# Identification of an Endosomal Antigen Specific to Absorptive Cells of Suckling Rat Ileum

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*Abstract.* A membrane fraction enriched in apical endosomal tubules was isolated from absorptive cells of suckling rat ileum and used as an immunogen to generate anti-endosome monoclonal antibodies. By immunofluorescence, one of these antibodies bound exclusively to the region of the apical endocytic complex in ileal absorptive cells, but not to other cell types. Immunoblot analysis showed the antigen as a diffuse 55–61-kD band which was highly enriched in the endosome fraction over whole-cell homogenate. The antigen appears to be an intramembrane glycoprotein: it partitioned primarily in the detergent phase after TX-114 extraction, and shifted to 44 kD after chemical deglycosylation. EM immunocytochemistry showed that the antibody bound to the luminal side of endosomal tubule membranes, a portion of endosomal vesicle membranes, and in endocytic pits of apical plasma membranes. However, it did not bind to multivesicular bodies, the giant lysosome, or other organelles. Immunocytochemistry after uptake with adsorbed or soluble tracer proteins showed that the antigen labeled portions of both prelysosomal pathways previously described in these cells (Gonnella, P. A., and M. R. Neutra, 1984, *J. Cell Biol.*, 99:909–917). The function of this glycoprotein is not known, but inasmuch as it has been detected only in absorptive cells of suckling rat ileum, it may serve a function specific to these cells. Nevertheless, this endosomal antigen, designated glycoprotein (gp) 55–61, will serve as a useful marker for exploring membrane dynamics in early stages of the endocytic pathway.

During endocytosis, receptors and ligands are delivered to a heterogeneous system of intracellular compartments collectively termed endosomes. Acidification of the lumen of these compartments results in dissociation of many receptor–ligand complexes, so that sorting of ligands and receptors toward different destinations may occur (for review, see refs. 1, 41, 58). Endosomal compartments in many cell types include clear vesicles with long tubular extensions (24, 39), and it is in the tubules that receptors are thought to accumulate and to be sorted according to their specific itineraries such as recycling or transepithelial transport (20). It is not known, however, whether the biochemical composition of the tubular extensions differs from that of the vesicular portion.

Although endosomes have special morphologic and functional features distinct from other intracellular compartments, it is not clear to what extent endosome membranes have a unique biochemical composition and can be considered a bonafide organelle. Subcellular fractions have been obtained that are enriched in endosomal vesicles, as identified by the presence of endocytosed tracers (50, 62) or enrichment in specific ligands or receptors (14, 16, 29, 42, 44, 61), and the protein composition of these fractions has been compared to that of plasma membrane and lysosomes. Some studies have provided evidence that the composition of endosome membrane differs from plasma membrane, but others have not found clear differences (2, 14, 29, 42, 44, 50, 62). Thus it is not yet established whether endosomes are relatively permanent organelles or are transient structures, formed by coalescence of incoming plasma membrane vesicles and rapidly disassembled by recycling of some membranes and transport of the remainder to lysosomes (24). To resolve this issue, information on the exact composition and turnover of membranes from the distinct subcompartments of the endosome system is needed, but such studies have been hampered by the difficulty of obtaining large amounts of highly purified vesicular or tubular endosomal membranes from cells and by the lack of endosome-specific biochemical or immunologic markers.

The absorptive epithelium of neonatal rat ileum provides a rich source of endosomal tubules. It consists of highly polarized cells that take up large amounts of milk proteins from the intestinal lumen. These cells contain an extensive tubulovesicular membrane system in the apical cytoplasm called the "endocytic complex" (13, 33). We have shown that this complex is analogous to endosomes of other cells in that it is an internal compartment that receives endocytosed ligands but lacks acid phosphatases (21). The tubular portions of ileal cell endosomes are unusual in that their luminal surfaces bear distinct periodic arrays, visible by electron microscopy (31,

64), that were dissociated from the membrane and shown to contain *N*-acetylhexosaminidase activity (27, 28). We have taken advantage of the abundance and polarization of this membrane system, and the presence of the morphologic marker, to isolate subcellular fractions enriched in tubular endosomal membranes. This preparation was used to generate monoclonal antibodies directed against the apical endocytic complex of ileal cells. We found that apical endosomes of absorptive cells in the neonatal rat ileum contain a unique membrane antigen whose distribution and properties are described in this report.

## Materials and Methods

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

### Subcellular Fractionation

16-d-old Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were killed, and 5-cm segments of ileum were excised, inverted onto sharpened wooden applicator sticks, and rinsed in ice-cold PBS. Inverted segments were incubated in cell dissociation medium containing 200 mM sucrose, 76 mM $Na_2HPO_4$, 10 mM $KH_2PO_4$, 60 mM NaOH, 20 mM EDTA, pH 7.4, with 0.5 mM phenylmethylsulfonyl fluoride, for 15 min. All steps were performed at 4°C and all solutions contained 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 2 μg/ml leupeptin, 1 μg/ml aprotinin, and 2 μg/ml antipain. Sticks were transferred to fresh cell dissociation medium and shaken to release epithelial sheets, which were then passed twice through an 18-gauge needle. Single cells were pelleted and resuspended in buffer containing 10 mM Hepes (Research Organics, Inc., Cleveland, OH), 5 mM $MgSO_4$, 1 mM $NaN_3$, and 1 mM EGTA, pH 7.5, incubated for 15 min, and repelleted. This pellet and the cells on top of the buffer were resuspended in 3 ml of brush border stabilization buffer containing 75 mM KCl, 5 mM $MgSO_4$, 10 mM imidazole, 1 mM EGTA, and 4 mM $NaN_3$, pH 7.0 (43), and homogenized for 90–210 s with a VirTis 45 homogenizer (VirTis Co., Inc., Gardner, NY). Release of brush borders was monitored by phase-contrast microscopy. This homogenate was brought up in brush border stabilization buffer and centrifuged for 10 min at 2,500 *g*. The pellet was resuspended in 60% sucrose, loaded beneath a discontinuous sucrose gradient of 30, 40, and 50%, and spun for 45 min at 96,000 *g* in a Beckman SW28 rotor (Beckman Instruments, Inc., Palo Alto, CA). The 30–40% and 40–50% interfaces, enriched in brush borders, were pooled, resuspended in stabilization buffer, and centrifuged for 30 min at 96,000 *g*. To solubilize brush border cytoskeleton and release the endocytic complex from microvillus membrane, the resulting pellet was resuspended in 3 ml of brush border destabilization buffer containing 10 mM imidazole, 5 mM $MgSO_4$, 500 mM KCl, 5 mM ATP, 1 mM EGTA, pH 7.2, for 10 min and then homogenized by passing it 40 times through a ball bearing homogenizer (0.0016-in gap) (Berni-Tech Engineering, Saratoga, CA) (3). This homogenate was loaded into 35% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) in stabilization buffer and spun at 28,000 *g* for 75 min in an SS34 rotor (Dupont/Sorvall, Wilmington, DE). 7-ml fractions were removed from the top of the gradient, resuspended in 10 mM Hepes, 250 mM sucrose, pH 7.2, and spun for 2 h at 96,000 *g*, and pellets were stored at −70°C. Densities were determined by comparison with a balance tube containing Percoll density marker beads.

As an alternative fractionation procedure, brush borders were collected from the discontinuous sucrose gradient described above, washed in destabilization buffer, and homogenized in destabilization buffer. The homogenate was diluted 1:1 with 0.5 M sucrose (final sucrose 1.03 g/ml) and loaded on top of a 1.08–1.22 g/ml continuous sucrose gradient with a 5-ml, 1.26 g/ml cushion, and centrifuged for 16 h at 82,500 *g* in a Beckman SW28 rotor. 2-ml fractions were collected from the bottom of the tube, specific gravities were determined with a refractometer, and enzyme assays were performed as below.

Ileal segments and pelleted samples from the fractionations were fixed and processed for electron microscopy as described previously (21).

### Enzyme Assays

Assays were done on fractions stored for <1 d at 70°C, or for <6 h at 4°C. 5′-nucleotidase activity was measured on 2 μg of protein according to the procedure of Widnell and Unkeless (63) as modified by Hubbard et al. (26), using 5′-adenosine monophosphate as substrate. *N*-acetyl-β-glycosaminidase activity was measured on 10 μg of protein according to Koldovsky and Palmieri (32), using *N*-acetyl-β-glucosaminide as substrate.

Protein was determined according to Lowry et al. (38) as modified by Bennett (6) or by the method of Bradford (9) using the Bio-Rad Laboratories (Richmond, CA) microassay procedure with BSA as standard.

