclonal antibody to examine the structure, subcellular localization, and intracellular pathway of this 95–105-kD lysosomal membrane glycoprotein. Some of the properties of the glycoprotein resemble those of other previously described 100–120-kD lysosomal membrane proteins in rat and mouse that are heavily glycosylated (7, 17) and are distributed in small quantities on endosomal and plasma membrane (28). The relationships among these antigens still remain to be determined.

The dynamics of the 95–105-kD lysosomal membrane glycoprotein were studied in chicken fibroblasts with a low pH–sensitive monoclonal antibody, CV24 IgG, as probe for surface and intracellular antigen. We found that CV24 IgG bound to surface antigen is rapidly internalized into endocytic vesicles and transported to lysosomes. Furthermore, surface antigen sites removed during endocytosis are rapidly replenished from pre-existing intracellular antigen pools. Finally, continued endocytosis of antigen with CV24 IgG does not change the number or steady state distribution of antigen molecules in cells. These results are consistent with a rapid and continuous cycling of antigen between lysosomes and/or endosomes and the cell surface.

Materials and Methods

Preparation of Monoclonal Antibody CV24

Monoclonal antibody CV24 (CV24 IgG) was generated from a fusion using

BALB/cJ mice immunized with a preparation of chicken liver coated vesicles. Material for the injection was kindly provided by Dr. T. Roth and colleagues (University of Maryland, Baltimore, MD). The coated vesicles were isolated by the method of Nandi et al. (24). The immunization protocol, cell fusion, hybridoma selection, and cloning procedures were as described by Fambrough and Bayne (9). Hybridoma CV24 was cloned in soft agar and grown as an ascites tumor in BALB/cJ mice. The monoclonal antibody secreted by this hybridoma was identified as an IgG by subunit analysis on SDS PAGE. Large quantities of CV24 IgG were purified from ascitic fluid by ammonium sulfate precipitation and ion exchange chromatography on DE52 (Whatman Chemical Separation Inc., Clifton, NJ). Purified IgG was stored at −70°C in 40 mM sodium phosphate, pH 6.8.

Binding of ^{125}I-CV24 IgG to a chicken liver coated vesicle preparation that had been size-fractionated by gel permeation chromatography (15) revealed the greatest antibody binding activity in column fractions containing large (>190-nm diam) uncoated membrane vesicles. CV24 IgG was therefore made against a contaminant in the coated vesicle preparation.

Cell Culture

Chicken fibroblast cultures were used in all experiments and obtained by mechanical dissociation of 11-d chicken embryo leg muscle and plating of the isolated cells in Falcon tissue culture dishes (Falcon Labware, Oxnard, CA). Adherent cells were grown to confluence and passaged from three to six times before use. Cells were maintained in Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with 4% chicken serum and 10% horse serum (MEM 410)' at 37°C in 5% CO_2.

Preparation of Labeled Proteins

Fluorescein-labeled CV24 IgG (F-CV24 IgG) and tetramethylrhodamine-labeled CV24 IgG (R-CV24 IgG) were prepared according to the procedure of Anderson and Fambrough (2). Fluorescein isothiocyanate was obtained from Molecular Probes, Inc. (Junction City, OR), and tetramethylrhodamine isothiocyanate was obtained from Research Organics, Inc. (Cleveland, OH). Rhodamine-labeled goat anti-mouse IgG was purchased from Cappel Laboratories (West Chester, PA). Sulforhodamine-labeled stearoyl-dextran (R-SD) was kindly given by Michael Koval, The Johns Hopkins University, Baltimore, MD, who prepared it as described in reference 43.

The conjugate between horseradish peroxidase (HRP) (Boehinger, Indianapolis, IN) and CV24 IgG (HRP-CV24 IgG) was prepared by the method of Wilson and Nakane (42). One or two peroxidase molecules were bound per IgG, as measured spectrophotometrically.

Iodination of CV24 IgG, C3/1 IgG, and wheat germ agglutinin was carried out with Iodogen (Pierce Chemical Co., Rockford, IL) according to the method of Salicinski et al. (31). Typically, 50–100 μg protein was iodinated with 1 mCi Na^{125}I (15 mCi/μg I, Amersham Corp., Arlington Heights, IL). Wheat germ agglutinin was iodinated in the presence of N-acetyl-glucosamine to preserve the active binding site of the lectin. Free iodine in the reaction mixtures was removed by chromatography on Dowex AG1-X2 ion exchange resin (BioRad Laboratories, Richmond, CA).

Fluorescence Microscopy

Cells were grown on 25-mm No. 1 glass coverslips in 35-mm culture dishes. For fixation, cells were treated with 1% formaldehyde, 0.02 M sodium phosphate, 0.15 M sucrose, pH 7.2, at 25°C for 10 min, and then washed for 15 min in Hanks' balanced salt solution containing 0.1% bovine serum albumin (HBSS/BSA) and 50 μg/ml lysine. Under these conditions, the cells remained impermeable to trypan blue (Gibco). Direct immunofluorescent staining was performed with 5–10 μg/ml F-CV24 IgG or R-CV24 IgG in Hepes-buffered MEM 10, pH 7.2 (Hepes MEM 10), in the presence or absence of 0.25% saponin (Calbiochem-Behring Corp., La Jolla, CA). Cells were incubated in this medium and then washed for 30 min in several changes of HBSS/BSA. Indirect immunofluorescent staining was performed on fixed cells as described in the figure legends. After washes in HBSS/BSA, coverslip cultures were mounted on depression slides in Hepes MEM 10 and examined with a Zeiss inverted microscope (Carl Zeiss Inc., Thornwood, NY) equipped with barrier

filters that prevented cross-over of fluorescein and rhodamine fluorescence. Micrographs were developed in Acufine developer (VWR Scientific, Philadelphia, PA). Nonspecific binding, determined by the addition of 50–100-fold excess unlabeled CV24 IgG to the labeling medium, was negligible.

^{125}I-CV24 IgG Binding Studies

Binding assays with ^{125}I-CV24 IgG were performed on 35-mm tissue culture dishes containing ~2 × 10^6 cells/dish. Specific binding was calculated by subtracting binding determined in the presence of 50- or 100-fold excess unlabeled CV24 IgG. Cell-associated radioactivity was measured with a Packard gamma-scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, IL) after the cells were dissolved in 2 ml of 1 N NaOH. The number of cells per culture dish was determined by direct counting on sister cultures stained with 2% Giemsa. For cells that were fixed before ^{125}I-CV24 IgG binding, the fixation protocol for fluorescence microscopy was used. Fixation had no substantive effect on the affinty of CV24 IgG for antigen, since the number of antibody binding sites measured at equilibrium on fixed cells was the same as on live cells at 4°C (data not shown). Our conditions for permeabilizing cells (0.25% saponin included in the antibody incubation medium) also had no substantive effect on antibody-antigen affinity (see Fig. 1).

Affinity Purification and Analysis of Biosynthetically labeled Antigen

Cell cultures grown on 150-mm dishes to near confluence (2 × 10^7 cells/dish) were incubated with methionine-free MEM 410 supplemented with 100–250 μCi/ml [^{35}S]methionine (1,400 Ci/mmol, Amersham Corp.) to label metabolically cellular proteins. After the labeling or chase period, the cultures were solubilized as described in figure legends or as follows. Pulse-chase cultures were solubilized with two 3.5-ml vol ice-cold extraction buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, and the protease inhibitors 2 mM benzamidine, 5 mM N-ethylmaleimide, and 1 mg/ml bacitracin [33]). Cell debris was then removed by sedimentation at 17,500 g for 1 h.

Antibody affinity chromatography was used to isolate CV24 antigen from the metabolically labeled cell extracts. CV24 IgG was coupled to CNBr-activated Sepharose-4B as recommended by the manufacturer (Pharmacia Fine Chemicals, Piscataway, NJ) or as described in reference 9 to generate immunobeads. Usually, 3 mg antibody was coupled to 1 g Sepharose. A volume corresponding to 50 μl packed CV24 IgG immunobeads was added to the cell extracts to precipitate all of the CV24 antigen. Before the addition of extract, immunobeads were incubated with horse serum to saturate nonspecific binding sites. The immunobead-containing extracts were incubated for 16 h at 4°C with constant gentle rocking. The immunobeads were then collected by sedimentation and washed as described previously (9). Antigen was eluted by incubation of the immunobeads in SDS gel sample buffer (3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 62 mM Tris-Cl, pH 6.8) at 100°C for 5 min.

Purified labeled CV24 antigen was analyzed by SDS PAGE in a 7.5% polyacrylamide gel (16). Molecular mass standards included β-galactosidase (116 kD), phosphorylase A (95 kD), BSA (68 kD), and ovalbumin (43 kD), all from Sigma Chemical Co., St. Louis, MO. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma Chemical Co.), destained, and treated for fluorography as previously described (9).

Immunoperoxidase Labeling and Electron Microscopy

Cells grown on 35-mm plastic tissue culture dishes were fixed with 1.6% formaldehyde, 0.25% glutaraldehyde, 2% sucrose, 0.02% saponin in 20 mM sodium phosphate, pH 7.2, for 20 min at 25°C. The cells were washed several times with HBSS/BSA containing 50 μg/ml lysine and then incubated with 5 μg/ml HRP-CV24 IgG in HBSS/BSA for 2 h at 25°C and washed in the same medium lacking antibody for 30 min at 4°C. The cells were next fixed in 1.6% glutaraldehyde, 0.8% paraformaldehyde, 5 mM $CaCl_2$ in 80 mM sodium cacodylate, pH 7.4, for 30 min at 25°C and washed in 0.1 M sodium phosphate, pH 6.5, for 1 h at 25°C. The cells were then incubated for 30 min in 0.1 M sodium phosphate, pH 6.5, containing 0.5 mg/ml diaminobenzidine (Aldrich Chemical Co., Milwaukee, WI) and 0.012% (vol/vol) H_2O_2 (12). After being washed for 5 min in 0.1 M sodium phosphate, pH 6.5, the cells were postfixed for 1 h at 25°C with 1% OsO_4 in 0.1 M sodium cacodylate, pH 7.4. The cells were then stained with 0.5% uranyl acetate for 1 h at 4°C, dehydrated in a graded ethanol series, and embedded in Epon. Thin sections were observed with a JEOL electron microscope.

