

# Endolyn-78, a Membrane Glycoprotein Present in Morphologically Diverse Components of the Endosomal and Lysosomal Compartments: Implications for Lysosome Biogenesis

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**Abstract.** A monoclonal antibody (2C5) raised against rat liver lysosomal membranes was used to identify a 78-kD glycoprotein that is present in the membranes of both endosomes and lysosomes and, therefore, is designated endolyn-78. In cultures of rat hepatoma (Fu5C8) and kidney cells (NRK), this glycoprotein could not be labeled with [<sup>35</sup>S]methionine or with [<sup>32</sup>P]inorganic phosphate but was easily labeled with [<sup>35</sup>S]cysteine and [<sup>3</sup>H]mannose. Pulse-chase experiments and determinations of endoglycosidase H (endo H) sensitivity showed that endolyn-78 is derived from a precursor of  $M_r$  58–62 kD that is processed to the mature form with a  $t_{1/2}$  of 15–30 min. The protein has a 22-kD polypeptide backbone that is detected after a brief pulse in tunicamycin-treated cells. During a chase in the presence of the drug, this is converted into an *O*-glycosylated product of 46 kD that despite the absence of N-linked oligosaccharides is effectively transferred to lysosomes. This demonstrates that the delivery of endolyn-78 to this organelle is not mediated by the mannose-6-phosphate receptor (MPR).

Immunocytochemical experiments showed that endolyn-78 is present in the limiting membranes and the interior membranous structures of morphologically

identifiable secondary lysosomes that contain the lysosomal hydrolase  $\beta$ -glucuronidase, lack the MPR, and could not be labeled with  $\alpha$ -2-macroglobulin at 18.5°C, a temperature which prevents appearance of endocytosed markers in lysosomes.

Endolyn-78 was present at low levels in the plasma membrane and in peripheral tubular endosomes, but was prominent in morphologically diverse components of the endosomal compartment (vacuolar endosomes and various types of multivesicular bodies) which acquired  $\alpha$ -2-macroglobulin at 18.5°C, and frequently contained substantial levels of the MPR and variable levels of  $\beta$ -glucuronidase. On the other hand, the MPR was very rarely found in endolyn-containing structures that were not labeled with  $\alpha$ -2-macroglobulin at the low temperature. Thus, the process of lysosomal maturation appears to involve the progressive delivery of lysosomal enzymes to various types of endosomes that may have already received some of the lysosomal membrane proteins. Although endolyn-78 would be one of the proteins added early to endosomes, other lysosomal membrane proteins may be added only to multivesicular endosomes that represent very advanced stages of maturation.

**T**HE biogenesis of a lysosome involves the joining of both luminal and membrane components to form a fully functional mature organelle. In most cell types, this process cannot be completely dissociated from the phenomenon of endocytosis that brings to the lysosome extracellular material internalized at the cell surface. The endocytotic route involves passage of the interiorized material through several types of endosomes that differ in their rela-

tive acidity (Yamashiro and Maxfield, 1987), biochemical composition (Mueller and Hubbard, 1986; Schmid et al., 1988), morphological appearance, and closeness to the plasma membrane or Golgi apparatus (see Helenius et al., 1983; Pastan and Willingham, 1983; Geuze et al., 1984, 1985; Wall and Hubbard, 1985; Hopkins, 1986; Dunn et al., 1986; Schmid et al., 1988). It is not clear, however (see Helenius et al., 1983), whether the different kinds of endosomes represent a series of permanent structures that transfer their luminal content from one to another, and ultimately to lysosomes (vesicle shuttle model), or represent successive maturation stages in a biochemical and morphological membrane-

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remodeling process which culminates in the conversion of endosomes into lysosomes (maturation model) (see Helenius et al., 1983; Sahagian, 1984; Brown et al., 1986; Griffiths et al., 1988). In any case, it is known that lysosomal hydrolases are brought to lysosomes or prelysosomes from the *trans*-Golgi network (TGN)<sup>1</sup> (see Griffiths and Simons, 1986) by clathrin-coated vesicles (Friend and Farquhar, 1967; Schulze-Lohoff et al., 1985; Brown et al., 1986; Lemansky et al., 1987) in which the enzymes are bound to an integral membrane protein carrier, the mannose-6-phosphate receptor (MPR), that is capable of functioning in multiple cycles of hydrolase delivery (see Kornfeld, 1987). Significant amounts of the receptor, however, are not present in the membrane of the mature lysosome (Geuze et al., 1984, 1985; Brown et al., 1986; Griffiths et al., 1988). In the maturation model, the MPR, as well as the lysosomal hydrolases and membrane proteins, would be expected to be present in endosomes and the removal of the receptor from mature lysosomes would simply represent the completion of the maturation process. In a vesicle shuttle model that takes into account the absence of the MPR from lysosomes, on the other hand, the hydrolases would be brought by the receptor to a permanent prelysosomal compartment where they could be packaged together with the lysosomal membrane proteins into an organelle from which the MPR is excluded. Recently, an apparently stable prelysosomal structure was described (Griffiths et al., 1988) that contains lgp 120, a membrane protein characteristic of lysosomes (Lewis et al., 1985), and large amounts of the MPR. Since endocytosed material was found in this structure it was proposed that it represents a specialized endosome from which lysosomes already containing the endocytosed material must emerge.

In the maturation model for lysosome biogenesis, membrane proteins of the mature lysosome are derived from the late endosome but may reach this organelle by different routes. Some proteins may, in principle, be brought together with the lysosomal hydrolases in vesicles derived from the Golgi apparatus. Others may be contributed by the membranes of early endosomes and could, therefore, represent either plasma membrane proteins that were interiorized together with extracellular material or distinctive components of endosomes which are not derived from the cell surface (Schmid et al., 1988). Finally, some lysosomal membrane proteins that apparently are not found in endosomes (Lewis et al., 1985; Green et al., 1987) could reach the lysosome by direct transfer from the TGN, independently of the lysosomal hydrolases.

To elucidate the pathways by which membrane proteins are incorporated into lysosomes, it is necessary to identify individual lysosomal membrane proteins, to determine their distribution within different components of the cellular endomembrane system, and to directly follow the routes that these proteins take to reach the lysosome. Recently, through the use of monoclonal or polyclonal antibodies raised against purified lysosomal membrane fractions, several lysosomal membrane proteins have been identified, and aspects of their biosynthesis, their subcellular localization, and pathways of delivery to the lysosome have been studied (Reggio et al.,

1. *Abbreviations used in this paper:* Endo H, endo- $\beta$ -*N*-acetylglucosaminidase; MPR, mannose-6-phosphate receptor; MVBs, multivesicular bodies; *O*-glycanase, endo- $\alpha$ -*N*-acetyl-galactosaminidase; TGN, *trans*-Golgi network.

1984; Chen et al., 1985*a,b*; Lewis et al., 1985; Tougard et al., 1985; D'Souza and August, 1986; Brown et al., 1986; Barriocanal et al., 1986; Lippincott-Schwartz and Fambrough, 1986, 1987; Green et al., 1987; Griffiths et al., 1988). In general, these are all glycoproteins with a high content of complex carbohydrate chains that may serve to protect the polypeptides from proteolytic degradation in the lysosome. Some of these proteins have been reported to be also present at significant levels in the plasma membrane and in endosomes (Reggio et al., 1984; Tougard et al., 1985; Lippincott-Schwartz and Fambrough, 1986, 1987), whereas others have been reported to be essentially undetectable outside lysosomes (Chen et al., 1985*b*; Lewis et al., 1985; Green et al., 1987).

In this report, we describe a previously unrecognized membrane glycoprotein, endolyn-78, which is present in substantial amounts in lysosomes, as well as in endosomes, but is detectable only at low levels in the plasma membrane and the peripheral tubular endosomal compartment. Our findings suggest that lysosomal membrane proteins are first delivered to endosomes, which are transformed into lysosomes not only by the acquisition of lysosomal hydrolases but also by a remodeling or maturation process that affects the protein composition of their limiting membranes.

## Materials and Methods

Most reagents were purchased from Sigma Chemical Co. (St. Louis, MO), or from VWR Scientific (San Francisco, CA). [<sup>35</sup>S]Methionine (1,000 Ci/mmol), [<sup>35</sup>S]cysteine (1,000 Ci/mmol), [<sup>32</sup>P]inorganic phosphate (1 Ci/mmol), 2-[<sup>3</sup>H]mannose (25 Ci/mmol), and Bolton-Hunter reagent (2,000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Trasylol was obtained from Mobay Chemical Corp. (Pittsburgh, PA); tunicamycin from Calbiochem-Behring Corp. (La Jolla, CA); sheep anti-mouse IgG, rhodamine-conjugated goat anti-mouse IgG, and fluorescein-conjugated goat anti-rabbit IgG from Cappel Laboratories (Cochranville, PA); endo- $\beta$ -*N*-acetylglucosaminidase H (endo H) and neuraminidase from Boehringer-Mannheim Biochemicals (Indianapolis, IN); and endo- $\alpha$ -*N*-acetyl-galactosaminidase (*O*-glycanase) was purchased from Genzyme Corp. (Boston, MA). DME, RPMI 1640, Kaighn's modified F12, and horse, fetal calf, and calf sera were purchased from Irvine Scientific (Santa Ana, CA). Sprague-Dawley female rats (150–200 g) were obtained from Taconic Farms, Inc. (Germantown, NY) and Balb/c mice from the Jackson Laboratory (Bar Harbor, ME).