### Monoclonal Antibody Production

To remove highly immunogenic glycoproteins such as brush border hydrolases and mucins from absorptive cell surfaces before subcellular fractionation, inverted ileal segments were incubated for 1 h at 4°C in 0.12 mg/ml papain in PBS, pH 6.8. Enzymatic activity was stopped by a 15-min incubation in cell dissociation medium containing 9 mg/ml iodoacetamide and 0.5 mM phenylmethylsulfonyl fluoride, and epithelial sheets were then isolated in fresh dissociation medium. Endosome-enriched fractions were obtained from Percoll gradients as described above. Robertsonian RBF/DN mice (Jackson Laboratories, Bar Harbor, ME) were injected intraperitoneally with the enriched tubule fraction (1 mg of protein) in Freund's complete adjuvant. After 4 wk, mice were boosted with 1 mg of protein in Freund's incomplete adjuvant. 1 wk later, serum was collected by tail bleed and tested by immunofluorescence to confirm an immunologic response. After 6 wk, mice were injected with 400 μg of protein without adjuvant, and 4 d later, spleen cells were fused with FOX-NY myeloma cells using 50% polyethylene glycol 1400–1600 (Boehringer Mannheim Biochemicals, Indianapolis, IN) in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 10 mM Hepes, 2 mM supplemental glutamine, 1 mM Na pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM mercaptoethanol. Fusion products were plated into six 96-well culture plates in adenine-aminopterin-thymidine selection medium, consisting of the RPMI 1640-based medium described above, supplemented with 20% FBS (KC Biologicals, Lenexa, KS), 75 μM adenine, 0.4 μM aminopterin, 16 μM thymidine and 3 μM glycine, with a feeder layer of mouse thymocytes. After 2 wk, selection medium was replaced by growth medium consisting of the medium described above with 20% FBS but without adenine, aminopterin, thymidine, glycine, or mercaptoethanol.

Supernatants from 96-well plates were screened by dot blot assay of 0.2-μg aliquots of endosome membranes denatured in SDS sample buffer (34, without bromophenol blue) on squares of nitrocellulose (Bio-Rad Laboratories), using rabbit anti-mouse IgG conjugated to horseradish peroxidase or alkaline phosphatase (HyClone Laboratories, Logan, UT). Positive wells were then screened by immunofluorescence on 0.5-μm frozen sections, as described below. Wells of interest were cloned twice by limiting dilution and clones were screened on Western blots of the endosome fraction using a multiple-channel miniblotter (Immunetics, Cambridge, MA).

### Immunocytochemistry

*Light Microscopy.* Neonatal ileum was fixed in periodate-lysine-2% paraformaldehyde fixative (40) for 3 h at room temperature (RT).[1] Samples were then washed in PBS, infiltrated 30 min in 5% sucrose, overnight in 1 M sucrose and 1–2 h in 2.3 M sucrose. 2-$mm^3$ blocks were mounted on chucks coated with Tissue-Tek II (Lab-Tek Products, Naperville, IL), frozen in Freon-22, and stored in liquid nitrogen. Frozen sections 0.5–1 μm thick were cut on a Reichert FC-4 cryomicrotome (Reichert-Jung, Vienna, Austria), transferred to microscope slides coated with 1% gelatin containing 0.1% potassium chromosulfate, and stored at −20°C until use.

For immunostaining, slides were washed with PBS, quenched with 50 mM $NH_4Cl$ in PBS, and blocked with PBS containing 0.2% gelatin. Sections were incubated in primary antibody diluted with PBS/gelatin for 2 h at RT and then washed thoroughly in PBS/gelatin. Sections were incubated for 1 h at RT in rabbit anti-mouse IgG conjugated to rhodamine (preadsorbed with rat serum [Hyclone Laboratories]) diluted 1:50 with PBS/gelatin, rinsed, and mounted. Sections were photographed with a Zeiss PM3 light microscope using 4 × 5 Tri X-Pan film at ASA 1600 with ACU-1 development for fluorescence optics, or 4 × 5 in Ektapan film, ASA 100 with DK-50 development for Nomarski optics (all from Eastman Kodak Co., Rochester, NY).

*Electron Microscopy.* Blocks of ileum were fixed in 0.5% glutaralde-

1. *Abbreviations used in this paper*: CF, cationized ferritin; NF, native ferritin; PBS/Tw, 0.1% Tween-20 in PBS; RT, room temperature; TBST, Tris-buffered saline–Tween-20.

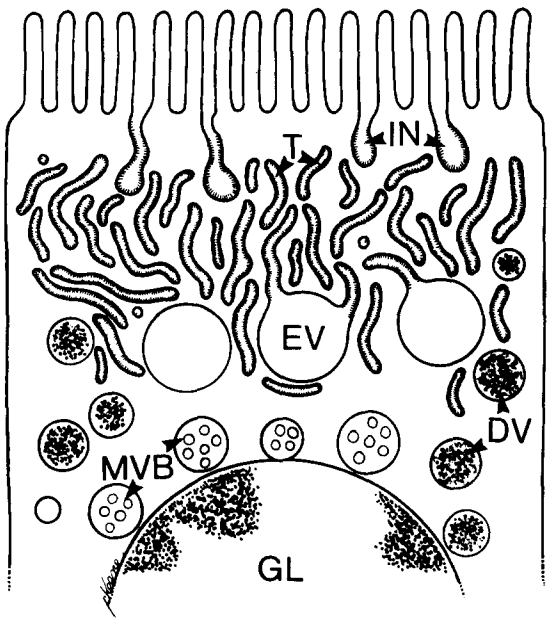



*Figure 1.* Diagram of the apical endocytic complex of an absorptive cell in suckling rat ileum. Milk proteins are endocytosed from deep invaginations (IN) between microvilli. Endocytosed ligands enter an extensive system of tubules (T) and endosomal vesicles (EV), and later enter multivesicular bodies (MVB) and the giant lysosome (GL). Soluble proteins collect in vesicles with dense content (DV) before delivery to the lysosome. Membrane-associated arrays are present on apical invaginations and tubules.

hyde, 2% paraformaldehyde, 0.1 M Na cacodylate, pH 7.4, for 90 min, dehydrated in ethanol with polyvinylpyrolidine and embedded in LR Gold resin at 4°C using benzoin methyl ether and UV light polymerization according to the supplier's instructions (Polysciences, Inc., Warrington, PA). 5-nm colloidal gold was made by the method of Muhlphordt (45) using tannic acid and sodium citrate as reducing agents. 10-nm colloidal gold was made by the method of Slot and Geuze (57). Protein A (Genzyme, Inc., Boston, MA) was adsorbed to gold at pH 7.4, and conjugates were stabilized with 0.01% polyethylene glycol (average mol wt 20,000), washed three times by centrifugation in an SS34 rotor (1 h at 30,000 *g* for 5-nm gold, and 1 h at 12,000 *g* for 10-nm gold), and stored in 50% glycerol at −20°C. Ultrathin sections were collected on nickel grids coated with Formvar and carbon. Immunocytochemistry was performed by the technique of Geuze et al. (19) as modified by Titus and Becker (59). Grids were quenched with PBS containing 10 mM $NH_4Cl$, blocked with Tris-buffered saline-Tween-20 (TBST) buffer (10 mM Tris-HCl, 500 mM NaCl, and 0.3% Tween-20, pH 7.2), containing 1% BSA, and incubated on drops of primary antibody (culture supernatant diluted 1:1 with TBST/BSA) for 1 h. After washing in TBST, grids were placed on a drop of rabbit anti-mouse IgG (Cappel Laboratories, Inc., Malvern, PA) diluted to 20 μg/ml in TBST/BSA for 1 h. Grids were incubated on a drop of protein A-gold for 30 min, washed in TBST, rinsed with distilled water, and poststained for 5 min with 2% aqueous uranyl acetate and for 1 min with Reynold's lead citrate. Control grids were incubated with monoclonal antibodies from the same fusion that were not directed against the endocytic complex.

### *SDS-Gel Electrophoresis and Immunoblotting*

Chemicals used in SDS-PAGE and immunoblotting were obtained from Bio-Rad Laboratories. Samples were prepared for gel electrophoresis by the method of Bartles et al. (4). Briefly, samples were solubilized with 0.17% (wt/vol) sodium deoxycholate, 0.12% (vol/vol) Emulphogene BC-720 (GAF Corporation, Wayne, NJ), and 0.02% SDS, and precipitated with 10% (wt/vol) TCA for 1 h at 4°C. Precipitates were extracted with 90% (wt/vol) acetone and 0.1 N HCl, resolubilized in sample buffer, and boiled for 3 min. SDS-PAGE was performed on discontinuous 7.5% or 5–15% gradient gels according to Laemmli (34). Molecular masses were estimated by comparison to standard proteins: myosin (200 kD), β-galactosidase (116.25 kD), phosphorylase B (92.5 kD), BSA (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD).