Percoll Density Gradient Fractionation

We followed the fractionation procedure of Rome et al. (29) with some

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1. *Abbreviations used in this paper:* endo H, endo-β-N-acetylglucosaminidase H; F-CV24 IgG, fluorescein-labeled CV24 IgG; HBSS/BSA, Hanks' balanced salt solution containing 0.1% bovine serum albumin; Hepes MEM 10, Hepes-buffered Eagle's minimal essential medium containing 10% horse serum; HRP, horseradish peroxidase; MEM 410, Eagle's minimal essential medium containing 4% chicken serum and 10% horse serum; R-CV24 IgG, tetramethylrhodamine-labeled CV24 IgG; R-SD, sulforhodamine-labeled stearoyl-dextran.

modifications. Confluent chick fibroblast cultures were dissociated with 0.25% trypsin in HBSS for 10 min at 37°C and washed twice in MEM 410. To ensure recovery from trypsinization, the cells were incubated at 37°C in MEM 410 for 2 h before Percoll fractionation. An alternate procedure for removing cells from culture dishes (i.e., scraping) resulted in the same subcellular distribution of CV24 antigen in Percoll gradients as was observed for the trypsinization protocol. All subsequent steps were carried out at 4°C. Approximately 5 × 10⁷ cells were washed twice in ice-cold 0.25 M sucrose, 1 mM EDTA, 10 mM Hepes, pH 6.8, resuspended in 2 ml of the same buffer, and subjected to nitrogen cavitation with a pressure of 30 psi applied for 10 min. The suspension of broken cells was collected and further disrupted with three gentle strokes of a Teflon pestle in a glass homogenizer. After centrifugation at 600 g for 10 min, the pellet was resuspended in 1 ml of the buffered sucrose solution, homogenized (two strokes), and centrifuged again. The pellet was extracted three additional times without further homogenization, and the supernatants obtained from the extracts were pooled.

The combined postnuclear supernatant (6 ml) was layered over 28 ml of a 30% Percoll (Pharmacia Fine Chemicals) solution previously layered over 4 ml of 2.5 M sucrose. The Percoll solution was prepared in 0.25 M sucrose, 29 mM NaCl, 1 mM EDTA, 10 mM Hepes, pH 6.8. The tubes were centrifuged in a Beckman VTi50 vertical rotor (Beckman Instruments Inc., Palo Alto, CA) at 28,500 rpm for 1 h at 4°C. 1-ml fractions were siphoned from the bottom of the tubes with an LKB fraction collector system (LKB Instruments, Inc., Gaithersburg, MD).

Enzyme and Marker Assays

Lysosomes. The lysosomal enzyme marker β-hexosaminidase was assayed on each Percoll gradient fraction with 4-methylumbelliferyl-β-D-galactoside as substrate. Incubation mixtures (0.4 ml total) contained 0.1 M sodium acetate, pH 4.4, 0.1% Triton X-100, 1.2 mM 4-methylumbelliferyl-β-D-galactoside (Research Products International Corp., Mt. Prospect, IL) and 50 μl of the gradient fraction to be assayed. After 30 min at 37°C the reaction was terminated by the addition of 1 ml of 0.5 M glycine, 0.5 M sodium carbonate, pH 10. The liberated 4-methylumbelliferone was measured in a fluorometer at 365 nm for excitation and 450 nm for emission. Results are expressed in arbitrary units of fluorescence.

Endocytic Vesicles. Cells were incubated with bovine ¹²⁵I-β-galactosidase (kindly provided by G. Sahagian, Tufts University, Boston, MA) at 4°C for 30 min and then warmed to 37°C for 3 min to allow internalization of the ligand by the cells (30). The cells were returned to 4°C and sedimented through HBSS containing 10% BSA. After a wash in buffered sucrose, the cells were broken by nitrogen cavitation and fractionated in a Percoll gradient. Radioactivity in each gradient fraction was measured.

Plasma Membrane. Cells were incubated with ¹²⁵I-wheat germ agglutinin (7 × 10⁶ cpm/ml) for 30 min (14, 30) and then sedimented through HBSS containing 10% BSA, all at 4°C. After several washes in buffered sucrose, the cells were broken by nitrogen cavitation and fractionated in a Percoll gradient. Radioactivity in each gradient fraction was measured.

Protein. Cells metabolically labeled for 12 h with [³⁵S]methionine were fractionated in a Percoll gradient, and the radioactivity from equivalent volumes of each fraction was measured.

CV24 Antigen. Cells were labeled with 100–250 μCi/ml [³⁵S]methionine for 12 h and then fractionated in a Percoll gradient. Each gradient fraction was solubilized in 1% Triton X-100 extraction buffer and incubated with CV24 immunobeads without prior removal of the Percoll. Washed immunoprecipitates were purified further by SDS PAGE and visualized by fluorography. The 100-kD region of the gel for each fraction was excised, incubated with NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL) overnight, neutralized with acetic acid, and then added to scintillation cocktail for measurement of radioactivity.

Results

Monoclonal Antibody CV24 Binding to Surface and Intracellular Sites on Chicken Fibroblasts

Binding studies with monoclonal ¹²⁵I-CV24 IgG on intact and permeabilized chicken fibroblast cultures indicated that CV24 IgG binding sites were primarily intracellular. Fig. 1 shows a binding isotherm and corresponding Scatchard plot for CV24 IgG binding to fixed cultures in the absence (*A*) and presence (*C*) of 0.25% saponin, respectively. This concentration of

saponin rendered the cells permeable to IgG without affecting antibody-antigen affinity. Antibody bound to a single class of high affinity binding sites (K_d = 6–8 nM) in both cases. Saponin treatment increased the number of antibody binding sites from 8,000 ± 800 to 380,000 ± 10,000. Thus, ~98% of the antigen recognized by CV24 IgG was intracellular.

Light microscope autoradiography of cells labeled with ¹²⁵I-CV24 IgG (at 4°C, before fixation) was performed to verify that surface antibody binding on fixed cells reflected actual antibody binding sites on cell surfaces rather than nonspecific binding due to fixation or binding to internal sites in a small number of leaky cells. A unimodal distribution of grain density per cell was observed across the cell population (data not shown).

Indirect immunofluorescent staining with CV24 IgG on intact and permeabilized chicken fibroblast cultures revealed the distribution of surface and intracellular antigen. Faint labeling over the entire cell surface was observed on all cells of formaldehyde-fixed cultures (Fig. 1 *B*). On permeabilized cells, however, bright labeling of numerous intracellular vesicles was observed (Fig. 1 *D*). These were scattered throughout the cytoplasm but appeared particularly abundant in the perinuclear region.

CV24 IgG binding to chicken fibroblast cultures was markedly affected by differences in pH and temperature. As shown in the pH-dissociation profile of Fig. 2 *A*, dissociation of ¹²⁵I-CV24 IgG from fixed cells was nearly complete at pH 4.8 after 1 h at 25°C. Acid sensitivity of CV24 IgG binding, therefore, provided a means for selectively removing cell surface-bound ¹²⁵I-CV24 IgG (see below). The rates of dissociation of ¹²⁵I-CV24 IgG from fixed cells at various temperatures are shown in Fig. 2 *B*. The half-time for antibody dissociation was 45 min at 37°C, 5 h at 25°C, and 50 h at 4°C.

A Primary Localization of CV24 Antigen in Lysosomal Membranes

Double labeling experiments performed with lysosomal markers on permeabilized chicken fibroblasts suggested that most of the intracellular structures labeled by fluorescent CV24 IgG were lysosomes. Staining by F-CV24 IgG on permeabilized cells co-localized almost completely with the distribution of acridine orange (Fig. 3), a vital dye that fluoresces orange-red upon accumulation in lysosomes and other acidic organelles (1, 23). Co-localization of CV24 fluorescence with the histochemical stain for acid phosphatase, an enzyme characteristic of some lysosomes, was not as good, although many structures labeled by CV24 IgG did contain the black histochemical reaction product (data not shown).