## Isolation of Lysosomes

A highly purified fraction of lysosomes was prepared from rat liver by the method of Wattiaux et al. (1978) and the luminal content and peripheral proteins were removed by alkaline washing (Fujiki et al., 1982). Trasylol (100 U/ml), phenylmethylsulfonyl fluoride (0.1 mM), leupeptin (0.5  $\mu$ g/ml), and chymostatin (0.5  $\mu$ g/ml) were added to all solutions.

## Monoclonal Antibody Production

For the production of monoclonal antibodies a fraction of alkaline-washed lysosomal membranes containing 100  $\mu$ g protein was dissolved in 0.5 ml PBS containing 0.5% SDS and 1%  $\beta$ -mercaptoethanol, boiled for 2–3 min, and emulsified with Freund's complete adjuvant. Female Balb/c mice received an initial intraperitoneal/subcutaneous injection of 100  $\mu$ g antigen with subsequent boosts, given every 4–6 wk for a period of 8 mo, containing the antigen (100  $\mu$ g) emulsified with incomplete Freund's adjuvant. 3 d after the final boost, the animals were killed, their spleens removed, dissociated, and a cell suspension prepared for fusion with P<sub>3</sub>U<sub>1</sub> myeloma cells according to the procedure of the St. Groth and Scheidegger (1980). Cells were plated in 96-well microtiter dishes and hybridomas secreting monoclonal antibodies recognizing lysosomal membrane proteins were identified by ELISA (Cobbold and Waldmann, 1981). Polyclonal antisera against  $\beta$ -glucuronidase, cathepsin D, or ribophorin I were prepared as previously described (Rosenfeld et al., 1982; Marcantonio et al., 1982).

## Cell Culture

The rat hepatocyte cell line, clone 9, and the hepatoma-derived Fu5C8 line were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere in Kaighn's modified F12 medium supplemented with 5% fetal calf serum and antibiotics. NRK-52E cells (obtained from the American Type Culture Collection, Rockville, MD) were cultured at 37°C in a 10% CO<sub>2</sub> atmosphere in DME supplemented with 5% calf serum and antibiotics.

## Immunofluorescence Microscopy

Clone 9 cells were plated on round glass coverslips (#1, 12 mm) and fixed with 2% formaldehyde in PBS for 30 min. Some coverslips were incubated with chloroquine (50 μM) 2–3 h before fixation. After permeabilization with 0.2% Triton X-100, the coverslips were incubated overnight at 4°C with undiluted hybridoma culture fluid containing monoclonal antibody, washed in PBS containing 0.2% gelatin, and incubated with rhodamine-conjugated goat anti-mouse IgG. For double labeling, coverslips were subsequently incubated overnight at 4°C with either rabbit anti-rat-β-glucuronidase or rabbit anti-rat-cathepsin D (at 1:2,000 dilution), washed, and then incubated with fluorescein-conjugated goat anti-rabbit IgG. Coverslips were mounted in Gelvatol (Monsanto Co., St. Louis, MO) and examined with a Leitz Orthoplan Microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with a Wild camera (Wild Heerbrugg Instruments, Inc., Farmingdale, NY).

## Labeling and Preparation of Cell Extracts

Monolayers of NRK or Fu5C8 cells were grown in 35-mm dishes to a density of ~5 × 10<sup>6</sup> cells per dish. Before labeling, the cultures were rinsed with cysteine-free RPMI and incubated in this medium for 1 h to allow for the depletion of the intracellular cysteine pool. They were then incubated with [<sup>35</sup>S]cysteine (125 μCi/ml) in cysteine-free medium supplemented with 5% dialyzed fetal calf serum for various times, and when necessary, the labeling period was followed by a chase in complete medium. In experiments using tunicamycin, confluent cultures were incubated with this drug (4 μg/ml) for 1 h in complete medium and for 1 h in cysteine-free medium containing tunicamycin before adding [<sup>35</sup>S]cysteine (125 μCi/ml) and tunicamycin. After labeling, monolayers were washed twice with Moscona's PBS (Moscona, 1961) and scraped off the dish into an SDS solution (20 mM Tris-HCl, pH 7.4, 2% SDS). The samples were sonicated 10–20 s in a Sonifier cell disruptor equipped with a microtip (Heat Systems-Ultrasonics, Inc., Farmingdale, NY), boiled for 2 min and centrifuged (2 min at 15,000 g) in an Eppendorf table top centrifuge (model 5412; Brinkmann Instruments Co., Westbury, NY) to remove insolubilized material. The supernatant was diluted fourfold with 50 mM Tris-HCl (pH 8.0), 190 mM NaCl, 6 mM EDTA, 2.5% Triton X-100, 0.02% NaN<sub>3</sub>, and 100 U/ml Trasylol (Solution A).

For labeling oligosaccharides, NRK cells preincubated for 20 min in DME glucose-free medium were labeled for 18 h at 37°C in DME supplemented with 0.25 mM glucose and [<sup>3</sup>H]mannose (100 μCi/ml) and then chased for 1 h in isotope-free medium. [<sup>32</sup>P]Inorganic phosphate labeling was carried out as described (Lemansky et al., 1985).

## Iodination of Cell Fractions

Purified plasma membranes (Croze and Morre, 1984) and lysosomes were extracted with alkali, sedimented, and resuspended in PBS. The resuspended samples, containing 500 μg of protein, were iodinated with 250 μCi of Bolton-Hunter reagent as described by the manufacturer (New England Nuclear). Unincorporated iodine was removed by G-25 Sephadex column chromatography and samples containing 50,000 cpm of trichloroacetic acid-precipitable radioactivity were analyzed by immunoprecipitation and PAGE, as described below.

## Immunoprecipitation

Cell extracts were incubated overnight at 4°C with either undiluted culture medium from a hybridoma or with IgG (5 μg/ml) purified from that medium by protein A-Sepharose affinity chromatography (Hjelm et al., 1972). After incubation, immunocomplexes were recovered using protein A-Sepharose CL-4B beads as previously described (Rosenfeld et al., 1982). For reduction-alkylation, immunoprecipitated material was treated with 100 mM DTT before incubation with 30 mM iodoacetamide for 1 h on ice. Samples were dialyzed overnight against PBS, concentrated in a Speed Vac (Savant Instruments, Inc., Hicksville, NY) and solubilized in electrophoresis buffer (Laemmli, 1970) before analysis by SDS-PAGE.

## Endoglycosidase Digestion

The protein recovered by immunoprecipitation from [<sup>35</sup>S]cysteine-labeled NRK cells was eluted from protein A-Sepharose beads by boiling in 0.5% SDS for 4 min. For endo H digestion, the supernatants were diluted with 1 M citrate-phosphate buffer (pH 5.0) to a final concentration of 50 mM, adjusted to 0.2% SDS, and incubated overnight at 37°C with 0.2 U/ml of the enzyme. For O-glycanase digestion, immunoprecipitated protein was eluted with 0.5% SDS, 1.0% NP-40, 25 mM EDTA, 1.0% β-mercaptoethanol, 100 mM NaP (pH 6.1), and incubated for 1 h at 37°C with neuraminidase (1 U/ml) to remove sialic acid. O-glycanase (4 mU) was then added and incubation continued overnight at 37°C. All samples were adjusted to 2.0% SDS in Laemmli's buffer for analysis by SDS-PAGE.

## Subcellular Fractionation

Fu5C8 cells grown to confluency in 60-mm dishes, were labeled for 1 h with [<sup>35</sup>S]cysteine, and chased in complete medium for 4 h. The cells were scraped off the dishes, recovered by centrifugation (1,000 g for 10 min), and resuspended in 1 ml of 0.25 M sucrose. The resulting suspension was subjected to nitrogen cavitation (500 psi for 5 min at 4°C) and centrifuged for 3 min at 750 g to remove unbroken cells and nuclei. 1 ml of the postnuclear supernatant was mixed with 9 ml of 22% isosmotic Percoll in a Ti 50 high speed ultracentrifuge tube (Beckman Instruments, Inc., Palo Alto, CA). After centrifugation (100,000 g at 4°C for 25 min), fractions (0.3 ml) were collected and either used directly for analysis or frozen at -20°C. Aliquots were used to measure the activity of marker enzymes β-hexosaminidase (Lippincott-Schwartz and Fambrough, 1986), 5'-nucleotidase (Croze and Morre, 1984), α-glucosidase (Michael and Kornfeld, 1980), or for immunoprecipitation with antiendolyn-78 or antiribophorin I antibody (Rosenfeld et al., 1984). Immunoprecipitates were analyzed by PAGE and fluorography and fluorographs were scanned with an LKB Instruments, Inc. (Gaithersburg, MD) ultrascan densitometer.