For immunoblotting, proteins were electrophoretically transferred to nitrocellulose (60), rinsed briefly in $dH_2O$, blocked with PBS containing 3% BSA and 0.1% Tween-20 for 1 h at RT, and rinsed briefly with 0.1% Tween-20 in PBS (PBS/Tw). Blots were then incubated in culture supernatant diluted 1:1 in PBS/Tw for 1 h at RT, rinsed in PBS/Tw, and incubated in goat anti-mouse IgG conjugated to alkaline phosphatase (HyClone Laboratories), diluted 1:1,000 in PBS/Tw containing 1% BSA, for 1 h at RT. Blots were rinsed with PBS/Tw and water, and developed with 0.6 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.6 mg/ml nitro blue tetrazolium in 0.15 M Tris buffer, pH 8.8.

To separate integral membrane proteins from peripheral, water-soluble proteins, 400-μg aliquots of endosome membrane fractions were extracted with Triton X-114 by the procedure of Bordier (8). Proteins in Triton X-114 pellets and aqueous supernatants were subjected to SDS-PAGE and immunoblotting.

For chemical deglycosylation, samples were precipitated and extracted as for SDS-PAGE, resuspended in 0.8% (wt/vol) SDS, 10 mM $NH_4HCO_3$, pH 8.5, by bath sonication, frozen, and lyophilized overnight. Deglycosylation was carried out for 3 h according to the method of Edge et al. (18) as modified by Bartles et al. (4).

To determine whether antibodies were binding to a carbohydrate epitope, Western blots were oxidized with periodic acid prior to immunoblotting as described by Woodward et al. (65).

### *Leupeptin Uptake*

Suckling rats were anesthetized by intraperitoneal injection of 0.15 ml of 25% urethane in PBS, and 3-cm ligated loops of ileum were injected with 50 μg/ml leupeptin in PBS. Loops were incubated 1 h in vivo, removed, and fixed for 90 min in 2% paraformaldehyde, 0.1% glutaraldehyde, in 0.1 M sodium cacodylate, pH 7.4. Ileal segments were then embedded in LR Gold resin and EM immunocytochemistry was performed as described above.

### *Endocytosis of Tracer Proteins*

Cationized ferritin (CF) (Miles Laboratories, Inc., Elkhart, IN), was diluted to 5.5 mg/ml with PBS. Native ferritin (NF) (cadmium-free, Polysciences, Inc.) was diluted to 25 mg/ml with PBS. Tracers were injected into ligated ileal loops and incubated for 30 min in vivo. Loops were then removed, fixed, and processed as in the leupeptin experiments. To clearly differentiate between ferritin and protein A–gold–antibody complexes, 10-nm gold was used.

## *Results*

### *Isolation of Endosome Membranes*

The apical "endocytic complex" of suckling rat ileal absorptive cells consists of an extensive system of tubulocisternae and vesicles concentrated in a narrow region between the microvillous border and the large, supranuclear lysosomal vacuole (Fig. 1). The membranes of the entire tubulocisternal system, as well as the deep plasma membrane invaginations between microvilli, bear regular arrays of dense projections on their luminal surfaces (Fig. 2) but none of the vesicles shows such arrays. Previous investigators used the membrane-bound arrays and their enyzmatic activity to identify membranes of the endocytic complex in subcellular fractions of mucosal scrapings from suckling rat ileum (28, 31).

We obtained high yields of the tubulocisternal portion of the endocytic complex for biochemical analysis and antibody production by a multistage fractionation procedure. For antibody production, antigenic apical surface glycoproteins, including brush border hydrolases and mucins, were removed enzymatically before EDTA-mediated release of epithelial sheets and subsequent isolation of brush borders. Under the stabilizing conditions used, the extensive brush border cy-

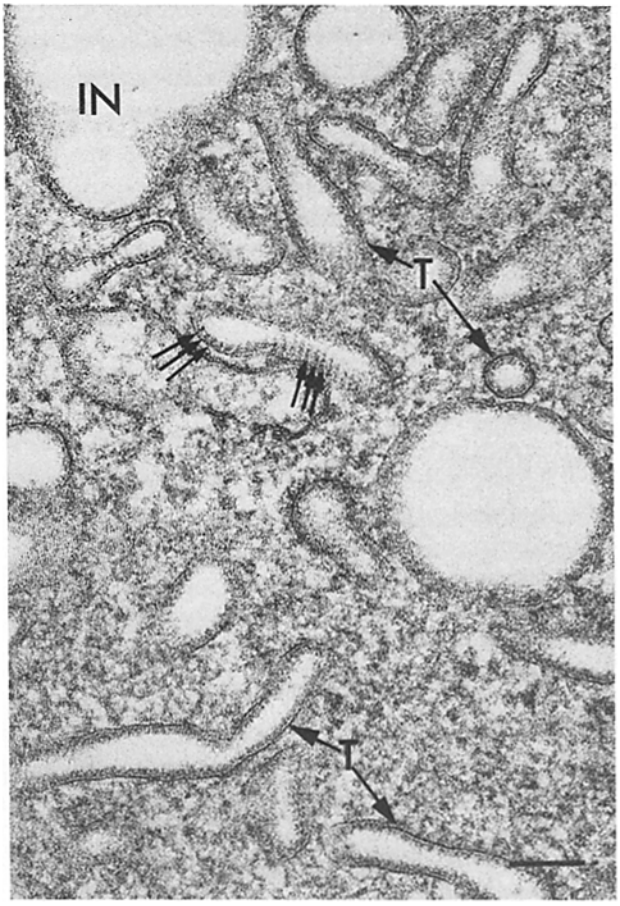



*Figure 2.* Apical portion of neonatal ileal absorptive cell. Membrane arrays (*arrows*) are seen on the luminal aspect of plasma membrane invaginations (IN) and endosomal tubules (T). Bar, 0.1 μm.

toskeleton maintained the association of much of the endocytic complex with the brush border during the first fractionation steps. Subsequent disruption of the brush border was not achieved using standard homogenization protocols such as dounce homogenization; release of endosomes from microvillous membranes required solubilization of the cytoskeleton followed by passage through a ball-bearing homogenization device (3).

Electron microscopy of the fraction of density 1.018–1.030 from the final Percoll gradient (as determined by Percoll density standards) showed primarily tubular and vesicular membrane profiles (Fig. 3 *A*). Principal contaminants recognized in this fraction included microvilli and long membrane fragments. The majority of membrane profiles exhibited distinctive surface arrays identical to those on tubular endosomes of intact cells (Fig. 3 *b*). The arrays were often on the external surface of isolated tubules, suggesting that the tubular compartments had inverted during the isolation procedure.

### *Protein Composition of Endosome Fraction*

SDS-PAGE of the endosome fraction showed that this preparation contained several components that were enriched over whole-cell homogenate (Fig. 4). After solubilization of the fraction in an aqueous solution of Triton X-114 at 4°C followed by repeated phase separation at 37°C, several of the enriched bands (apparent molecular masses 140–150, 85, and 35 kD) partitioned primarily in the detergent phase, indicating that they may be major integral membrane proteins. Because other membranes were present in the fraction, however, none of these bands could be positively ascribed to endosomal tubules. The 150-kD band, for example, corresponds in molecular mass to lactase, an integral glycoprotein known to be abundant in microvillus membranes of suckling rodents (48, 49).

### *Monoclonal Antibodies*

The endosome fraction was used as immunogen to produce mouse/mouse hybridomas. Of 576 wells exhibiting growth, 191 were positive by dot-blot assay of SDS-denatured membrane protein, using goat anti-mouse IgG conjugated to alkaline phosphatase or horseradish peroxidase as the detection system. 42 strongly positive wells were screened by immunofluorescent staining of 0.5-μm frozen sections, 27 showed staining of the region of apical cytoplasm known to contain the endocytic complex. Although many of these also showed staining of microvilli, basal-lateral surfaces or other intracellular regions initially, some of these patterns were lost during expansion of the cultures, leaving four cultures that exhibited fluorescence exclusively in the apical endosome region. These were cloned twice and screened on Western blots. One of these reacted strongly on Western blots and was therefore selected for further study. After the second cloning, sister clones exhibited identical staining patterns by immunofluorescence and recognized identical bands on Western blots.