Immunoelectron microscopy performed with HRP-CV24 IgG on saponin-permeabilized fibroblasts revealed membrane staining of multivesicular bodies and numerous large vacuoles containing HRP-positive membrane fragments and myelin figures (Fig. 4). The diaminobenzidine reaction product appeared to be associated primarily with the inner surface of the labeled vacuole membranes, suggesting that the CV24 antigenic determinant is on the luminal surface of these vacuole membranes. The endoplasmic reticulum, mitochondria, cytoplasm, and nuclei showed no detectable HRP reaction product. The plasma membrane and the Golgi apparatus showed very little, if any, labeling, despite the existence of some CV24 IgG binding sites. Lack of significant labeling of
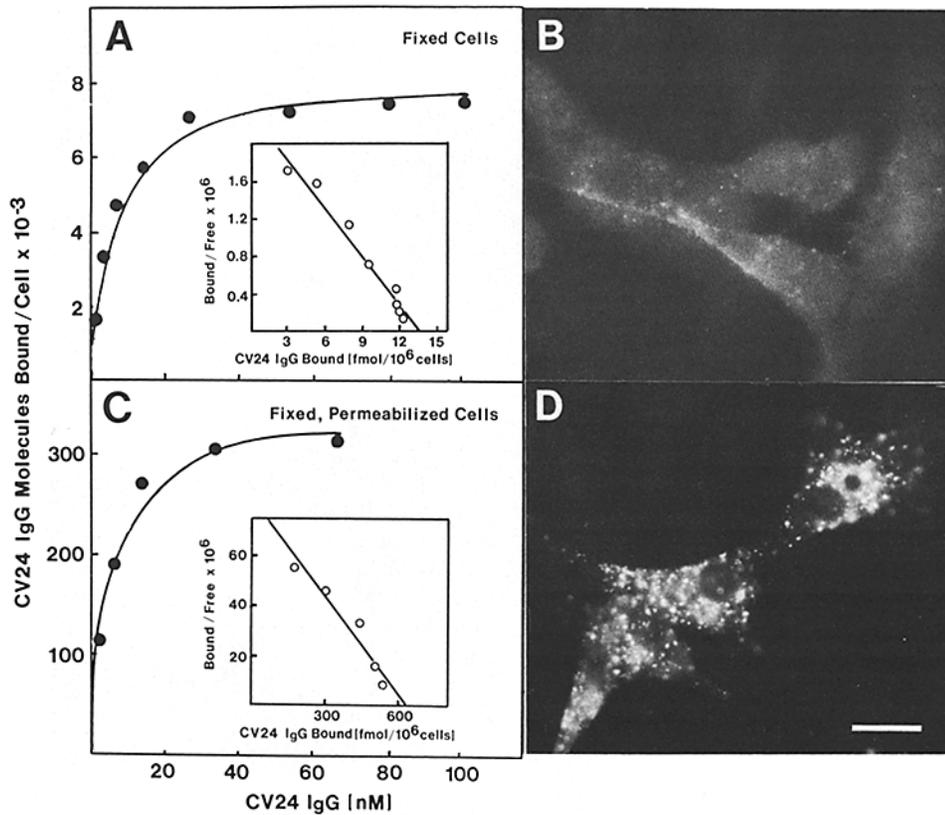
Figure 1. CV24 IgG binding to intact and permeabilized chicken fibroblasts. (*A* and *C*) Binding of ^{125}I-CV24 IgG. Specific binding, expressed as antibody binding sites per cell, was measured on formaldehyde-fixed cells with increasing concentrations of ^{125}I-CV24 IgG in Hepes MEM 10 for 2 h at 25°C in the absence (*A*) or presence (*C*) of 0.25% saponin (as described in Materials and Methods). A Scatchard plot of the data is shown in the insets. All points are averages of duplicate binding measurements, which in all cases were within 10% of each other. (*B* and *D*) Indirect immunofluorescent staining. Cells were fixed as above and incubated with 5 μg/ml CV24 IgG in Hepes MEM 10 for 2 h at 25°C in the absence (*B*) or presence (*D*) of 0.25% saponin. The cells were then washed in HBSS/BSA, incubated with rhodamine-labeled goat anti-mouse IgG, and prepared for microscopy as described in Materials and Methods. The slightly granular labeling pattern observed on fixed cells (Fig. 1 *B*) is probably due to cross-linking of antibody-antigen complexes at the cell surface by second antibody. Cross-linking of CV24 antibody-antigen complexes into aggregates occurs on live cells after incubation with second antibody even at 4°C (not shown). Bar, 10 μm.

these two structures indicated that the peroxidase histochemical technique was not sensitive enough to detect the relatively low concentrations of antigen in these membranes. Similar problems with peroxidase histochemistry have been reported by others (28).

That CV24 antigen is localized primarily in lysosomal membranes was confirmed by analysis of its distribution after subcellular fractionation of organelles by centrifugation in Percoll gradients (Fig. 5). The distribution of CV24 antigen was nearly identical to the distribution profile for the lysosomal enzyme marker, β-hexosaminidase. Approximately 80% of CV24 antigen was found in the major peak of lysosomal enzyme activity, well separated from other organellar markers on the gradient. The rest (20%) migrated as a broad peak in the region containing several other organelle markers in addition to β-hexosaminidase, including markers for endosomes and plasma membranes.

Small Quantities of CV24 Antigen in Endocytic Vesicle Membranes

To determine whether CV24 antigen occurs in other membranes of the endocytic pathway in addition to lysosomes and the plasma membrane, its distribution was compared with the staining pattern of a fluorescently labeled plasma membrane marker internalized into endocytic vesicles by chicken fibroblasts. We used R-SD (43, 44) to label the plasma membrane of chick fibroblasts by incubating the cells with the marker at 4°C. The resultant fluorescent pattern of the fibroblasts showed diffuse surface labeling, and Percoll gradient fractionation of cells so labeled resulted in a peak of fluorescence at the position of plasma membranes labeled with ^{125}I-wheat germ agglutinin. Fibroblasts labeled with R-SD at 4°C and then warmed to 37°C for 7 min showed a punctate fluorescence pattern (Fig. 6*A*) indicative of interiorization of the R-SD into endocytic vesicles. Subcellular fractionation of these cells on Percoll gradients showed that the marker R-SD peak was shifted from the position of plasma membranes to the slightly higher density position of endocytic vesicles (data not shown). Thus, R-SD can be used as a marker for endocytic vesicles in chick fibroblasts incubated with the dextran for brief periods at 37°C. To determine whether CV24 antigen occurs in endocytic vesicle membranes, we looked for co-localization of fluorescence in cells incubated with R-SD and then stained with F-CV24 IgG. Fig. 6*A* shows the fluorescent distribution of R-SD on fibroblasts after labeling at 4°C and warming to 37°C for 7 min. Numerous small fluorescent spots
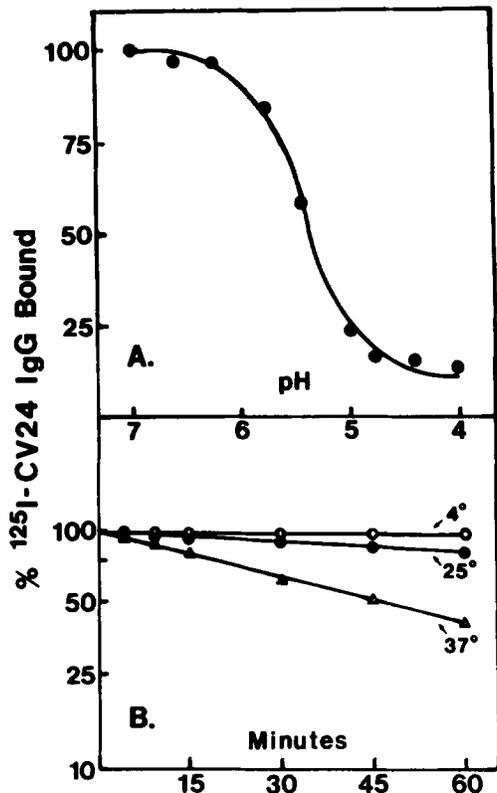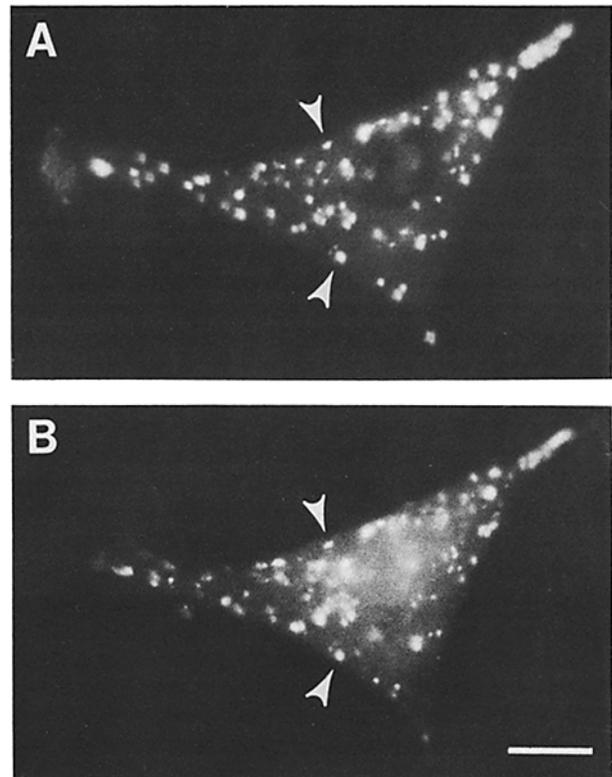
Figure 2. Effect of pH and temperature on the dissociation of ^{125}I-CV24 IgG bound to chicken fibroblasts. Cultured chicken fibroblasts were fixed with formaldehyde, rinsed briefly in HBSS/BSA, incubated with 3 μg/ml ^{125}I-CV24 IgG in HBSS/BSA for 2 h, and then washed in HBSS/BSA at 4°C for 30 min. (A) pH effect. Cells were reincubated in HBSS/BSA at the indicated pHs for 1 h at 25°C. The medium was removed and the cells were solubilized in 1 N NaOH. Cell-associated and released radioactivity were then measured. The quantity of ^{125}I-CV24 IgG that remained bound to cells after incubation at each pH is plotted as a percentage of ^{125}I-CV24 IgG bound after incubation at pH 7. (B) Temperature effect. Cells were reincubated in HBSS/BSA at the indicated temperatures. At specified times, medium was removed from the cells and counted for released radioactivity. At the end of the experiment, cells were solubilized with 1 N NaOH and cell-associated radioactivity was measured. The quantity of ^{125}I-CV24 IgG bound at each time point is plotted as a percentage of total ^{125}I-CV24 IgG bound at time zero. All points are averages of triplicate binding measurements after subtraction for nonspecific binding (determined by adding 50-fold excess unlabeled CV24 IgG to the labeling medium).

Figure 3. Co-distribution of CV24 IgG binding with acridine orange uptake in chicken fibroblasts. Cells were incubated with 2 μg/ml acridine orange (Sigma Chemical Co.) in MEM 410 for 10 min at 37°C, washed in HBSS/BSA at 4°C to remove unabsorbed dye, and photographed using rhodamine epifluorescence (A). The cells were then fixed with formaldehyde, incubated with 5 μg/ml F-CV24 IgG in the presence of 0.25% saponin for 1.5 h at 25°C, and washed in HBSS/BSA. The same cells were then rephotographed with fluorescein epifluorescence (B). Arrowheads show examples of coincidence of acridine orange and F-CV24 IgG staining. Control cells incubated with acridine orange or F-CV24 IgG alone in the manner described above showed no fluorescence in the contrasting optical channel. Bar, 10 μm.

presumably corresponding to endocytic vesicles were distributed throughout the cytoplasm. To reveal the distribution of CV24 antigen, the cells shown in Fig. 6A were then fixed, permeabilized, and stained with F-CV24 IgG. Staining by F-CV24 IgG (Fig. 6B) overlapped with many of the vesicles carrying R-SD. Since numerous other vacuoles not containing the internalized compound were labeled by F-CV24 IgG these results suggest that only small quantities of CV24 antigen reside in endocytic vesicle membranes.