## Receptor-mediated Endocytosis of α-2-Macroglobulin

NRK cells were incubated at 4°C for 60 min in serum-free media containing α-2-macroglobulin conjugated to 20-nm gold particles. The temperature was then raised to either 18.5°C for 30, 45, and 180 min, or to 37°C for 5, 10, 20, 90, and 180 min. Samples were then washed with ice cold PBS and fixed for conventional EM or for immunolabeling on frozen thin sections as described below.

## Immunogold Labeling of Frozen Thin Sections

Pellets of NRK or clone 9 cells were fixed in formaldehyde (2–3.5%) / glutaraldehyde (0.5–2%) mixtures in 0.1 M sodium cacodylate (pH 7.4) buffer. Frozen thin sections (Tokuyasu, 1980), were processed for single labeling of endolyn-78 using the monoclonal antibody 2C5, followed by sheep anti-mouse IgG conjugated to 10-nm colloidal gold particles. In double-labeling experiments, after labeling for 2C5, the samples received either rabbit anti-rat β-glucuronidase IgG or rabbit anti-MPR IgG, followed by protein A-gold (5 nm). Sections were postfixed in glutaraldehyde and osmium tetroxide, positively stained with aqueous uranyl acetate (Ivanov, I. E., manuscript in preparation), embedded in LR White resin (London Resin Co., Ltd., Rasingstoke, Hampshire, England) as described by Keller et al. (1984), and observed at 80 kV with a Phillips EM 301 electron microscope.

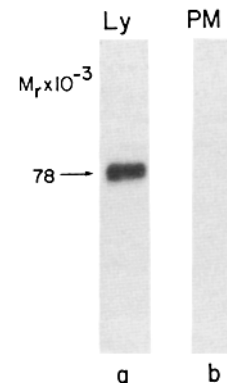
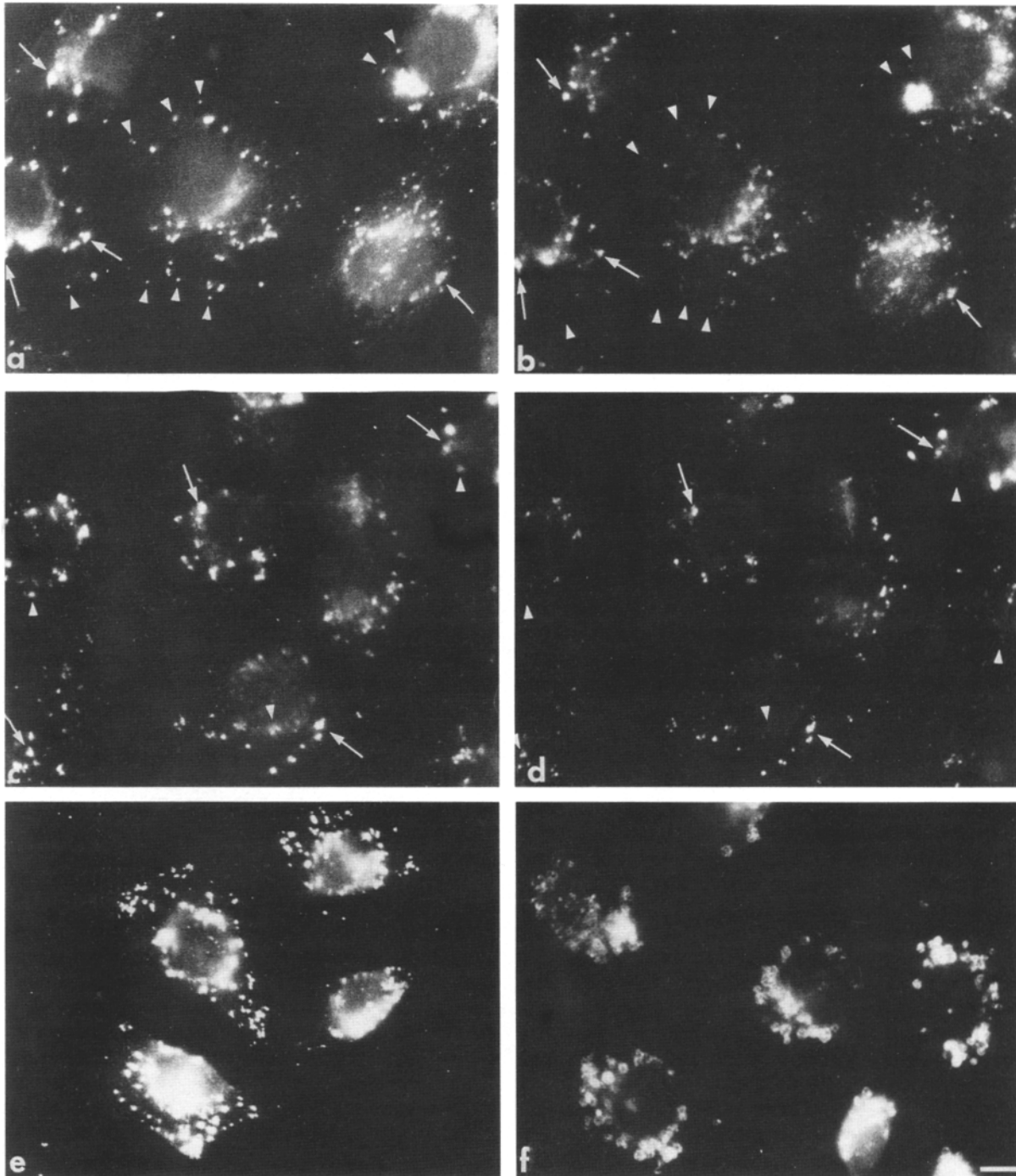


Figure 1. The monoclonal antibody to 2C5 recognizes a 78-kD protein present in lysosomes but undetectable in a plasma membrane fraction. Alkaline-washed lysosomal membranes (lane a) and a plasma membrane fraction (lane b) isolated from rat liver were dissolved in PBS and the samples were iodinated with <sup>125</sup>I-Bolton-Hunter reagent before immunoprecipitation with the monoclonal antibody 2C5. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography.



**Figure 2.** Endolyn-78 is present in vesicular structures that contain lysosomal hydrolases and swell after chloroquine treatment. (a-d) Clone 9 rat hepatocytes were processed for double-labeling immunofluorescence with monoclonal antibody 2C5 (a and c) and either rabbit anti-rat  $\beta$ -glucuronidase (b) or rabbit anti-rat cathepsin D (d). Affinity-purified rhodamine-conjugated, goat anti-mouse IgG (a and c) or fluorescein-conjugated, goat anti-rabbit IgG (b and d) were used as secondary antibodies. Many lysosomes, identified by their labeling with anti- $\beta$ -glucuronidase or anti-cathepsin D (b and d, arrows), are also labeled with the monoclonal antibody 2C5 (a and c, arrows). However, the monoclonal antibody also labels many vesicles (presumptive endosomes) (a and c, arrowheads) that are not labeled or are only very weakly labeled with antibodies to either lysosomal hydrolase (b and d, arrowheads). (e and f) Rat liver clone 9 cells were incubated in the absence (e) or presence (f) of chloroquine ( $50 \mu\text{M}$  for 2 h) and processed for immunofluorescence using the 2C5 monoclonal antibody. Bar,  $10 \mu\text{m}$ .

## Results

Supernatants of hybridomas generated by immunization of mice with a purified lysosomal membrane fraction were screened by ELISA for antibodies that recognize compo-

nents of the lysosomal membrane. This yielded, in addition to antibodies that recognized a 120-kD protein (not shown) which probably corresponds to the lgp 120 described by other investigators (Lewis et al., 1985), several monoclonal antibodies that recognized a protein of 78 kD that appeared

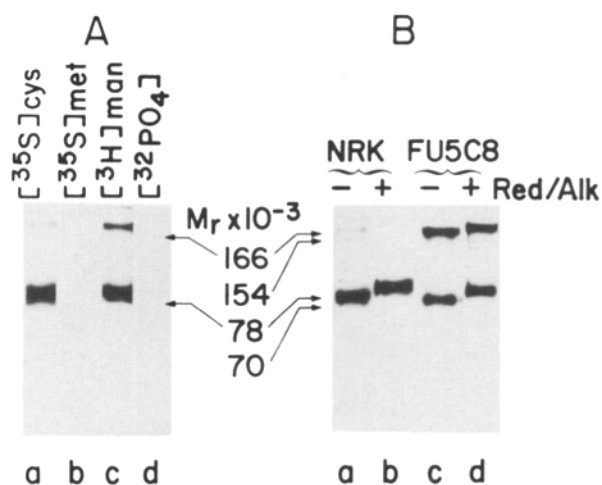
to be distinct from all other previously described lysosomal membrane proteins (see Barriocanal et al., 1986). One of the monoclonal antibodies (2C5) that recognized the 78-kD protein (Fig. 1), produced at high titers in cell culture, was selected for further studies. This antibody recognized the antigen in assays that are carried out under denaturing conditions, such as Western blotting and immunoprecipitation procedures, as well as in immunofluorescence with lightly fixed cultured cells, a test in which the native configuration of the protein is likely to be conserved. As shown in Fig. 1, the antigen recognized by this antibody is found in lysosomes but is not detectable in a plasma membrane fraction.