The immunofluorescence staining pattern of this antibody is shown in Fig. 5. A band of apical cytoplasm positioned below the microvillous border, corresponding to the region rich in endosomal tubules, was heavily labeled. This pattern was readily distinguished from the continuous band of fluorescence seen in sections incubated with monoclonal antilactase (provided by A. Quaroni, Cornell University), which stains only microvilli (48). The endosomal staining pattern also differed dramatically from that seen with a polyclonal antibody directed against a lysosomal membrane component (lysosomal glycoprotein [lgp-120] provided by I. Mellman and A. Helenius, Yale University, ref. 35). Anti-lgp-120 labeled the membrane of the giant vacuole and a cluster of small vesicles just above the vacuole that corresponded in position to acid phosphatase-positive multivesicular bodies (13, 21).

Endosomal antibody binding in neonatal ileum was restricted to epithelial absorptive cells; no labeling was seen in cells of the lamina propria or other tissue layers. The antibody did not bind to any structures in frozen sections of other periodate-lysine-2% paraformaldehyde-fixed epithelial tissues including jejunum, liver, and kidney of suckling rats, ileum and urinary bladder of adult rats, and ileum of adult rabbits.

### *Ultrastructural Localization of the Endosomal Antigen*

EM immunocytochemistry using ultrathin sections of LR Gold-embedded tissue revealed the distribution of the anti-

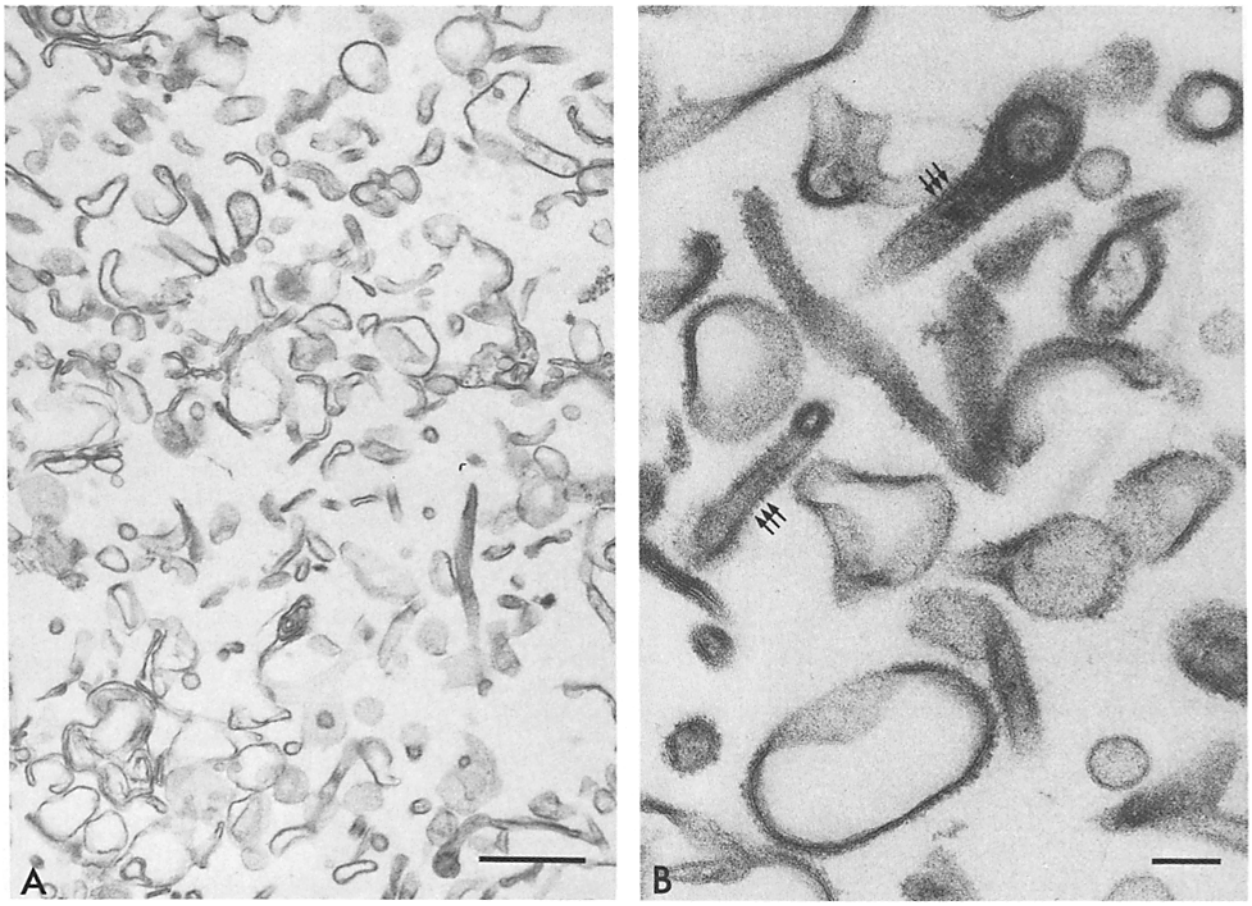



*Figure 3.* Endosome fraction obtained by Percoll gradient centrifugation. (*A*) The fraction consists primarily of tubular and vesicular membranes. Bar, 0.5 μm. (*B*) Membrane-associated arrays are seen as regular projections on the external aspect of the tubules (*arrows*), indicating that tubules inverted during the fractionation procedure. Bar, 0.1 μm.

gen within the apical endocytic complex. Antibody-binding sites, visualized by binding of rabbit anti-mouse IgG followed by protein A–colloidal gold, were located primarily in endosomal tubules (Fig. 6). Antigen was also present on some apical plasma membrane invaginations and in some (but not all) clear endosomal vesicles. In all these locations, binding sites were associated with the luminal side of the membrane. Multivesicular bodies, dense-content vesicles, and giant lysosomal vacuoles were not labeled. No binding sites were observed on microvilli or basolateral cell surfaces, or in other intracellular compartments such as Golgi complex cisternae, Golgi complex-associated vesicles, or rough endoplasmic reticulum.

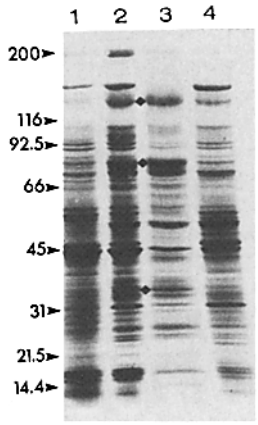
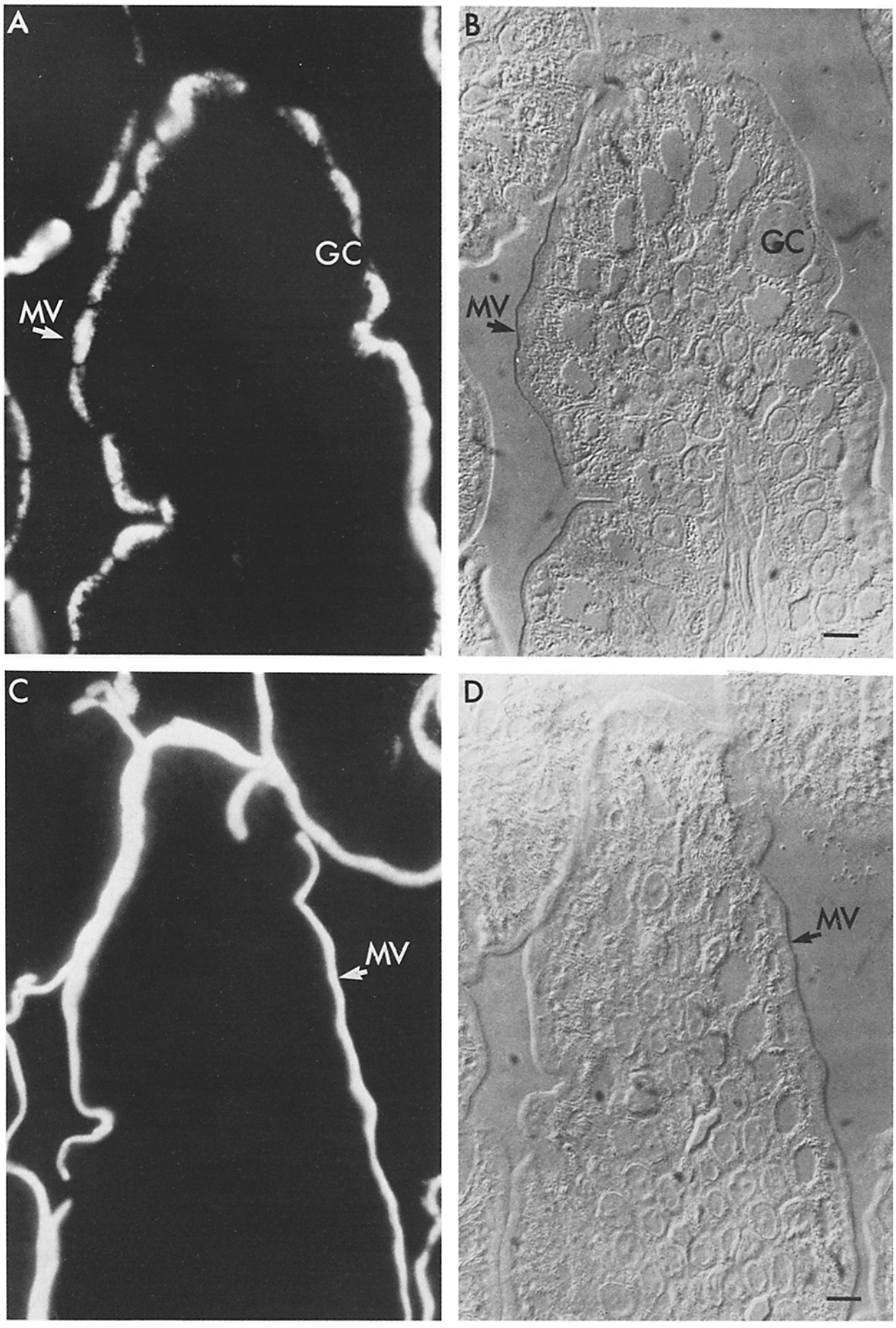