Rapid Internalization and Reappearance of CV24 Antigen in the Plasma Membrane

The presence of CV24 antigen in membranes throughout the endocytic pathway (including the membranes of lysosomes

and endocytic vesicles, and the plasma membrane) raised the possibility that this molecule might circulate through these compartments. To test this hypothesis directly, surface antigen was labeled with CV24 IgG, and its movement in living cells was studied by examining the movement of bound antibody.

When chicken fibroblasts were labeled with R-CV24 IgG at 4°C for 3 h (Fig. 7A), a faint, uniformly distributed fluorescent staining pattern was observed. The inclusion of 100-fold excess unlabeled CV24 IgG in the incubation medium abolished this fluorescent labeling pattern. After the cells were warmed to 37°C for 5 min, numerous small fluorescent spots became concentrated in the perinuclear region (Fig. 7B). Low pH treatment of these cells to dissociate surface-bound antibody (see below) did not affect the distribution of fluorescence, indicating that antibody was internalized to cytoplasmic vesicles.

The kinetics of internalization of surface-bound CV24 IgG were studied by measuring the time course for development of resistance to low pH stripping of ^{125}I-CV24 IgG bound to cell surfaces, after cells were warmed to 37°C from 4°C. The
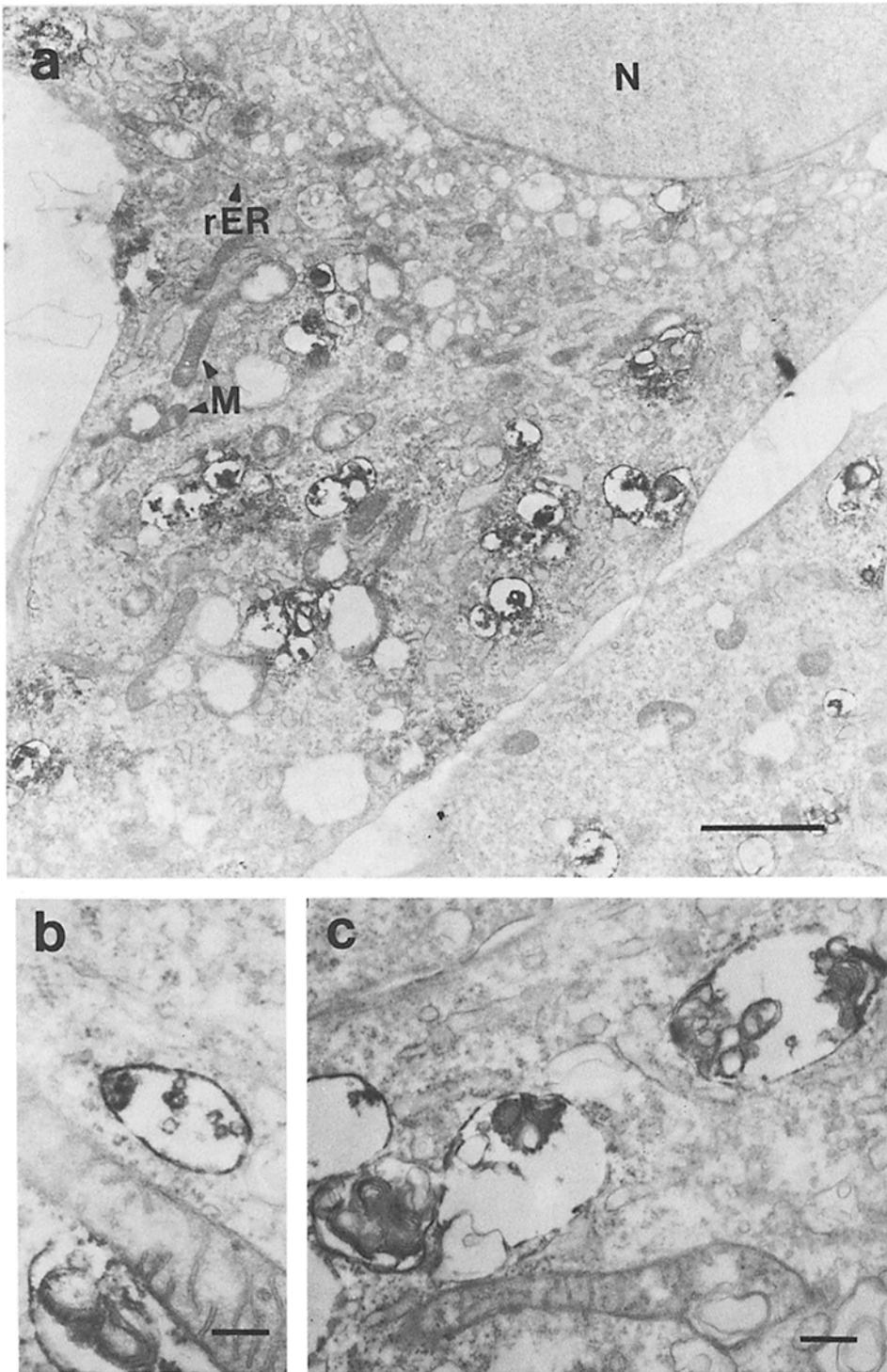
Figure 4. Immunoperoxidase labeling of chicken fibroblasts. Cells were fixed with formaldehyde in buffer containing 0.02% saponin, incubated with 5 µg/ml HRP-CV24 IgG for 2 h at 25°C, washed, fixed with glutaraldehyde, reacted with diaminobenzidine/H_2O_2, and then prepared for electron microscopy as described in Materials and Methods. (*a*) An overview of the cytoplasm of a chicken fibroblast cell showing the distribution of HRP reaction product. *M*, mitochondria; *N*, nucleus; *rER*, rough endoplasmic reticulum. Bar, 2 µm. (*b* and *c*) HRP-positive vacuoles at higher magnification. Bar, 0.2 µm. Controls performed on cells incubated with HRP-CV24 IgG in the presence of 100-fold excess unlabeled CV24 IgG or on cells not exposed to the conjugate were free of staining, indicating specific binding of the HRP-CV24 IgG (not shown).

results, shown in Fig. 8, are consistent with the internalization of ^{125}I-CV24 IgG by first-order kinetics with a half-time of 2 min. Rapid uptake of CV24 IgG by cells did not occur at 4°C. In parallel experiments, a different monoclonal antibody, C3/1 IgG (40), bound to cell surfaces at 4°C showed a very slow development of resistance to low pH stripping upon warming of cells to 37°C (Fig. 8). Thus, rapid internalization of CV24 IgG by cells appeared to be a distinct characteristic of this monoclonal antibody.

Internalization of CV24 IgG bound to cell surfaces should deplete the plasma membrane pool of CV24 antigen. To examine whether the plasma membrane is replenished with CV24 antigen after CV24 IgG uptake, we warmed cells to 37°C that had CV24 IgG bound to their surfaces and then assayed for new antigen sites with ^{125}I-CV24 IgG on the fixed cells. As shown in Fig. 9, the appearance of CV24 IgG binding sites in the plasma membrane upon warming of the cells occurred with a half-time of ~2 min. No new antigen sites appeared at 4°C. Since dissociation of CV24 IgG from antigen at the cell surface at 37°C occurs with a half-time of 50 min
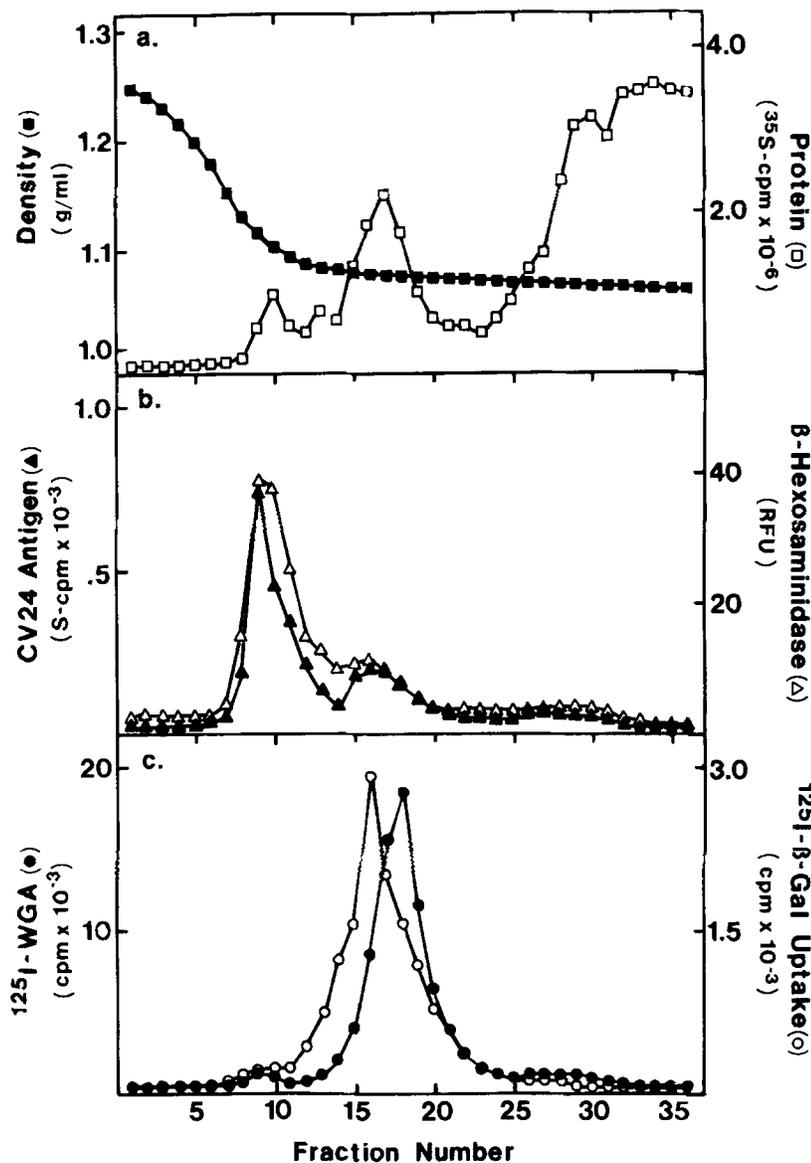
Figure 5. Distribution of CV24 antigen on Percoll density gradients. Chicken fibroblast cultures were metabolically labeled with [³⁵S]methionine for 12 h, disrupted by nitrogen cavitation, and the postnuclear supernatant was fractionated in 30% Percoll. Total antigen was then isolated from each fraction, and the location of different subcellular organelles was determined by enzyme and marker assays (see Materials and Methods). The density profile (■) and distribution of ³⁵S-protein (□) across the gradient are shown in a. b shows the distribution of the lysosomal marker β-hexosaminidase (△), and of CV24 antigen (▲). c shows the distribution of the plasma membrane marker, ¹²⁵I-wheat germ agglutinin (bound to cells at 4°C) (●) and the endosomal marker, ¹²⁵I-β-galactosidase (after 3 min of endocytosis at 37°C) (○). The data in c were derived from two separate Percoll gradients prepared at the same time and identically to the Percoll gradient used in a and b, in order to avoid counting overlap between the two isotopes.