The lysosomal location of the antigen recognized by the 2C5 antibody was demonstrated by double-labeling immunofluorescence using rabbit antibodies to the lysosomal hydrolase  $\beta$ -glucuronidase or cathepsin D to label lysosomes. In a cell line of hepatocyte origin (clone 9), the 2C5 monoclonal antibody labeled numerous cytoplasmic vesicles and vacuoles which were also labeled with the antibodies to the lysosomal hydrolases (Fig. 2, a-d). However, the monoclonal antibody also labeled other vesicular structures distributed throughout the cytoplasm, which did not contain detectable levels of the lysosomal hydrolases and are, therefore, likely to represent endosomes. That a large proportion of the antigen recognized by the 2C5 monoclonal antibody was, indeed, present in the membranes of lysosomes, and probably also in the membranes of endosomes, was demonstrated by the fact that in cells treated with chloroquine, a drug that increases intralysosomal and endosomal pH and leads to the swelling of these organelles (Brown et al., 1986), the monoclonal antibody labeled intensely the periphery of the swollen vacuoles (see Fig. 2, e and f). The localization of the antigen recognized by 2C5 in lysosomes and endosomes was definitively established by immunoelectron microscopy experiments (see below).

We propose that proteins present in both the endosomal and lysosomal membrane compartments be designated endolyns (*endosomal and lysosomal proteins*) and, therefore, will refer to the 78-kD antigen recognized by the monoclonal antibody 2C5 as endolyn-78.

### Biochemical Characterization and Biosynthesis of Endolyn-78

To further characterize endolyn-78, NRK cells were metabolically labeled with various radiolabeled precursors, and immunoprecipitates obtained from cell lysates with the 2C5 monoclonal antibody were analyzed by SDS-PAGE. Endolyn-78 could only be labeled in cells incubated with [<sup>35</sup>S]cysteine or [<sup>3</sup>H]mannose but not in cells incubated with [<sup>35</sup>S]methionine or [<sup>32</sup>P]inorganic phosphate (Fig. 3 A). In contrast, ribophorins I and II,  $\beta$ -glucuronidase, and a microsomal esterase, all labeled much more intensely with methionine than with cysteine (not shown). Thus, endolyn-78 is a glycoprotein that appears to lack phosphate and has a high cysteine to methionine ratio. Although, when disulfide bonds in the immunoprecipitated proteins were reduced with DTT or mercaptoethanol, the antigen migrated in SDS gel electrophoresis as a band of 78 kD, a second band of ~166 kD was also frequently observed (Fig. 3 A, a and c). These 78- and 166-kD bands probably represent monomeric and dimeric forms of the same protein since both were again observed when the material in either band was excised from the

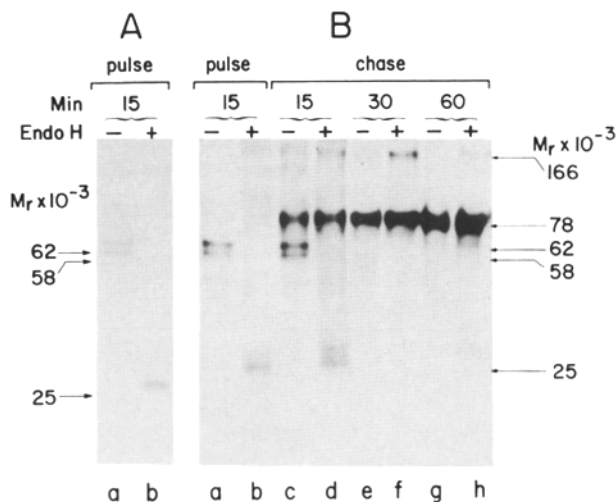


**Figure 3.** Endolyn-78 is a cysteine-rich glycoprotein with a low methionine content that exists in monomeric and dimeric forms. (A) NRK cells were incubated with [<sup>35</sup>S]cysteine (lane a), [<sup>35</sup>S]methionine (lane b), [<sup>32</sup>P]inorganic phosphate (lane d) for 4 h, or [<sup>3</sup>H]mannose (lane c) for 18 h, and endolyn-78 was immunoprecipitated from the detergent-solubilized cell lysates. Immunoprecipitates were analyzed directly by SDS-PAGE and fluorography. (B) NRK (lanes a and b) or Fu5C8 (lanes c and d) cells were labeled with [<sup>35</sup>S]cysteine for 4 h and endolyn-78 was immunoprecipitated from detergent-treated lysates. The immunoprecipitates were either analyzed directly (lanes a and c) by SDS-PAGE followed by fluorography or were treated with 100 mM DTT and alkylated with iodoacetamide before analysis (lanes b and d). Note that the proportion of monomeric to dimeric forms recovered from the two cell types is different.

gel, incubated at 37°C, and rerun under the same conditions. When, after reduction, the immunoprecipitated protein was alkylated to prevent reformation of any disulfide bonds, the relative amounts of the monomeric and dimeric forms were not affected but the electrophoretic mobility of each form was significantly reduced (Fig. 3 B).

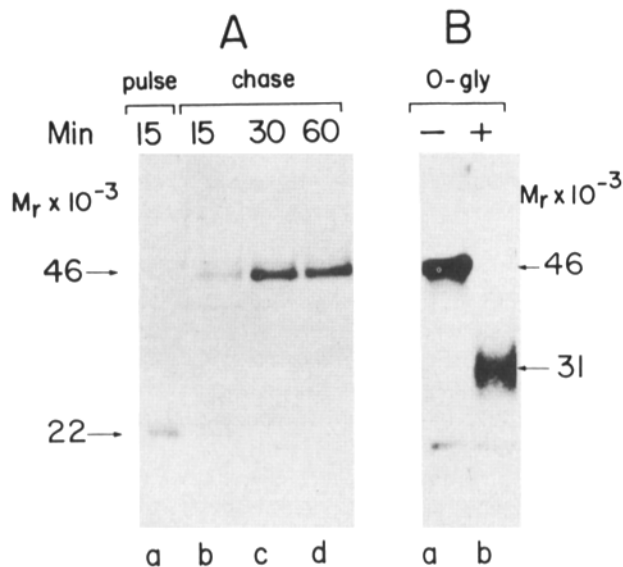
Its efficient labeling with [<sup>3</sup>H]mannose, together with the known fact that other lysosomal membrane proteins are rich in carbohydrate (e.g., Lewis et al., 1985; Barriocanal et al., 1986; Fambrough et al., 1988), prompted us to undertake a more detailed analysis of the glycoprotein nature of endolyn-78. When cultured cells were pulse-labeled for 15 min with [<sup>35</sup>S]cysteine, immunoprecipitation yielded two closely migrating products of ~58 and 62 kD (Fig. 4 A). These were converted with a half life of <15 min to the mature monomeric 78-kD and dimeric 166-kD forms (Fig. 4 B). The 58- and 62-kD precursors contain large amounts of high mannose oligosaccharide chains, since endo H treatment converted them to a single polypeptide of ~25 kD (Fig. 4, A and B). This suggests that both precursors only differ in the extent of their N-glycosylation.