*Figure 4.* SDS-PAGE of proteins in whole cells and endosome fraction (5–15% gradient gel, Coomassie Blue stain). Lane 1, homogenate of whole ileal absorptive cells; lane 2, endosome-enriched fraction from Percoll gradient (shown in Fig. 3); lane 3, Triton X-114 extract of endosome fraction (detergent pellet); lane 4, aqueous supernatant from Triton X-114 extraction. Of the bands that appear to be enriched in the endosome fraction, a subset (*diamonds*) partitions into TX-114 and thus may be intramembrane proteins.

In those large clear endosomal vesicles that were labeled, antibody-binding sites were often confined to the apical pole of the vesicle, the side at which tubular connections are generally located (Fig. 6). Fortuitous planes of section that passed through endosomal vesicles and their tubular extensions showed that some vesicles which were devoid of binding sites were continuous with tubules rich in binding sites (Fig. 7). To inhibit protease activity of the lysosomal system (17, 54), the absorptive epithelium was allowed to take up leupeptin from the ileal lumen in vivo for 1 h before fixation and EM immunocytochemistry. Under these conditions, the ultrastructural distribution of the antigen was unaltered and no binding sites were detected in the lysosomal compartment. This suggests that the antigen is indeed absent from lysosomal membranes of control cells, and its absence by im-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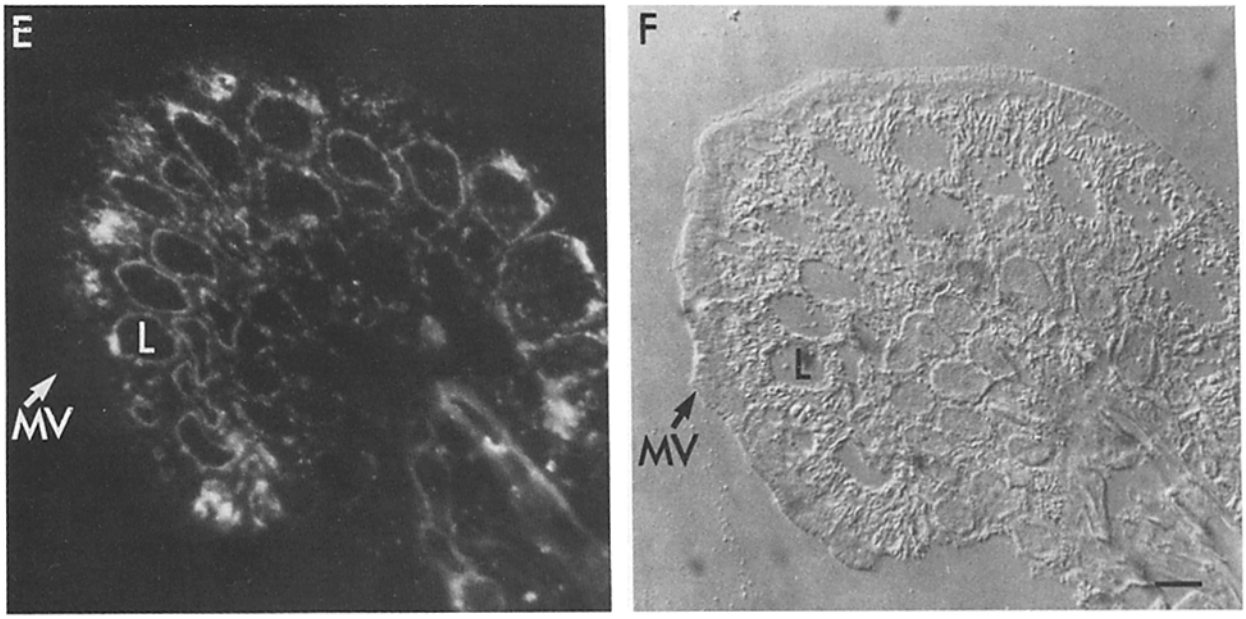


*Figure 5.* Immunofluorescent staining and matched Nomarski images of 1-μm frozen sections of neonatal ileum. Bar, 10 μm. *Opposite*: (*A* and *B*) Monoclonal antibody raised against the endosome fraction from neonatal ileum. A diffuse band just below the microvillous border (MV) and above the giant lysosome is labeled. Tubular and vesicular endosomes are concentrated in this region. GC, goblet cell. (*C* and *D*) Monoclonal antilactase (gift of Dr. Andrea Quaroni, ref. 50). Immunoreactive lactase is concentrated in microvillus membranes (MV) but not in the endocytic complex. *Above*: (*E* and *F*) Polyclonal antibody raised against lgp 120, a glycoprotein antigen of lysosomal membrane (gift of Drs. Ira Mellman and Ari Helenius, ref. 35). The distribution of lgp 120 in giant lysosome (L) and associated lysosomal vesicles does not appear to overlap with that of the endosomal antigen shown in A/B. MV, microvilli.

munocytochemistry is not due simply to enzymatic digestion of the membrane surface epitope by lysosomal proteases (16).

To visualize the distribution of the antigen along the two separate prelysosomal pathways previously demonstrated for soluble and membrane-bound protein tracers (21), EM immunocytochemistry was performed after uptake of either NF (a fluid-phase tracer) or CF (an adsorptive tracer). The membrane compartments at early stages of both endosomal pathways showed antibody binding: most but not all small apical vesicles that contained NF and apical tubules that contained CF were labeled by the antibody (Fig. 8, *A* and *B*). In contrast, larger vesicles with dense content that collect and concentrate NF after endocytosis were not labeled (Fig. 8 *A*). Large clear endosomes that receive CF after uptake were partially labeled or unlabeled as described above (Fig. 7). The multivesicular bodies and the giant lysosome still did not label, although both structures contained CF and NF.

### *Properties of the Antigen*

To identify the endosomal antigen recognized by the antibody, immunoblot analysis was performed after SDS-PAGE separation of endosomal tubule proteins. The antibody recognized a broad band of apparent molecular mass ~55–61 kD (Fig. 9). This antigen did not correspond to any of the major bands of the whole endosome fraction, or the Triton X-114 extract of this fraction as seen by Coomassie Blue staining of polyacrylamide gels (Fig. 4). The antigen was present in whole-cell homogenates of isolated ileal epithelium and showed the same mobility in gels of this starting material as in the final membrane fraction, indicating that proteolysis had not occurred during the membrane isolation procedures. Antigen in the endosome fraction was significantly enriched over whole cell homogenate (Fig. 10, lanes *1* and *2*). The antigen partitioned primarily in the detergent phase during Triton X-114 phase separation (Fig. 10, lane *3*) suggesting that it is an integral membrane protein. However, a smaller portion of the immunoreactivity consistently partitioned into the aqueous phase (Fig. 10, lane *4*).