(see Fig. 2B) and CV24 IgG is internalized by cells with a half-time of 2 min, these results suggest ¹²⁵I-CV24 IgG is binding to new antigen sites at the cell surface rather than to pre-existing sites from which unlabeled antibody has dissociated.

Replacement of cell surface CV24 antigen was also detected on cells incubated with CV24 IgG in the presence of the protein synthesis inhibitor cycloheximide (data not shown). Thus, replenishment of CV24 antigen at the cell surface does not require de novo synthesis of antigen.

Continuous Uptake and Lysosomal Degradation of CV24 IgG

The rapid replacement of CV24 antigen sites in the plasma membrane was not diminished by exposure of fibroblasts to CV24 IgG for many hours. Under these conditions there was continuous, selective, temperature-sensitive uptake of CV24 IgG. Internalized ¹²⁵I-labeled CV24 IgG was degraded within ~30 min, and the radiolabeled degradation product (presumably ¹²⁵I-tyrosine) was released into the culture medium (Fig. 10A). Cell-associated CV24 IgG reached a steady state in

which most of this undegraded antibody was in cytoplasmic vacuoles (Fig. 10B). The absence of significant surface labeling on these cells is probably due to the relatively slow rate of association of antibody and antigen at the cell surface in comparison to the subsequent rate of internalization. When degradation of internalized antibody was blocked by leupeptin (an inhibitor of some lysosomal proteases [18, 19]), large quantities of antibody accumulated in cytoplasmic vacuoles (Fig. 10 D). The amount of antibody accumulated in leupeptin-treated cells was equivalent to the sum of the cell-associated and degraded antibody in control cells (Fig. 10, A and C). After 8 h, several times the number of total cell surface antibody binding sites had accumulated in the leupeptin-treated cells. The distribution of internalized antibody in leupeptin-treated cells corresponded almost exactly to the distribution of CV24 antigen (Fig. 11). Continuous exposure of cells to CV24 IgG had no detectable effect on the steady state distribution of antigen in cells. Cells incubated with CV24 IgG for 8 h had the same number of surface and intracellular antibody binding sites as did control cells (data not shown).
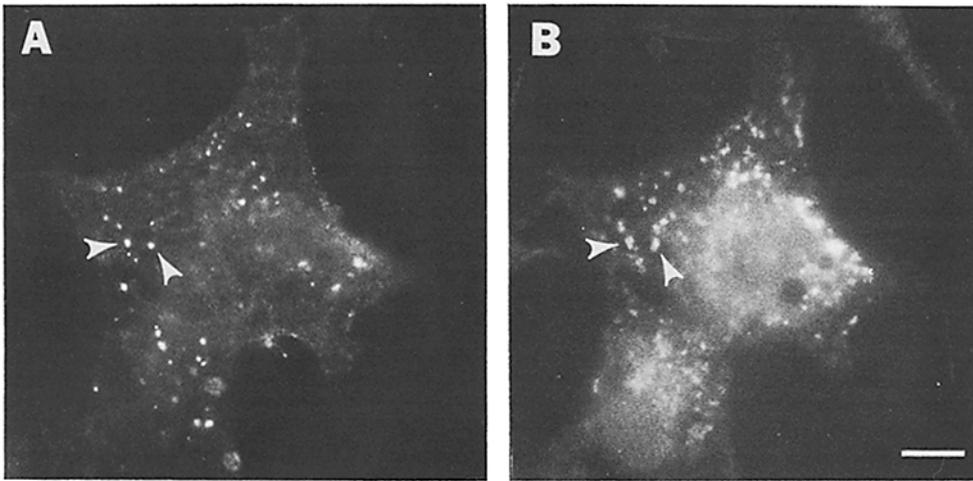
Figure 6. Co-distribution of CV24 antigen with sites of stearoyl-dextran uptake in chicken fibroblasts. Cells were incubated with 1 mg/ml R-SD in Hepes MEM 10 at 4°C for 30 min and then warmed to 37°C for 7 min. Afterwards the cells were washed in Hepes MEM 10, fixed with formaldehyde, and incubated in Hepes MEM 10 containing 5 μg/ml F-CV24 IgG, 0.25% saponin for 1.5 h at 25°C. The cells were then washed in HBSS/BSA and prepared for microscopy. (*A*) Cell photographed with rhodamine epifluorescence. (*B*) The same cell photographed with fluorescein epifluorescence. Arrowheads point to examples of the co-localization of these two markers. No channel cross-over of rhodamine and fluorescein fluorescence was observed on cells incubated as described above with R-SD or with F-CV24 IgG alone. Bar, 10 μm.
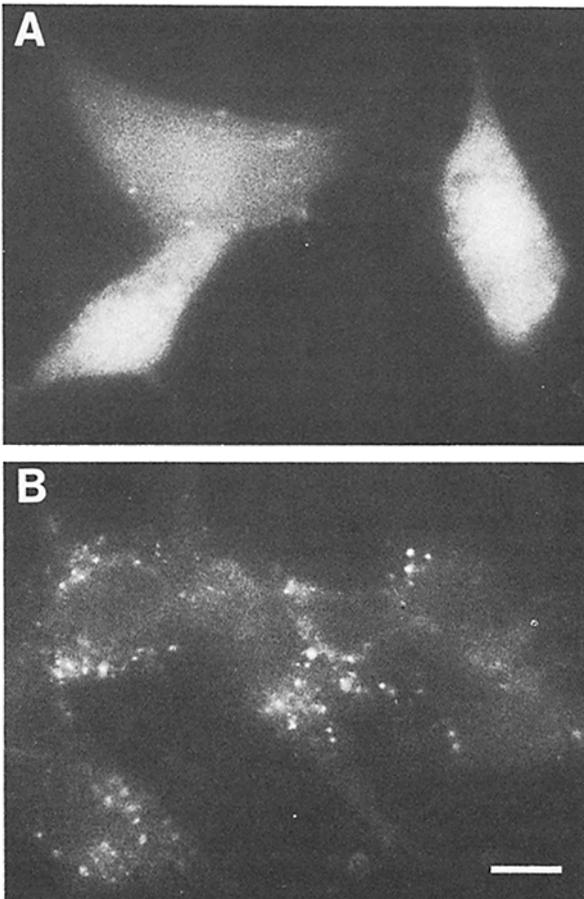

Figure 7. Internalization of surface-bound R-CV24 IgG at 37°C. Cell cultures were incubated with 10 μg/ml R-CV24 IgG in Hepes MEM 10, pH 7.2, for 3 h at 4°C, and washed extensively in unlabeled medium at 4°C. The cells were then reincubated in unlabeled medium at 37°C for 5 min. (*A*) Cells photographed after incubation at 4°C and washing. (*B*) Cells photographed after warming to 37°C for 5 min. Bar, 10 μm.
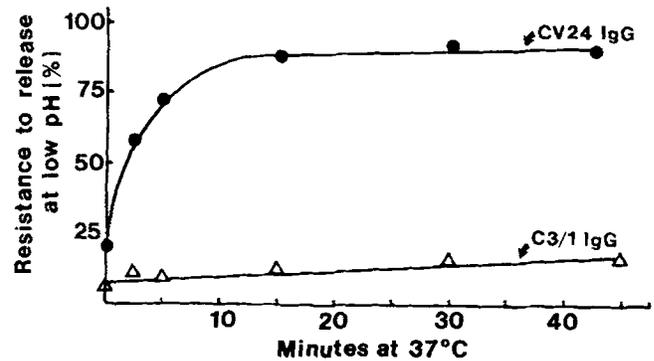
Figure 8. Time course for internalization of surface-bound CV24 IgG at 37°C. Chicken fibroblast cultures were incubated for 4 h at 4°C with 1 μg/ml of either ^{125}I-CV24 IgG or ^{125}I-C3/1 IgG, a monoclonal antibody that recognizes a different cell surface determinant than does CV24 IgG (see reference 40). After being washed at 4°C in unlabeled medium, the cells were warmed to 37°C for the indicated times and then treated with ice-cold 0.1 M acetic acid, 1.5 M NaCl for 5 min (13) to remove surface-bound IgG. The radioactivity that remained associated with cells after the low pH treatment was plotted as a percentage of the total radioactivity associated with the cells before warm-up at 37°C. All data points were corrected for nonspecific binding (addition of 50-fold excess unlabeled antibody to the incubation mediums) and represent the average of duplicate binding measurements.