The extensive glycosylation of endolyn-78 was also apparent when the labeled protein was recovered from cells treated with tunicamycin, an inhibitor that blocks the acquisition of N-linked oligosaccharides. After a 15-min pulse with [<sup>35</sup>S]cysteine, a labeled polypeptide of 22 kD was immunoprecipitated by the 2C5 antibody (Fig. 5 A). The slight molecular mass difference between this polypeptide (22 kD) and that generated by endo H treatment from the pulse-labeled poly-



**Figure 4.** Recently synthesized endolyn-78 contains large amounts of N-linked oligosaccharides. (A) Immunoprecipitates obtained with the 2C5 antibody from NRK cells labeled for 15 min with [<sup>35</sup>S]cysteine were analyzed directly (lane a) or after treatment with endo H (lane b) by SDS-PAGE. (B) After labeling for 15 min, cultures were chased for the times indicated. Immunoprecipitates from cell lysates were analyzed as in A.

peptide produced in control cells (25 kD, Fig. 4) can be attributed to the presence in the latter of the proximal asparagine-linked *N*-acetylglucosamine moieties. During a chase period, the 22-kD polypeptide was converted with a  $t_{1/2} < 15$  min into a protein of 46 kD (Fig. 5 A). This conversion results from the acquisition of O-linked oligosaccharide chains,



**Figure 5.** Endolyn-78 contains O-linked as well as N-linked oligosaccharides. (A) NRK cells were pretreated with tunicamycin and labeled with [<sup>35</sup>S]cysteine for 15 min, followed by a chase for the times indicated, with inhibitor still present. Immunoprecipitates obtained from detergent lysates were analyzed by SDS-PAGE and fluorography. (B) NRK cells pretreated with tunicamycin were labeled with [<sup>35</sup>S]cysteine for 4 h in the presence of the inhibitor. Immunoprecipitated endolyn-78 was analyzed by SDS-PAGE before (lane a) or after (lane b) treatment with *O*-glycanase.

since treatment of the 46-kD protein with *O*-glycanase reduced its electrophoretic mobility to 31 kD (Fig. 5 B). The difference in relative molecular mass between this polypeptide and that produced during a brief pulse in cells treated with tunicamycin (22 kD) is likely to be due to the presence in the former of residual O-linked oligosaccharides, such as GlcNAc $\beta$ (1-3)-GalNAc-Ser/Thr or GalNAc-Ser/Thr, that are not removed or are inefficiently removed, respectively, by the *O*-glycanase (Lamblin et al., 1984).

Based on the difference in electrophoretic mobility between the product that accumulates in the presence of tunicamycin and the high mannose form of endolyn-78 found in briefly labeled normal cells, it can be estimated (Fambrough et al., 1988) that the protein contains  $\sim 12$ –13 asparagine-linked oligosaccharides. It should be noted that these oligosaccharides appear to play a role in the dimerization of endolyn-78 since the dimeric form was not observed in extracts from tunicamycin-treated cells (Fig. 5).

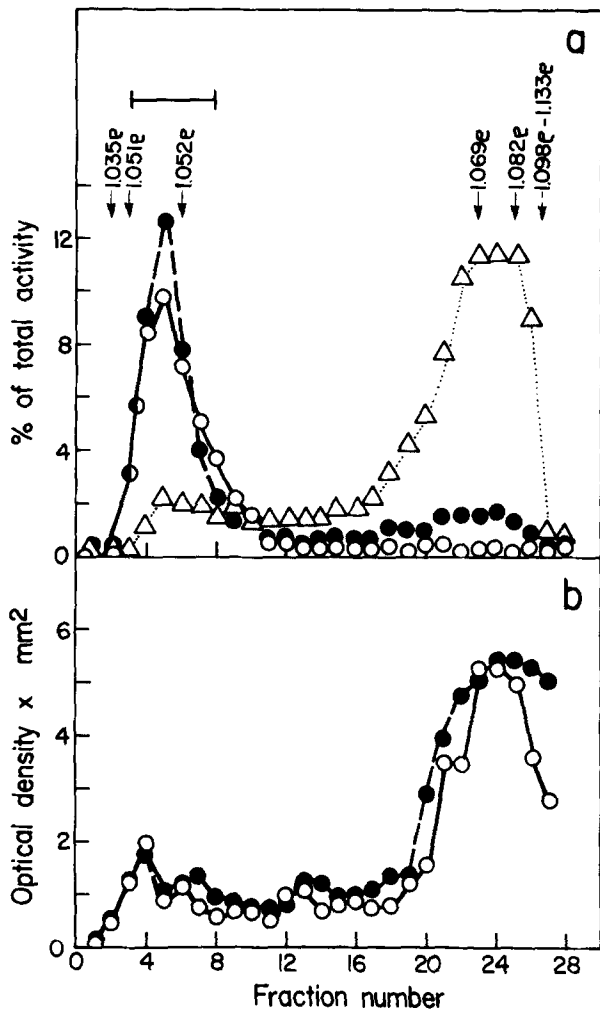
The [<sup>3</sup>H]mannose-labeled oligosaccharide chains present in glycopeptides obtained by pronase digestion of the monomeric and dimeric forms of mature endolyn-78 were analyzed by concanavalin A-Sepharose chromatography and in both cases the predominant oligosaccharide structures were of the tri- and tetraantennary type (Krusius et al., 1976), with no high mannose oligosaccharide chains (results not shown).

#### **Recovery of Endolyn-78 in a Lysosomal Fraction: Addition of N-Linked Oligosaccharides Is not Required for Targeting the Protein to Lysosomes**

The subcellular distribution of endolyn-78 was examined (Fig. 6 b) by isopycnic centrifugation in Percoll gradients, to separate lysosomes from endosomes and other organelles in extracts obtained from cells labeled for 1 h with [<sup>35</sup>S]cysteine and chased for 4 h. The distribution of labeled endolyn-78 was compared with that of early endosomes, labeled with Lucifer Yellow during a 5-min incubation *in vivo*, and of several marker enzymes characteristic of the plasma membrane (5'-nucleotidase), endoplasmic reticulum ( $\alpha$ -glucosidase), and lysosomes ( $\beta$ -hexosaminidase; Fig. 6 a). The lysosomal fraction, recovered at a density of 1.069–1.082 g/ml, contained the bulk of the  $\beta$ -hexosaminidase activity and 50% of the labeled immunoprecipitable endolyn. The remainder of the labeled endolyn-78 was distributed throughout the gradient, with a small peak (12% of the total) found at a density of 1.035–1.052 g/ml. This region contained the endosomes that had recently ingested Lucifer Yellow, as well as the plasma membrane and endoplasmic reticulum fractions. To determine if N-linked oligosaccharides play a role in the targeting of endolyn-78 to lysosomes, an extract from cells labeled in a similar manner but in the presence of tunicamycin was fractionated in a Percoll gradient (Fig. 6 b). It was found that the form of endolyn that lacks N-linked oligosaccharides had the same distribution as the normal product. It, therefore, can be concluded that the sorting mechanism that addresses endolyn-78 to lysosomes does not rely on the presence of the mannose-6-phosphate marker or other features of the N-linked oligosaccharides.

#### **Electron Microscopic Localization of Endolyn-78**

The nature of the cytoplasmic structures containing endolyn-78 was examined in a cell line of hepatocyte origin (clone 9)



**Figure 6.** Recovery of endolyn-78 in a lysosomal fraction: tunicamycin treatment does not prevent the incorporation of newly synthesized endolyn-78 into lysosomes. (a) Lysates of Fu5C8 cells were fractionated by centrifugation in isoosmotic Percoll gradients and 0.3-ml fractions were collected for analysis. The distributions of 5'-nucleotidase (●),  $\alpha$ -glucosidase (○), and  $\beta$ -hexosaminidase ( $\Delta$ ), as markers for the plasma membrane, ER, and lysosomes, respectively, are expressed as percent of total activity. The region containing endosomes, labeled *in vivo* by incubating the cells with Lucifer Yellow for 5 min immediately before cell fractionation, is marked with a line segment (—|—). (b) Fu5C8 cells were labeled with [ $^{35}$ S]cysteine for 1 h in the presence (●) or absence (○) of tunicamycin and incubated for 4 h in chase medium. Cell lysates were fractionated as in (a) and the radioactivity in endolyn-78, recovered by immunoprecipitation from each fraction, was determined by densitometric scanning of the exposed x-ray film. The isopycnic density (g/ml) across the gradient was calculated from the position of marker beads.

after double labeling with rabbit antibodies to the lysosomal hydrolase  $\beta$ -glucuronidase and the monoclonal antibody 2C5 (Fig. 7). The distribution of both antigens is given in Table I, which shows that 48% of the  $\beta$ -glucuronidase and 35% of the endolyn-78 were found in morphologically identifiable lysosomes. Most of these structures were labeled with both antibodies but, whereas  $\beta$ -glucuronidase was mainly located in the luminal content, endolyn-78 was found on the inner