To determine whether this antigen is a glycoprotein, the membrane preparation was subjected to chemical deglycosylation with tetramethanesulfonic acid, a treatment that removes both *N*-linked and *O*-linked sugars with the exception of *N*-linked *N*-acetylglycosamine residues attached to asparagine (18). When deglycosylated in this way and then immunoblotted, the molecular mass of the protein shifted from 58 kD (average) to 44 kD (Fig. 10, lanes *5* and *6*). Two lines of evidence indicate that the antibody recognizes a polypeptide epitope of the antigen rather than an oligosaccharide. First, after chemical deglycosylation, it was still possible to identify the antigen by immunoblotting (Fig. 10, lane *6*). Second, periodate oxidation of Western blots before exposure to antibody, a procedure that oxidizes carbohydrate moieties of glycoproteins and often destroys antibody binding to carbohydrate epitopes (65), did not affect the binding of the antibody to Western blots (not shown).

In an attempt to further characterize the membranes that contain this antigen, we applied the homogenate of isolated brush borders to continuous sucrose gradients instead of the Percoll gradients used for most of these studies. Immunoblot analysis, enzyme assays, and EM of fractions from sucrose gradients showed that plasma membranes (including micro-

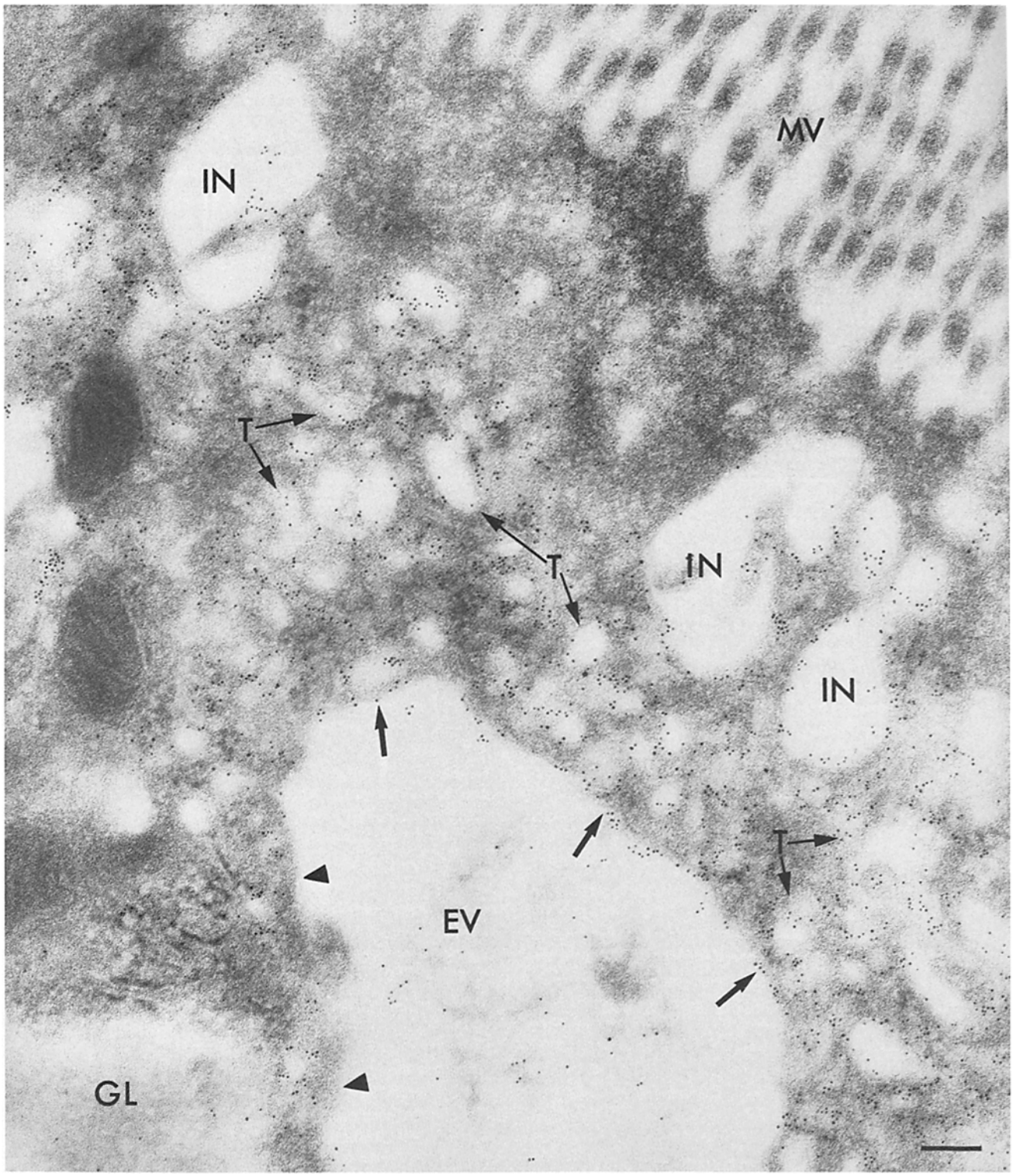


*Figure 6.* Localization of endosomal antigen by electron microscope immunocytochemistry: LR Gold sections labeled sequentially with monoclonal antibody, rabbit anti-mouse IgG, and protein A–5-nm gold. Antigen is concentrated in endosomal tubules (T). The endosomal vesicle (EV) is labeled on the apical side (*arrows*) but not on the basal side (*arrowheads*). Plasma membrane invaginations (IN) are partially labeled. Colloidal gold particle distribution indicates that antibody-binding sites are on the luminal side of these membranes. Microvilli (MV) and giant lysosome (GL) are not labeled. Bar, 0.1 μm.

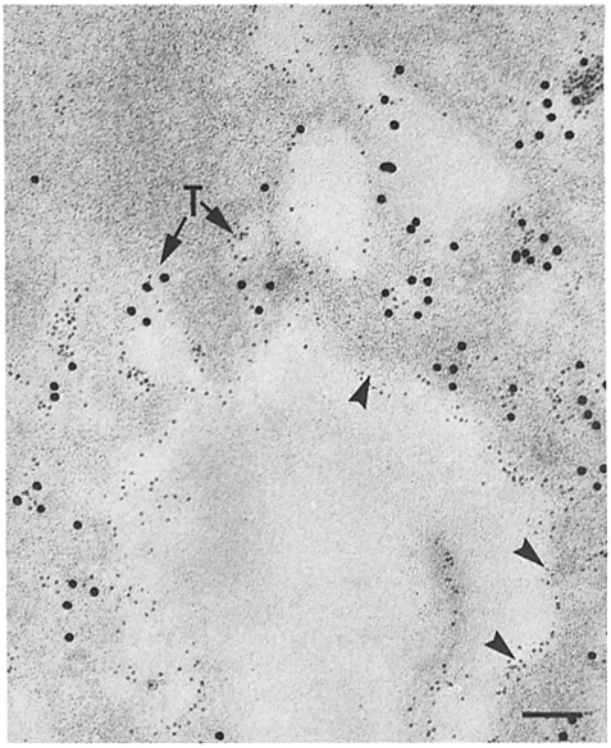


*Figure 7.* An endosomal vesicle with attached endosomal tubules (T) in a section immunolabeled as in Fig. 6, except that 10-nm protein A–gold was used. The cells were incubated with CF for 30 min at 37°C to label the endocytic pathway. The tubule membranes (T) are labeled by anti-endosome monoclonal antibody, but the membrane of the vesicle is devoid of label (*arrowheads*). Bar, 0.1 μm.

villi), and endosomal membranes were poorly separated on these gradients. Plasma membrane enzymatic markers (5′-nucleotidase, alkaline phosphatase) eluted over a broad density range (1.11–1.17 g/ml) that included antibody-binding activity at densities 1.15–1.17 g/ml (Fig. 11). A lower-density protein peak (1.05–1.09 g/ml) contained no membranes but was rich in *N*-acetylhexosaminidase activity, the enzyme present in membrane-associated arrays of ileal absorptive cells. In that the giant lysosome and soluble lysosomal proteins had been removed during the initial brush border isolation procedure, this enzyme activity was most likely due to the nonintegral arrays released from membranes of the endocytic complex. The lack of antibody binding activity in these same light fractions suggests that the antigen is not part of the arrays. This is consistent with TX-114 data suggesting that it is an integral membrane glycoprotein.