Biochemical Characteristics of CV24 Antigen

The molecular species recognized by CV24 IgG was isolated by immunoprecipitation from detergent extracts of chicken fibroblasts labeled with [^{35}S]methionine. Analysis by SDS PAGE showed a single labeled protein of 95–105 kD on SDS polyacrylamide gels (Fig. 12, lane *3*) that was found only in the membrane fraction of these cells. The apparent molecular weight was not changed by reduction. Labeled antigen represented ~0.01% of the total labeled protein in the membrane fraction of the cells. When immunoprecipitation of CV24 antigen was performed on labeled cell extracts that were
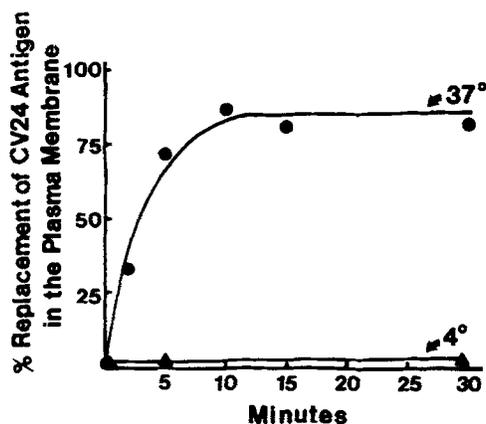
Figure 9. Replenishment of CV24 antigen sites in the plasma membrane. Chicken fibroblast cultures were incubated for 4 h at 4°C with 20 μg/ml of CV24 IgG in Hepes MEM 10. After being washed at 4°C in unlabeled medium, the cells were either kept at 4°C or warmed to 37°C for the indicated times and then fixed with formaldehyde at 4°C. Unlabeled CV24 antigen at the cell surface was then bound with ^{125}I-CV24 IgG (5 μg/ml for 2 h). Radioactivity associated with cells at different warm-up periods was plotted as a percentage of the total CV24 antigen sites measured on fixed cells that had no prior exposure to CV24 IgG. All data points were corrected for nonspecific binding (addition of 50-fold excess unlabeled antibody to the incubation mediums) and represent the average of duplicate binding measurements.

solubilized in Triton X-114, phase separation by the method of Bordier (4) resulted in the complete partitioning of CV24 antigen into the detergent phase, with no traces of it in the aqueous phase (Fig. 12, lanes 6 and 7). CV24 antigen, therefore, has the properties of an integral membrane protein.

The polydispersity (95–105 kD) of CV24 antigen revealed by SDS PAGE suggested that it was modified posttranslationally. To test this possibility, pulse-chase analysis of antigen was performed with chicken fibroblasts that were labeled with [^{35}S]methionine for 15 min and then chased in unlabeled medium (Fig. 13). Isolation of CV24 antigen after the earliest period revealed a 90-kD precursor form. Conversion to the mature (95–105 kD) form of antigen occurred with a half-time of ~15 min. To determine whether the conversion from precursor to mature form of antigen reflected processing of N-linked oligosaccharides from high-mannose to complex type, antigen from the pulse-chase experiment was treated with endo-β-N-acetylglucosaminidase H (endo H). Endo H removes high mannose N-linked oligosaccharides (39). Digestion of newly synthesized CV24 antigen with endo H for 20 h revealed two distinct bands of molecular weights 48,000 and 50,000, and a less apparent ladder of bands, each with molecular weights less than that of the precursor form of antigen. The lowest molecular weight (48,000) form of the bands generated by endo H can be presumed to represent CV24 polypeptide with all N-linked oligosaccharides removed. This should be the size of the core polypeptide of CV24 antigen, providing that only N-linked oligosaccharide chains occur on the precursor form. In contrast to digestion of precursor CV24 antigen, digestion of the mature form of antigen by endo H decreased its apparent molecular weight by only 10,000 and produced a broad band on SDS gels. These results suggest that the CV24 antigen has multiple N-
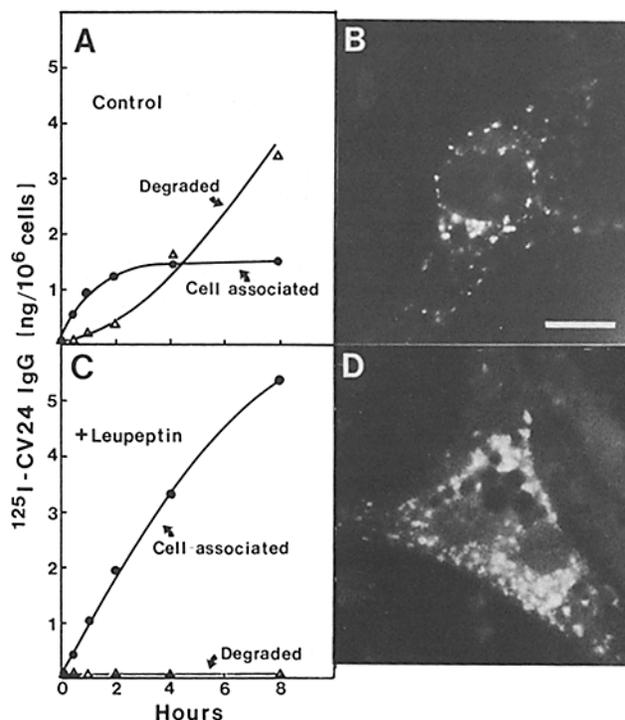
Figure 10. Binding and degradation of CV24 IgG on chicken fibroblasts in the presence and absence of leupeptin. (*A* and *C*) Cell cultures were incubated at 37°C in medium containing ^{125}I-CV24 IgG (2 μg/ml) in the absence (*A*) or presence (*C*) of leupeptin (20 μM) (Sigma Chemical Co.). After the indicated times at 37°C, the antibody-containing medium was removed from the cells and analyzed for ^{125}I-degradation products by the method of Goldstein and Brown (11). No degradation products were released by cells incubated in the above fashion at 4°C. The cells were then washed in HBSS/BSA at 4°C for 30 min, and cell-associated radioactivity was measured by dissolving the cells in 1 N NaOH and counting in a spectrometer. Each value was corrected for nonspecific binding by subtracting the values determined in the presence of 50-fold excess unlabeled CV24 IgG. The averages of triplicate binding measurements were plotted. (*B* and *D*) Cell cultures were incubated at 37°C for 8 h in medium containing F-CV24 IgG (2 μg/ml) in the absence (*B*) or presence (*D*) of 20 μM leupeptin. The cells were washed extensively at 4°C in unlabeled medium before fluorescence microscopic examination. Bar, 10 μm.

linked oligosaccharide chains per antigen polypeptide and that most of these chains are processed to complex type in the mature protein.

Lactoperoxidase-catalyzed surface iodination of intact chicken fibroblasts at 4°C followed by immunoprecipitation with CV24 IgG was performed to compare CV24 antigen isolated from the plasma membrane with intracellular antigen. ^{125}I-labeled surface CV24 antigen displayed the same heterogenous molecular weight of 95,000–105,000 on SDS gels that was characteristic of metabolically labeled intracellular antigen (data not shown).

Discussion

Subcellular Distribution of CV24 Antigen

In the present study we identified a lysosomal membrane glycoprotein of 95–105 kD in chicken cells using monoclonal antibody CV24 IgG. Evidence for the predominant lysosomal
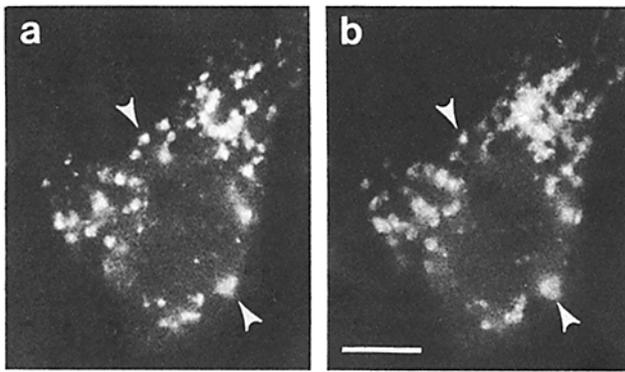
Figure 11. Co-distribution of F-CV24 IgG internalized from the cell surface with intracellular CV24 antigen. Chicken fibroblast cultures were incubated for 8 h at 37°C in Hepes MEM 10, pH 7.2, containing 2 μg/ml F-CV24 IgG and 20 μM leupeptin. After being washed extensively in unlabeled medium, the cells were fixed with formaldehyde, incubated in medium containing 5 μg/ml R-CV24 IgG, 0.25% saponin for 2 h, washed, and prepared for microscopy. (a) Cell photographed with fluorescein epifluorescence. (b) Same cell photographed with rhodamine epifluorescence. Arrowheads show examples of the co-distribution of the two antibody labeling patterns. Controls performed on cells incubated without R-CV24 IgG or F-CV24 IgG in the manner described above displayed no fluorescence in the contrasting optical channel (not shown). Bar, 10 μm.

location of the glycoprotein included extensive co-localization of CV24 monoclonal antibody binding with sites of acridine orange uptake; co-distribution of the antigen predominantly with a lysosomal marker after subcellular fractionation; and the presence of immunoreactive antigen on the luminal sides of structures resembling lysosomes at the ultrastructural level. CV24 antigen appears to be a major component of lysosomal membranes since ~300,000 antibody binding sites per cell (i.e., 80% of the total antibody binding sites per cell; see Figs. 1 and 5) can be assigned to lysosomes in chicken fibroblasts. This corresponds to a site density in lysosomal membranes of ~3,000 molecules/μm^2 assuming one antibody binding site per molecule and 100 μm^2 lysosomal membrane per cell (36–38).

In addition to residing in lysosomes, small quantities of CV24 antigen reside in other cellular organelles. Approximately 8,000 antibody binding sites were on the cell surface, 2% of the total antibody binding sites. Localization of some CV24 IgG binding sites to sites of stearoyl-dextran uptake in cells incubated with the membrane marker for 7 min at 37°C indicated that antigen also resides in endocytic vesicle membranes.