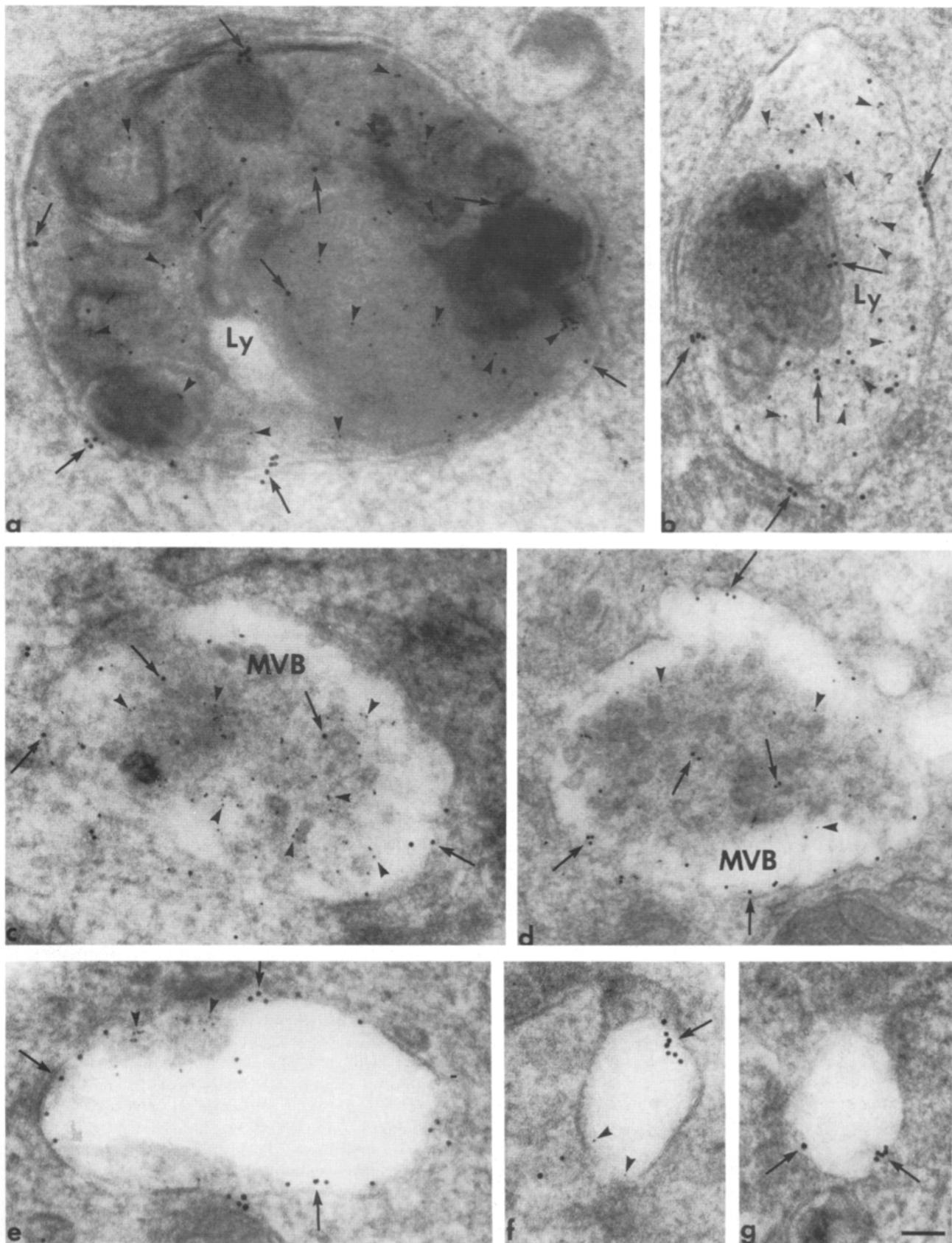
aspect of the limiting membrane, as well as in the luminal content (Fig. 7, *a* and *b*). Vacuolar endosomes and vacuoles that because of their content of internal membrane vesicles could be designated as multivesicular bodies (MVBs), contained  $\beta$ -glucuronidase in varying, but generally much lesser amounts (11% of the total, see Table I) than lysosomes. These structures also contained endolyn-78 (23% of the total, Table I), which in the MVBs was predominantly on the outer aspects of the membranes of the interiorized vesicles (Fig. 7, *c* and *d*). Many small and medium-sized cytoplasmic vacuoles with clear lumen, presumably vacuolar endosomes, were devoid of  $\beta$ -glucuronidase (Fig. 7 *g*) or contained low amounts of this lysosomal hydrolase (Fig. 7, *e* and *f*) but contained endolyn-78, in agreement with the observations made by double-labeling immunofluorescence (Fig. 2). Significant amounts of  $\beta$ -glucuronidase and endolyn ( $\sim 35\%$  of each, Table I) were also found in tubulovesicular elements near the Golgi apparatus and throughout the cytoplasm, but the two labels were rarely found in the same elements (not shown).

#### **Presence of Endolyn-78 in Endosomes: Colocalization with Internalized $\alpha$ -2-Macroglobulin**

To directly confirm the endosomal nature of the nonlysosomal vacuolar structures containing endolyn-78, and to establish the distribution of this protein within the structurally and functionally complex endosomal compartment, the distribution of endolyn-78 was correlated with that of the endocytic marker  $\alpha$ -2-macroglobulin (Table I) in NRK cells, which express the  $\alpha$ -2-macroglobulin receptor (Pastan et al., 1977).

The structures labeled by  $\alpha$ -2-macroglobulin-gold conjugates at various times after the administration of this marker are shown in Fig. 8. Within a few minutes after interiorization in coated pits (Fig. 8 *a*), the ligand was found within branching narrow tubules (40–80 nm) (Fig. 8, *b* and *c*) or small vacuoles (0.3  $\mu$ m) (Fig. 8, *c* and *d*), which presumably correspond to the most peripheral region of the endosomal system (see Helenius et al., 1983; Hopkins, 1986). Soon thereafter (10 min), the marker was also found in larger (0.5–0.8- $\mu$ m) vacuoles, which were usually empty and were designated vacuolar endosomes. Some structures of this type contained a few internalized membrane vesicles and may be regarded as incipient MVBs (Fig. 8 *e*). At these early stages the ligand remained closely apposed to the inner aspect of the limiting membranes or to the surface of the interiorized vesicles. At 20 min after administration,  $\alpha$ -2-macroglobulin began to accumulate in the lumen of the vacuoles which, to a varying extent, were filled with small vesicles and tubules, as well as with more complex membranous structures and some amorphous material that may represent the result of autophagy (Fig. 8, *f* and *g*). Because of their time of labeling and location within the cell, and because the ligand was already released from its site of attachment on the membrane, these structures may correspond to juxtannuclear or "late endosomes" (Helenius et al., 1983; Hopkins, 1986). Because of their characteristic morphology, however, many of them could also be described as MVBs. At later times,  $\alpha$ -2-macroglobulin appeared in large membrane-bounded bodies (0.5–2.0  $\mu$ m) with the dense and heterogeneous luminal content characteristic of secondary lysosomes (Fig. 8, *g* and *h*).

The distribution of endolyn-78 in structures containing en-



**Figure 7.** Colocalization of endolyn-78 and  $\beta$ -glucuronidase in lysosomes and endosomes of clone 9 cells. Endolyn-78 was immunolocalized with 10-nm gold particles (*arrows*) and  $\beta$ -glucuronidase with 5-nm gold particles (*arrowheads*). (*a* and *b*) Typical lysosomes with a heterogeneous content that includes vesicles, membrane whorls, and multilamellar structures are labeled for endolyn-78 (*arrows*) in the limiting membranes, as well as internally.  $\beta$ -glucuronidase (*arrowheads*) is found throughout the luminal content of lysosomes. (*c* and *d*) In MVBs



Table I. Distribution of Endolyn-78,  $\beta$ -Glucuronidase, MPR, and Endocytosed  $\alpha$ -2-Macroglobulin in Cultured Cells\*

Cell	Marker	Temperature	Plasma membrane	TGN + cytoplasmic vesicles	Tubular endosomes	Vacuolar endosomes + MVB	MVB/Ly + lysosomes	Total
		$^{\circ}\text{C}$	%	%	%	%	%	<i>n</i>
Clone 9	Endolyn-78	37.0	5	37	‡	23	35	1,998
	$\beta$ -glucuronidase	37.0	5	36	‡	11	48	1,463
NRK	Endolyn-78	37.0	6	22	3	21	48	646
		18.5	16	27	2	19	36	607
	MPR	18.5	6	56	0	30	8	718
	$\alpha$ -2-macroglobulin-gold conjugates	18.5	31	0	36	32	<1	376
		37.0	3	0	8	24	65	432

\* NRK cells were incubated with  $\alpha$ -2-macroglobulin-gold conjugates (20 nm) for 3 h at either 37 or 18.5°C before fixation. Clone 9 cells were fixed without prior incubation with  $\alpha$ -2-macroglobulin. Cells were processed for immunogold labeling on frozen thin sections to localize the different markers. For each marker, gold particles were counted over randomly selected areas and the percentage in each subcellular component was tabulated.

‡ The very low percentages found in this compartment are included in the TGN + cytoplasmic vesicles figures.