## Discussion

We have used a membrane fraction enriched in apical endosomal tubules of absorptive cells from suckling rat ileum to obtain an anti-endosome monoclonal antibody. This antibody recognizes a luminally exposed epitope of an intramembrane component located primarily in endosomal tubules, but is also present in a portion of endosomal vesicle membranes and in endocytic pits of apical plasma membrane. The antibody binds to the peptide portion of a glycoprotein of apparent $M_r$ 55–61 kD, designated gp 55–61, that appears to be unique to absorptive cells of neonatal ileum.

The endosomal compartment, through which receptors and ligands move after endocytosis, is now recognized as a morphologically heterogeneous system consisting of distinct subcompartments (24). Extensive tracer studies of hepatocytes, fibroblasts, and other cells have shown that ligand–receptor complexes rapidly enter a system of smooth vesicles with multiple tubular extensions and later, ligands pass into multivesicular endosomes that lack lysosomal hydrolase activity (19, 39, 47, 66). Immunocytochemical data suggest that sorting and clustering of receptors and budding of receptor-rich vesicles for return to the plasma membrane may occur in the tubular extensions whereas the vesicular portions serve as collecting vesicles for dissociated ligands (20). Multivesicular endosomes seem to be largely devoid of recycling receptors (19, 44) and can ultimately fuse with Golgi complex-derived vesicles that carry lysosomal hydrolases (10).

Our results are consistent with the idea that this morphologic and functional specialization within the endosomal system may reflect biochemical differences in membrane composition. Little information is available on the molecular features that distinguish endosomes from other organelles or that underlie functionally distinct subcompartments such as endosomal tubules. Whether tubulovesicular endosomes represent relatively stable, long-lived organelles, or simply transient, short-lived structures formed by coalescence of incoming plasma membrane-derived vesicles, is still controversial (24). Subcellular fractionation studies of hepatocytes and KB carcinoma cells, in which endosomes were identified by endocytosed markers, reported biochemical features that distinguished membranes of early endosome compartments from lysosomes and plasma membrane, including absence of marker enzymes, high cholesterol content, and/or a distinct protein profile on SDS gels (2, 14, 29, 42, 62). Highly-enriched liver endosome fractions obtained by an horseradish peroxidase/diaminobenzidine density-shift procedure were dramatically depleted in marker enzymes for plasma membrane, lysosomes, and Golgi complex (50). In other studies, however, iodination of endosome membranes revealed a subsample of plasma membrane constituents but no unique endosomal protein components (46). Apparent discrepancies among these studies could be due to variations in cell type or more likely to differences in the sampling of endosome systems obtained by different fractionation procedures. When documented morphologically, some "endosome fractions" were dominated by multivesicular bodies (50) and others by clear vesicles (14). In only one study were fractions enriched in tubulovesicular endosomes and MVEs analyzed separately (44); these authors recognized that selective loss of tubular extensions during fractionation could still have affected the results obtained. The lack of endogenous markers for all or part of the endosome system continues to retard progress in elucidating the composition and dynamics of this complex compartment. If endosomes were entirely transient, however, such markers would not be expected to exist. Our results indicate that endosome marker proteins may exist and can potentially shed new light on endosomal membrane dynamics.

Absorptive cells of suckling rat ileum provide a unique

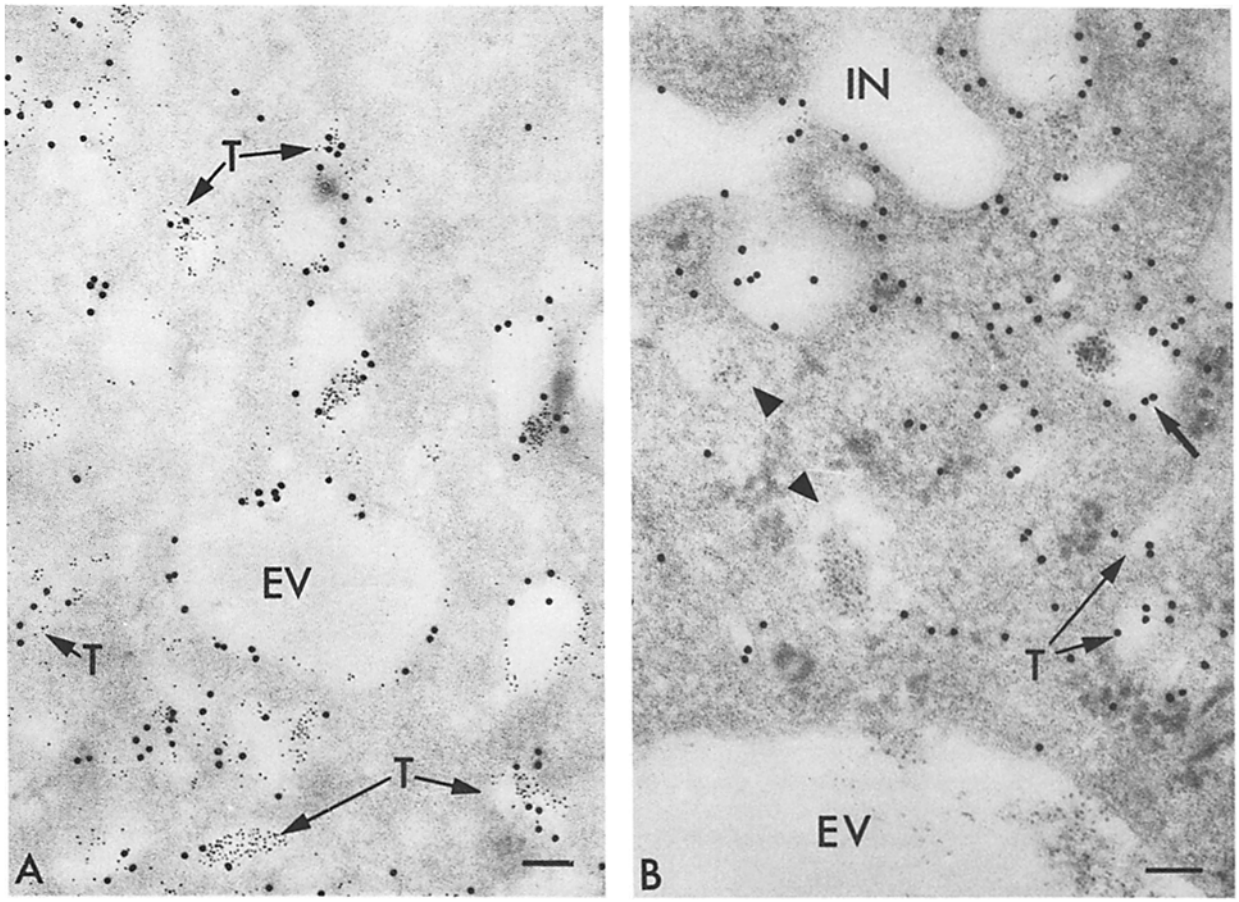


*Figure 8.* Immunolocalization of the endosomal antigen after uptake of CF or NF from the intestinal lumen in vivo. Processed as in Fig. 6, but with 10-nm protein A-gold. Bar, 0.1 μm. (*A*) CF. Endosomal tubules (T) contain the absorptive tracer, and are also labeled with anti-endosome antibody. A small endocytic vesicle (EV) labeled with CF is also labeled with anti-endosome antibody. (*B*) NF. Endosomal tubules (T) show antibody binding but do not contain the soluble tracer. Some small vesicles containing NF are immunolabeled (*heavy arrow, right*) but others are not (*arrowheads*). The membrane of the NF-containing endosomal vesicle (EV) does not bind antibody.

system for study of tubulovesicular endosomes. The clear polarization of this endosomal system and the stabilizing influence of the brush border cytoskeleton on the apical cell pole allowed us to separate brush border-associated endosomes from lysosomes and other parts of the cells. Subsequent steps released endosomal membranes, and those derived from the tubules were readily distinguished by their luminal arrays from endosomal vesicles (which lack arrays) and other contaminating organelles, but not from the invaginated domains of apical plasma membrane (on which arrays also reside). Although these arrays are not integral, they provide a valuable ultrastructural landmark in that endosomal tubules of other cell types, if vesiculated or detached from endosomal vesicles during homogenization, have no distinguishing features and cannot be recognized in fractions.

Although ileal cell endosomal tubules have unique features (28, 31, 55, 64), they are comparable in important respects to endosomal tubules of other cell types. Membrane-bound ligands rapidly enter ileal cell tubules after endocytosis and subsequently enter clear endosomal vesicles, multivesicular bodies, and the giant vacuole, a typical lysosome-directed pathway (21). The physiologic milk peptides, nerve growth factor and epidermal growth factor, are endocytosed into the tubules and in part transported across the epithelium (23, 56), suggesting that these tubules, like their counterparts in neonatal jejunal cells (52) and hepatocytes (20, 25), may be a site for sorting of specific receptor-bound ligands out of the lysosome-directed pathway.