## Circulation of CV24 Antigen through the Endocytic Pathway

The most interesting feature of CV24 antigen, thus far, is its apparent rapid circulation between cell surfaces and intracellular membranes. When cell surface antigen was tagged with CV24 IgG, rapid specific uptake into endocytic vesicles and transport of CV24 IgG to lysosomes were observed. This process continued undiminished for many hours on cells continuously exposed to CV24 IgG, suggesting a constant exchange of surface and intracellular pools of antigen. Since the addition of cycloheximide to cells did not block this process, the exchange of CV24 antigen in the plasma mem-
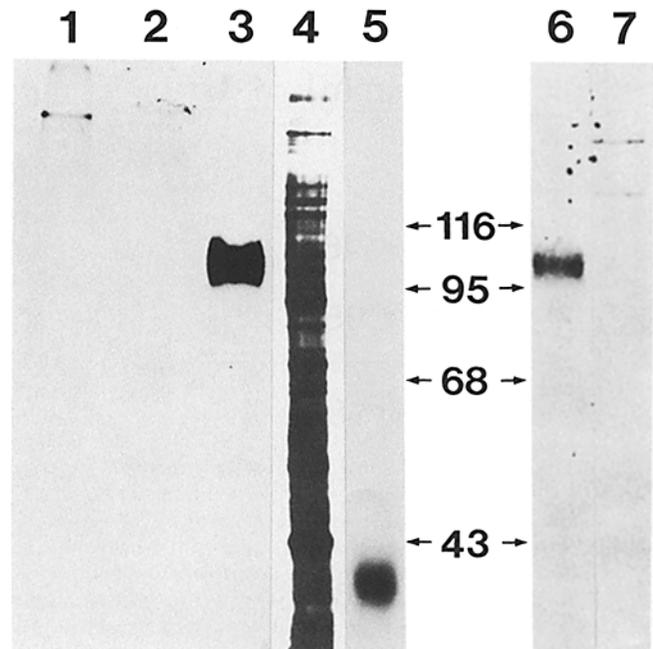
Figure 12. Biochemical characterization of CV24 antigen. Lanes 1–5, metabolic labeling with [^{35}S]methionine. Chicken fibroblast cultures were labeled with [^{35}S]methionine for 12 h. After the conditioned medium was removed, cultures were washed and homogenized in low ionic strength buffer (0.01 M sodium phosphate, pH 7.2, containing a set of protease inhibitors: 1 mM EDTA, 2 mM benzamidine, 5 mM N-ethylmaleimide, 1 mg/ml bacitracin [33]). The homogenate was centrifuged at 27,000 g and the pellet was extracted in the same buffer with 0.5% Triton X-100 added. The extract was clarified at 100,000 g. Affinity chromatography with CV24 IgG immunobeads (lanes 1–3) and C3/1 IgG immunobeads (lane 5) was performed on the conditioned medium (lane 1), the post-27,000 g supernatant (soluble cellular content) (lane 2), and the detergent-solubilized membrane fraction (lanes 3 and 5) followed by SDS PAGE and fluorography. The electrophoretic pattern of the detergent extract of membranes is shown in lane 4. Lanes 6 and 7, solubility in Triton X-114. Chicken fibroblast cultures were labeled with [^{35}S]-methionine for 15 h, washed, homogenized in low ionic strength buffer, and centrifuged at 27,000 g for 1 h. The pellet was resuspended at 4°C in buffer containing 1% Triton X-114, 0.15 M NaCl, 1 mM EDTA, 0.01 M sodium phosphate, pH 7.2. The solution was partitioned into a detergent and aqueous phase to separate membrane and hydrophilic proteins according to the method of Bordier (4). Affinity chromatography with CV24 IgG immunobeads performed on the detergent (lane 6) and the aqueous (lane 7) phase of the solution was followed by analysis with SDS PAGE and fluorography.

brane must occur from pre-existing intracellular pools of antigen. The process through which antigen sites are replenished at the cell surface during CV24 IgG uptake had a half-time of ~2 min. This was equivalent to the rate of internalization at 37°C for CV24 IgG bound to cell surfaces. Thus, movement of CV24 antigen both to and from the cell surface is rapid, reminiscent of the movement of recycling receptors (including LDL, α2 macroglobulin, and asialoglycoprotein receptors [6, 37]).

We do not have direct evidence for the intracellular transit of CV24 antigen in the absence of CV24 IgG. Several observations, however, lead us to believe that CV24 antigen moves through the vacuolar membrane system of chicken fibroblasts constitutively. First, the total number and steady-state sub-

Control

0 15 30 45 60 [min]

Endo H

0 15 30 45 60

←116→

← 95 →

← 68 →

← 43 →
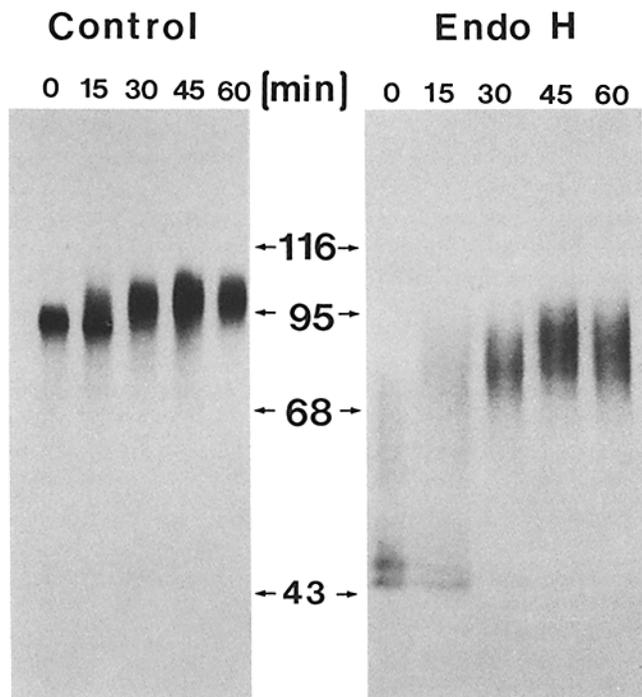
Figure 13. Processing of CV24 antigen and cleavage by endo H. Chicken fibroblast cultures were pulse-labeled with 150 μCi/ml [³⁵S] -methionine in methionine-free medium for 15 min and chased with methionine-containing medium for the indicated times. CV24 antigen was purified with CV24 IgG immunobeads as described in Materials and Methods. Half of the purified sample was made 50 mM Na acetate, pH 5.8, 0.4% SDS, 2.5% β-mercaptoethanol and then boiled for 5 min. Endo H (Miles Scientific Inc., Naperville, IL) at 44 μ/ml was added and the sample was incubated at 37°C for 15 h. The other half of each sample remained untreated. Both sets of samples were then analyzed by SDS PAGE and fluorography. Cleavage by endo H was determined to be specific since CV24 antigen treated as described above for the endo H samples but without the enzyme showed the same migration pattern as untreated antigen. Moreover, a different unglycosylated polypeptide (the alpha-subunit of the (Na⁺ + K⁺)-ATPase [9]), when treated with the enzyme as described above, showed no change in molecular weight on SDS polyacrylamide gels (data not shown).

cellular distribution of CV24 antigen molecules in cells was unaffected by exposure of cells to CV24 IgG, suggesting that down-regulation of surface CV24 antigen by cross-linking with divalent CV24 IgG does not occur. Second, the appearance of CV24 antigen sites at the cell surface occurred with the same kinetics as CV24 antigen-antibody internalization, consistent with a steady state cycling of antigen between surface and intracellular compartments. Finally, despite heterogeneity in its molecular weight, CV24 antigen isolated from different subcellular fractions off of Percoll gradients showed the same level of polydispersity on SDS gels, consistent with the continuous circulation of antigen between different subcellular pools.

The intracellular pathway by which CV24 antigen moves to and from the cell surface is not clear at this time. Our result showing the transport of CV24 IgG to lysosomes is not evidence for the movement of antigen from cell surface to lysosomes, since antibody could dissociate from antigen after internalization (for example, in the acidic compartment of the endosome). Because there are at least two intracellular pools of CV24 antigen (in lysosomal and endosomal membranes), antigen could move to and from the cell surface from either one or both of these compartments. Preliminary results indicate that the half-life of CV24 antigen in these cells is long (~35 h). Since half of all surface antigen sites are replaced after 2 min and surface antigen represents 2% of total antigen, the equivalent of the entire antigen pool in these cells moves to the cell surface every 200 min. All of the CV24 antigen molecules in the cell, therefore, could cycle many times to and from the cell surface before being degraded. Not all CV24 antigen molecules in the cell necessarily cycle to and from the cell surface, however. If only a small portion of the total internal pool of CV24 antigen (e.g., that present in endocytic vesicle membranes) cycles, then those antigen molecules would cycle much more frequently.

Biochemical Characteristics

Biochemical characterization of CV24 antigen revealed it to be an integral membrane glycoprotein carrying N-linked oligosaccharides. The antigen was synthesized as a 90-kD precursor that was processed to a mature form of 95–105 kD with a half-time of 15 min. The reduction in molecular weight to 48,000 of the precursor form of CV24 antigen after endo H cleavage indicated there were numerous high mannose oligosaccharide chains per polypeptide. Most of these oligosaccharide chains were converted to complex type, since mature CV24 antigen was only slightly sensitive to endo H digestion. CV24 antigen therefore can be presumed to be core glycosylated in the rough endoplasmic reticulum, transported to the Golgi apparatus, and terminally glycosylated like other membrane glycoproteins.

In addition to its high oligosaccharide content, CV24 antigen appears to be differentially glycosylated. When antigen was eluted from discrete subregions of its 95–105-kD band on SDS gels and re-electrophoresed, each fraction retained its own electrophoretic mobility and displayed a residual level of polydispersity (data not shown). Since the core polypeptide of CV24 antigen migrates as a sharp band of 48,000 on SDS gels (Fig. 13), differential glycosylation probably accounts for the diffuse migration of antigen during SDS PAGE. The differential glycosylation of antigen is probably unrelated to the multiple locations and age of antigen in cells, since there was no difference in the average molecular weight of antigen isolated from different subcellular compartments off of density gradients and from lactoperoxidase iodinated cell surfaces. Also, mature [³⁵S]methionine-labeled antigen isolated after a 35-h chase in unlabeled medium had a molecular weight identical to that of mature antigen isolated immediately after labeling.

The biological significance for the extensive glycosylation of CV24 antigen is unknown. Glycosylation could serve to protect lysosomal membrane constituents from degradation by hydrolytic enzymes (17). Alternatively, it may be important for targeting of membrane components to lysosomes. Since CV24 antigen cannot be labeled with [³²P]phosphate (data not shown), some carbohydrate signal other than the mannose-6-phosphate recognition marker (22, 25, 34) would be required for targeting of these molecules to lysosomes.