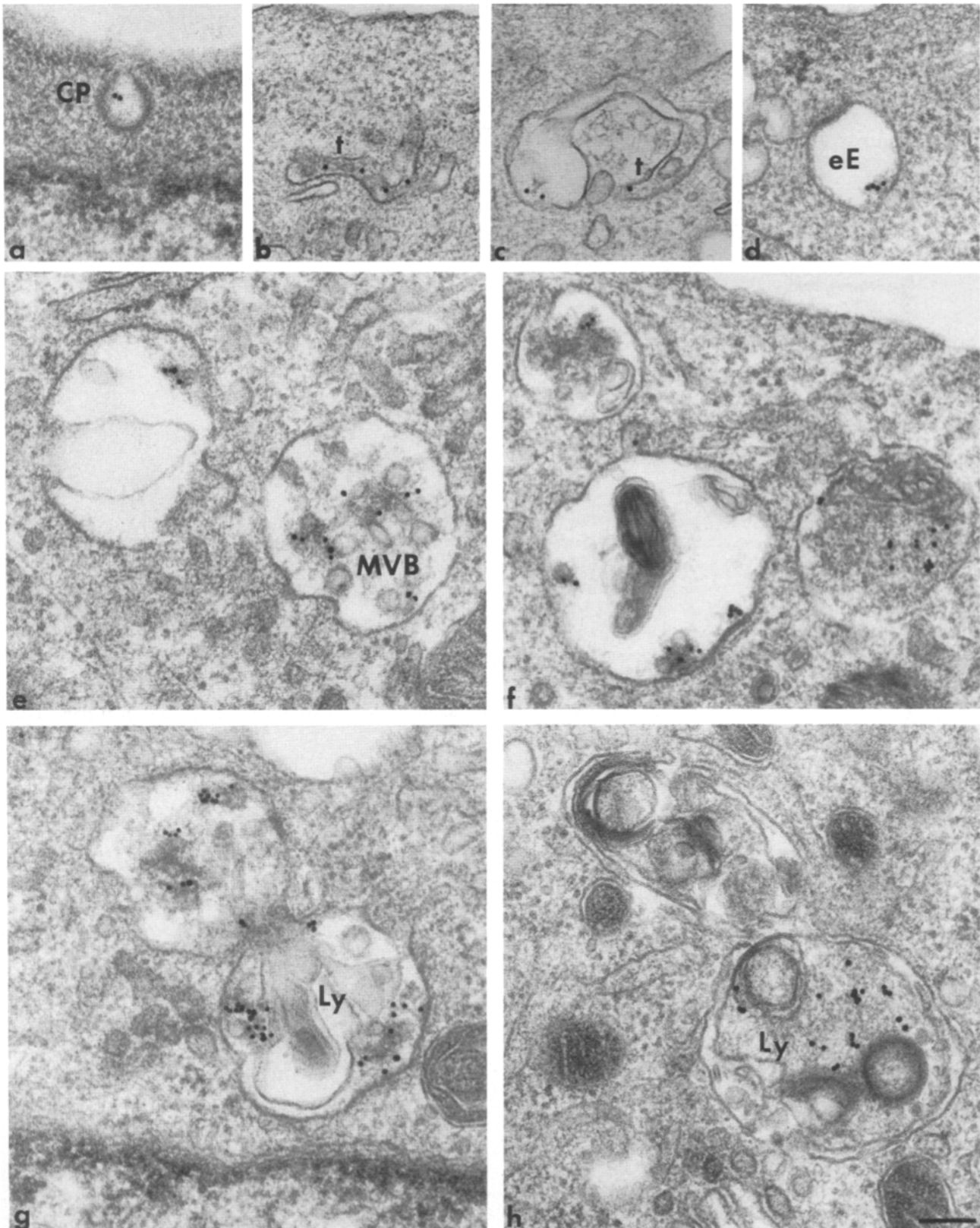
docytosed  $\alpha$ -2-macroglobulin-gold conjugates (20 nm) was determined in NRK cells that had been incubated with the endocytic marker for 20 min, 90 min, or 3 h and were processed for immunogold labeling using the 2C5 antibody, which was localized with a second antibody conjugated to 10-nm gold particles (Fig. 9). It was observed that the coated vesicles through which  $\alpha$ -2-macroglobulin enters the cell (Fig. 9 a), and the small peripheral tubular elements in which it was found soon thereafter (not shown, but see Fig. 10 that illustrates endocytosis taking place at 18.5°C), contained very low amounts of endolyn-78 (Table I). Higher amounts of this antigen were found, however, in larger vacuolar endosomes in which the  $\alpha$ -2-macroglobulin was still associated with the inner aspect of the vacuolar surface (Fig. 9, b and c). MVBs containing  $\alpha$ -2-macroglobulin also contained substantial amounts of endolyn-78 in the surrounding membranes, as well as in the interiorized membrane vesicles and tubules (Fig. 9, d-f). As expected, in secondary lysosomes, where at later times  $\alpha$ -2-macroglobulin appeared in large quantities, endolyn was present in both the peripheral membrane and within the lumen (Fig. 9, f and g).

Endosomes can be operationally defined as those vacuolar structures which receive and accumulate an endocytic marker, such as  $\alpha$ -2-macroglobulin, at low temperatures (18.5°C) that do not permit its entrance into lysosomes (Dunn et al., 1980). We found that in NRK cells incubated for 45 min at 18.5°C with  $\alpha$ -2-macroglobulin, 90% of the endosomal structures that had received the tracer were tubules near the cell surface, which contained very low amounts of endolyn-78 (Fig. 10 a). After a longer incubation (3 h, Fig. 10, b-e), however, typical vacuolar endosomes and MVBs that were

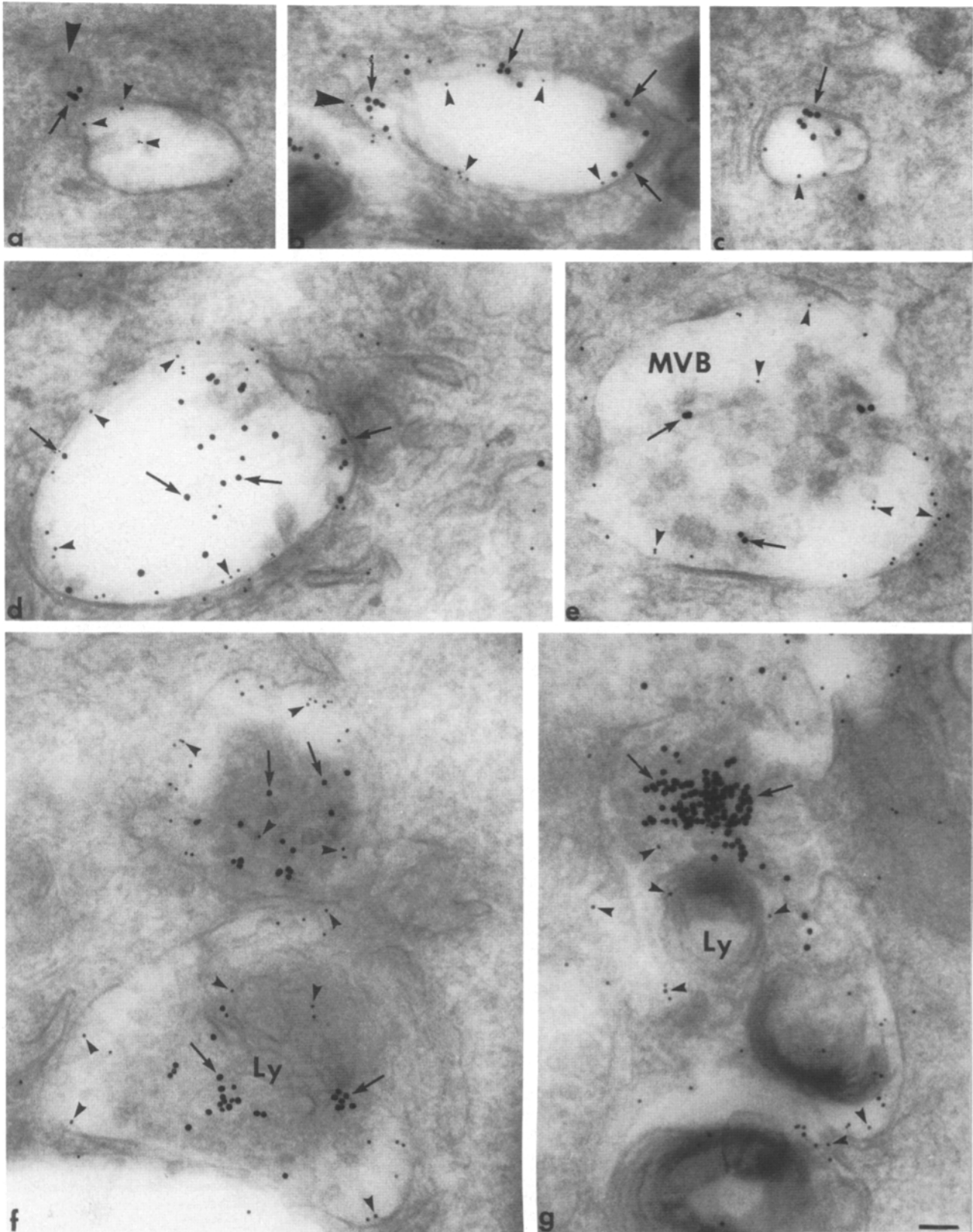
located deeper in the cell and in aggregate represented ~50% of the structures labeled with  $\alpha$ -2-macroglobulin contained 19% of the endolyn molecules (Table I). The presence of endolyn-78 in these structures definitively established that this protein is a component of the endosomal compartment. Even after 3 h at 18.5°C, the endocytic marker was not present in morphologically identifiable lysosomes and only very rarely (<1%, Table I) it was found in a class of MVBs that, in addition to internalized vesicles, contained membrane whorls or larger membrane inclusions (which may represent the result of autophagy) and, for that reason, are labeled MVB/Ly in Fig. 10, c and d. At this time, lysosomes and MVB/Ly together contained 36% of the endolyn molecules (Table I). It should be noted (Table I) that the prolonged incubation at 18.5°C led to a significant increase in the percentage of endolyn molecules at the cell surface (from 6 to 16%), which may be accounted for by a concomitant diminution in the percentage of endolyn molecules found in lysosomes.