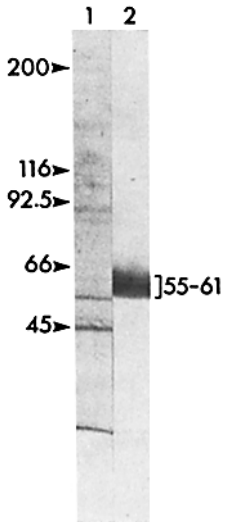


*Figure 9.* Identification of the endosomal antigen on immunoblot. Endosome fraction proteins were separated on a 7.5% polyacrylamide gel, transferred to nitrocellulose, and stained with amido black (lane *1*) or monoclonal antibody and secondary antibody conjugated to alkaline phosphatase (lane *2*). The antigen appears as a broad band ranging in molecular mass from 55 to 61 kD. Molecular mass standards are in kilodaltons.

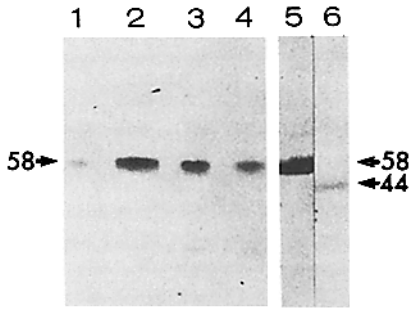


*Figure 10.* Immunoblot characterization of the endosomal antigen. The antigen is highly enriched in the endosomal fraction (lane *2*) when compared with an equal protein load of whole-cell homogenate (lane *1*). The antigen partitions preferentially into the TX-114 detergent pellet (lane *3*), but a significant amount partitions into the aqueous phase (lane *4*). When deglycosylated with tetramethanesulfonic acid, the apparent molecular mass shifts from 58 kD (average) (lane *5*) to 44 kD (lane *6*).

We have now identified an integral membrane glycoprotein (gp 55–61) that is present in ileal cell endosomal tubules, apical plasma membrane pits, and a portion of clear endosomes, but absent from most vesicular endosomal membranes, multivesicular bodies, and lysosomes. It is thus restricted to membranes functioning at early stages of the endocytic process, stages at which sorting of membrane domains and receptor clusters are thought to occur. The role of this glycoprotein is unknown, but because it has thus far been detected only in absorptive cells of suckling rat ileum, it may well serve a function specific to these cells such as receptor-mediated uptake of some unidentified milk component. Its highly restricted distribution, however, differs from other known receptors (20). In intestine, Fc receptors that mediate uptake of maternal IgG from milk in neonatal jejunum are distributed on apical microvilli and pits, throughout the apical endosome pathway, and on basal-lateral plasma membranes (53). Ileal cell endosomal tubules, with their unusual luminal arrays and intramembrane particle patterns, may well have other unidentified functions in addition to ligand uptake. Future studies may reveal the specialized functions of these membranes and the role of gp 55–61 in this system. Meanwhile, the antigen serves as a valuable membrane marker in this cell.

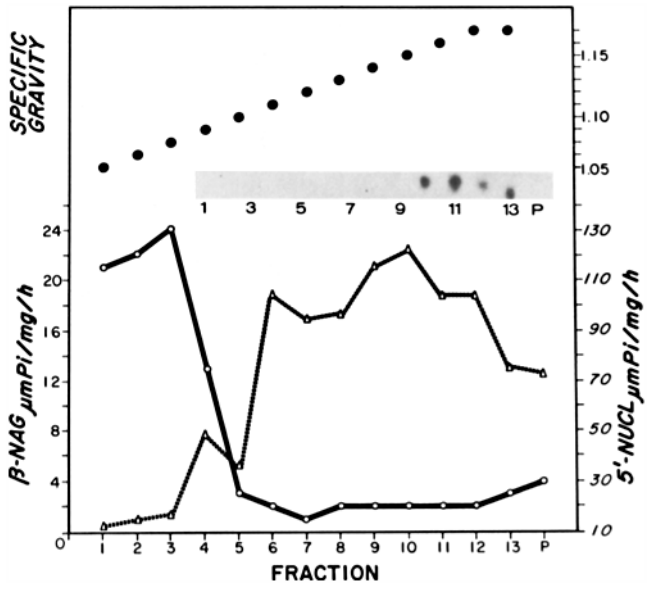


*Figure 11.* Enzymatic and immunoblot profile of brush border homogenate, fractionated by sucrose gradient centrifugation. β-*N*-acetylglucosaminidase (β-NAG, *solid line*), an enzyme present in the luminal arrays associated with endosomal tubules of ileal absorptive cells (27, 28), was present in low-density fractions. The lack of membranes in these light fractions suggests that the arrays had been released during centrifugation. Plasma membrane marker enzymes 5′ nucleotidase (5′ mNUCL, *dotted line*) and alkaline phosphatase (not shown) codistributed over a broad, heavier-density range. Endosomal antigen gp 55–61 was present in a subset of these heavier, membrane-rich fractions and thus was not associated with the β-NAG–containing arrays.

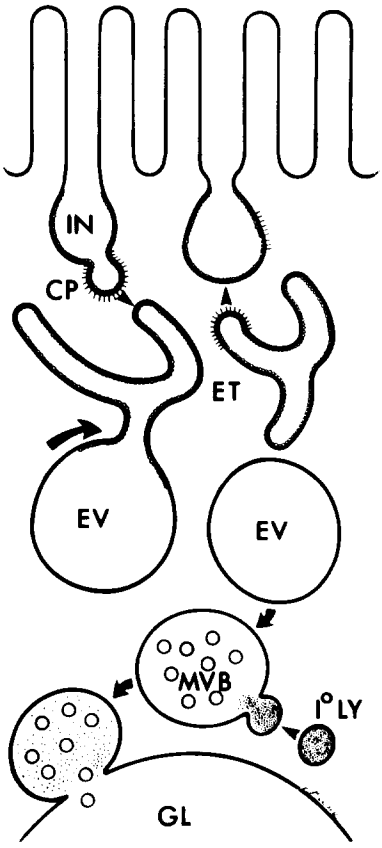


*Figure 12.* Diagram showing distribution of endosomal antigen gp 55–61 and possible movements of antigen and membrane in endocytic complex of ileal absorptive cell. The antigen (*clear circles* on luminal membrane face) resides primarily in endosomal tubules (ET); it is also present in portions of apical plasma membrane invaginations (IN). There are clathrin-coated pits (CP) on invaginations and tubules, suggesting vesicular traffic to and from these membrane domains during endocytic activity. Antigen resides on apical side of endosomal vesicles (EV) adjacent to tubular connections, but may be segregated into tubular membranes (*curved arrow*). Vesicles may then detach from tubular system, become multivesicular bodies (MVB), acquire lysosomal hydrolases from primary lysosomal transport vesicles (1°LY), and fuse with giant lysosome (GL).

Immunologic methods have previously been used to identify organelle-specific antigens in rough endoplasmic reticu-

lum, Golgi complex (37), and lysosome membranes (11, 35, 36, 51) and plasma membrane domains (5, 30). Lysosomal membrane antigens of unknown function have been used to follow the movement and turnover of these antigens in metabolic labeling studies (11, 36). Lipincott-Schwartz and Fambrough (36) recently showed that small but significant amounts of a lysosomal membrane antigen is also present on plasma membranes and that the antigen moves rapidly in and out of this "stable" organelle. In ileal cells, the tubular portions of endosomes may be relatively stable structures, but vesicles containing gp 55–61 may move to and from the antigen-positive pits between microvilli, a restricted plasma membrane domain from which endocytosis occurs (22). The polarized distribution of the antigen in endosomal vesicles, just adjacent to tubular extensions, and its absence from lysosomal membranes even when enzyme activity is suppressed by leupeptin, suggests that this membrane antigen is spared degradation by collecting in the tubular membranes. The large, clear endosomal vesicles containing luminal proteins but devoid of gp 55–61 might periodically detach from the tubular network and deliver both content and membrane to the lysosomal system. A similar model has been suggested by others for endosomal systems in liver and kidney proximal tubule cells (7, 12). According to the model shown in Fig. 12, the endosome compartment would have both semipermanent, recycling components and short-lived, transient components. Metabolic labeling studies of the gp 55–61 antigen are currently underway to test this model in the ileal absorptive cell system.

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