Comparison with Other Lysosomal Membrane Glycoproteins

Several heavily glycosylated lysosomal membrane proteins with molecular weights of 100,000–120,000 have recently been identified in rat and mouse (7, 17, 28). Since CV24 IgG does not cross-react into rat and mouse tissue, the relationship of CV24 antigen to these other lysosomal membrane glycoproteins awaits further study. Nevertheless, CV24 antigen resembles biochemically the lysosomal membrane glycoproteins described by Lewis et al. (17) and Chen et al. (7), since both are synthesized as 90-kD precursors and cleaved by endo H to yield 42–45-kD polypeptides. However, unlike CV24 antigen, these antigens are not detected in endocytic vesicle and plasma membranes of fibroblasts. A different lysosomal membrane glycoprotein of 100 kD described by Reggio et al. (28) has been detected in endocytic vesicle and plasma membranes. Of particular interest is that both CV24 antigen and the Reggio antigen are heavily concentrated along the ruffled border membrane of osteoclasts that secretes lysosomal enzymes into the bone resorbing area, whereas the lysosomal membrane protein described by Lewis et al. is absent (3).

Possible Roles for CV24 Antigen

We do not as yet know what role the CV24 antigen plays in lysosomal structure and function. Its presence on other structures of the endocytic pathway suggests a function not exclusively associated with lysosomes. Thus, we do not expect the CV24 antigen to be a specific component of the transport system(s) lysosomes use for removing the products of intralysosomal digestion. Neither do we expect the CV24 antigen to play a purely structural role in maintaining lysosomal membrane integrity.

The distribution of CV24 antigen on nonlysosomal membranes could be due to the incidental mixing of membrane proteins during membrane flow to lysosomes and have nothing to do with its function. This is unlikely, however, because other lysosomal membrane proteins, which should be affected in the same way, are not distributed like CV24 antigen on nonlysosomal membranes (7, 17). Thus, we are currently investigating whether CV24 antigen could be involved in the specific interaction or targeting of endocytic vesicles to lysosomes, or in some different membrane function common to lysosomal and other membranes.

References

1. Allison, A. C., and M. R. Young. 1964. Uptake of dyes and drugs by living cells in culture. *Life Sci.* 3:1407–1414.

2. Anderson, M. J., and D. M. Fambrough. 1983. Aggregates of acetylcholine receptors are associated with plaques of a basal lamina heparan sulfate proteoglycan on the surface of skeletal muscle fibers. *J. Cell Biol.* 97:1396–1411.

3. Baron, R., L. Neff, J. Lippincott-Schwartz, D. Louvard, I. Mellman, A. Helenius, and M. Marsh. 1985. Distribution of lysosomal membrane proteins in osteoclasts and their relationship to acidic compartments. *J. Cell Biol.* 101(5, Pt. 2):53a. (Abstr.)

4. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604–1607.

5. Bridges, K., J. Harford, G. Ashwell, and R. D. Klausner. 1982. Fate of receptor and ligand during endocytosis of asialoglycoproteins by isolated hepatocytes. *Proc. Natl. Acad. Sci. USA.* 79:350–354.

6. Brown, M. S., and J. L. Goldstein. 1979. Receptor-mediated endocytosis: insights from the lipoprotein receptor system. *Proc. Natl. Acad. Sci. USA.* 76:3330–3337.

7. Chen, J. W., T. Murphy, M. Willingham, I. Pastan, and J. T. August. 1985. Identification of two lysosomal membrane glycoproteins. *J. Cell Biol.* 101:85–95.

8. Deurs, B. V., and K. Nilausen. 1982. Pinocytosis in mouse L-fibroblasts: ultrastructural evidence for a direct membrane shuttle between the plasma membrane and the lysosomal compartment. *J. Cell Biol.* 94:279–286.

9. Fambrough, D. M., and E. K. Bayne. 1983. Multiple forms of (Na⁺ + K⁺)-ATPase in the chicken. *J. Biol. Chem.* 258:3926–3935.

10. Farquhar, M. G. 1983. Intracellular membrane traffic: pathways, carriers, and sorting devices. *Methods Enzymol.* 98:1–13.

11. Goldstein, J. L., and M. S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts: comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Cell Biol.* 249:5153.

12. Graham, R. C., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291–302.

13. Karin, M., and B. Mintz. 1981. Receptor-mediated endocytosis of transferrin in developmentally totipotent mouse teratocarcinoma stem cells. *J. Biol. Chem.* 256:3245–3252.

14. Kartner, N., N. Alon, M. Swift, M. Buchwald, and J. R. Riordan. 1977. Isolation of plasma membranes from human skin fibroblasts. *J. Membr. Biol.* 36:191–211.

15. Kelly, W. F., A. Passaniti, J. W. Woods, J. L. Daiss, and T. F. Roth. 1983. Tubulin as a molecular component of coated vesicles. *J. Cell Biol.* 97:1191–1199.

16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

17. Lewis, V., S. A. Green, M. Marsha, P. Vihko, A. Helenius, and I. Mellman. 1985. Lysosomal membrane glycoproteins. *J. Cell Biol.* 100:1839–1847.

18. Libby, P., and A. L. Goldberg. 1978. Leupeptin, a protease inhibitor, decreases protein degradation in normal and diseased muscles. *Science (Wash. DC).* 199:534–536.

19. Libby, P., S. Bursztajn, and A. L. Goldberg. 1980. Degradation of the acetylcholine receptor in cultured muscle cells: selective inhibitors and the fate of undegraded receptors. *Cell.* 19:481–491.

20. Lippincott-Schwartz, J., and D. M. Fambrough. 1983. Properties and dynamics of a lysosomal membrane glycoprotein. *J. Cell Biol.* 97(5, Pt. 2):411a. (Abstr.)

21. Merion, M., and W. S. Sly. 1983. The role of intermediate vesicles in the adsorptive endocytosis and transport of ligand to lysosomes by human fibroblasts. *J. Cell Biol.* 96:644–650.

22. Miller, A. L., B. C. Kress, R. Stein, C. Kinnon, H. Kern, J. A. Schneider, and E. Harms. 1981. Properties of N-acetyl-β-D-hexosaminidase from isolated normal and T-cell lysosomes. *J. Biol. Chem.* 256:9352–9362.

23. Moriyama, Y., T. Takano, and S. Ohkuma. 1982. Acridine orange as a fluorescent probe for lysosomal proton pump. *J. Biochem.* 92:1333–1336.

24. Nandi, P. K., G. Irace, P. P. van Jaarsveld, R. E. Lippoldt, and H. Edelhoch. 1982. Instability of coated vesicles in concentrated sucrose solutions. *Proc. Natl. Acad. Sci. USA.* 79:5881–5885.

25. 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.

26. Ohkuma, S., Y. Moriyama, and T. Takano. 1982. Identification and characterization of a proton pump on lysosomes by fluorescein isothiocyanate-dextran fluorescence. *Proc. Natl. Acad. Sci. USA.* 79:2758–2762.

27. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327–3331.

28. Reggio, H., D. Bainton, E. Harms, E. Coudrier, and D. Louvard. 1984. Antibodies against lysosomal membranes reveal a 100,000 molecular weight protein that cross-reacts with purified H⁺, K⁺ ATPase from gastric mucosa. *J. Cell Biol.* 99:1511–1526.

29. Rome, L. H., A. J. Garvin, M. M. Allietta, and E. F. Neufeld. 1979. Two species of lysosomal organelles in cultured human fibroblasts. *Cell.* 17:143–153.

30. Sahagian, G. G., and E. F. Neufeld. 1983. Biosynthesis and turnover of the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells. *J. Biol. Chem.* 258:7121–7128.

31. Salicinski, P. R. P., C. McLean, J. E. Sykes, V. V. Clement-Jones, and

P. J. Lowry. 1981. Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3,6-diphenyl glycoluril (Iodo-gen). Anal. Biochem. 117:136–146.

32. Schneider, D. 1983. ATP-dependent acidification of membrane vesicles isolated from purified rat liver lysosomes. J. Biol. Chem. 258:1833–1838.

33. Silman, I., L. DiGiamberardino, J. Lyles, J. Y. Couraud, and E. A. Barnard. 1979. Parallel regulation of acetylcholinesterase and pseudocholines-terase in normal, denervated and dystrophic chicken skeletal muscle. Nature (Lond.). 280:160–162.

34. Sly, W. S., and H. D. Fischer. 1982. The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. J. Cell Biochem. 18:67–85.

35. Sly, W. S., H. D. Fischer, A. Gonzalez-Noriega, J. H. Grubb, and M. Natowicz. 1981. Role of the 6-phosphomannosyl-enzyme receptor in intracel-lular transport and adsorptive pinocytosis of lysosomal enzymes. Methods Cell Biol. 23:191–214.

36. Steinman, R. M., S. E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis: a stereologic analysis. J. Cell Biol. 68:665–687.

37. Steinman, R. M., I. S. Mellman, W. A. Muller, and Z. A. Cohn. 1983. Endocytosis and the recycling of plasma membrane. J. Cell Biol. 96:1–27.

38. Steinman, R. M., J. M. Siler, and Z. A. Cohn. 1974. Pinocytosis in fibroblasts: quantitative studies in vitro. J. Cell Biol. 63:949–969.

39. Tarentino, A. L., and F. Maley. 1974. Purification and properties of an endo-β-N-acetylglucosaminidase from streptomyces griseus. J. Biol. Chem. 249:811–816.

40. Wakshul, E., E. K. Bayne, M. Chiquet, and D. Fambrough. 1983. Characterization of a plasma membrane glycoprotein common to myoblasts, skeletal muscle satellite cells, and glia. Dev. Biol. 100:464–477.

41. Wall, D. A., G. Wilson, and A. L. Hubbard. 1980. The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. Cell. 21:79–83.

42. Wilson, M. B., and P. K. Nakane. 1978. Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In Immunofluorescence and Related Staining Techniques. W. Knapp, K. Holubar, and G. Wick, editors. Elsevier/North-Holland Biomedical Press, Amsterdam, 215–224.

43. Wolf, D. E., P. Henkart, and W. Webb. 1980. Diffusion, patching and capping of stearoylated dextrans on 3T3 cell plasma membranes. Biochemistry. 19:3893–3904.

44. Wolf, D. E., J. Schlessinger, E. Elson, W. Webb, R. Blumenthal, and P. Henkart. 1977. Diffusion and patching of macromolecules on planar lipid bilayer membranes. Biochemistry. 16:3476–3483.