To obtain an insight into the process of lysosomal biogenesis, it was of interest to determine the distribution of endolyn-78 with respect to the MPR, which brings the lysosomal hydrolases to developing lysosomes. This was accomplished by double immunolabeling, using the 2C5 antibody to endolyn-78 and an antibody to the 215-kD MPR, in NRK cells that had been incubated with  $\alpha$ -2-macroglobulin at 18.5°C for 3 h. These conditions made it possible to distinguish between the endosomal compartment, which at this temperature receives the tracer, and the lysosomal one, from which the tracer is excluded. In these cells, about half of the MPR molecules were present in tubulovesicular structures found near the Golgi apparatus, probably TGN elements,

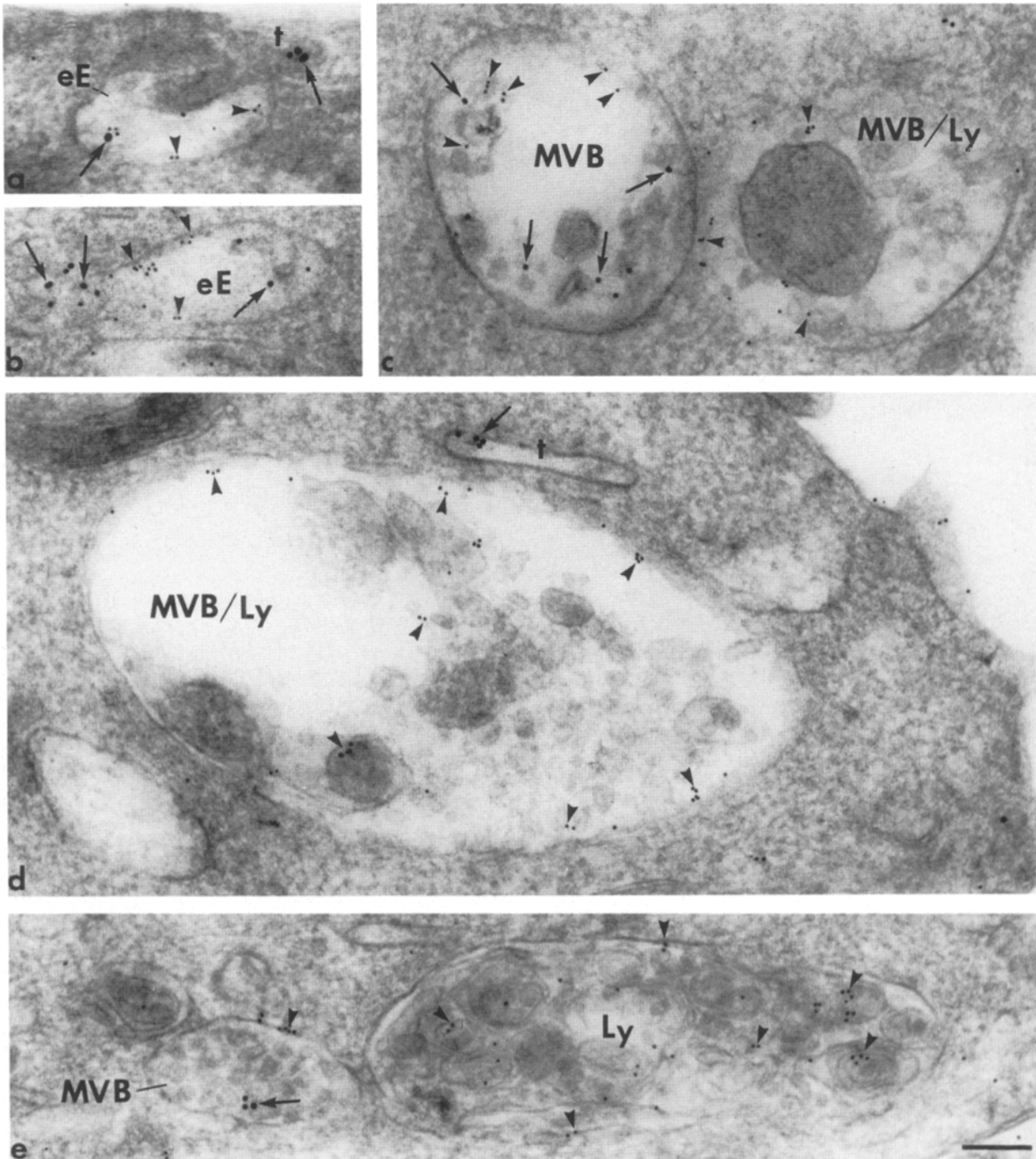
endolyn-78 is found in the limiting membranes and in the interior vesicles (arrows). The extent of labeling of individual MVBs with anti- $\beta$ -glucuronidase (arrowheads) is variable and some (d) show little labeling for the lysosomal enzyme (e, f, and g). Many vacuolar structures that, because of their size and mostly empty lumen, may correspond to early endosomes contain endolyn-78 (arrows) and either lack (g) or contain (arrowheads, e and f) low amounts of  $\beta$ -glucuronidase. Bar, 0.1  $\mu\text{m}$ .



**Figure 8.** Components of the endocytotic pathway followed by  $\alpha$ -2-macroglobulin-gold conjugates during their transfer to lysosomes in NRK cells. NRK cells were incubated at 37°C with  $\alpha$ -2-macroglobulin-gold conjugates (20 nm) for varying amounts of time and the cells were fixed in glutaraldehyde and processed for routine transmission electron microscopy. At early times (5 min, *a-d*) the label is present in coated pits (CP, *a*), branching narrow peripheral tubules (*t*, *b* and *c*) and small vacuoles, presumably early vacuolar endosomes (*eE*, *d*) which in limited areas of their membranes may still bear a fuzzy coat, presumably reflecting their recent fusion with a coated vesicle. In all these cases, the gold particles are closely apposed to the limiting membranes. At 10 min (*e*), gold particles are also found in larger endosomes and in MVBs in which the marker appears bound to internalized vesicles. At 20 min (*f*), label is found also in the lumen of large vacuoles that contain internal vesicles, as well as more complex membranous structures and some amorphous material. At this (*g*, 20 min) and later times (*h*, 90 min), the label appears with increasing frequency in membrane-bounded bodies with the heterogenous morphology characteristic of secondary lysosomes (*Ly*). Bar, 0.2  $\mu$ m.



**Figure 9.** Localization of endolyn-78 along the pathway of endocytosis of  $\alpha$ -2-macroglobulin. NRK cells incubated at 37°C for 20 (*a*, *b*, *d*, and *e*) or 90 (*c*, *f*, and *g*) min with  $\alpha$ -2-macroglobulin-gold conjugates (20 nm) were fixed and processed for frozen thin sectioning, and immunolabeling for endolyn-78 (10-nm gold particles) as described in Materials and Methods. In both *a* and *b* small, probably coated, vesicles (*large arrowheads*) containing only  $\alpha$ -2-macroglobulin appear to be fusing with preexisting endosomes. In *a*, the preexisting endosome contains only endolyn-78 (*small arrowheads*), whereas in *b* it contains both  $\alpha$ -2-macroglobulin (*arrows*) and endolyn-78 (*small arrowheads*). In early vacuolar endosomes (*b* and *c*), particles of both sizes are predominantly associated with the limiting membrane from which tubules and small vesicles sometimes appear to bud internally. In late endosomes (*d*), which frequently contain some vesicles, and in typical MVBs (*MVB*, *e*), many  $\alpha$ -2-macroglobulin conjugates (*arrows*) are no longer bound to the limiting membrane. In these structures endolyn-78 (*small arrowheads*) is associated not only with the limiting membranes, but also with membranes of internalized vesicles. Lysosomes (*Ly*, *f* and *g*), with characteristic complex internal membraneous structures contain large amounts of  $\alpha$ -2-macroglobulin (*arrows*) in their lumen and endolyn-78 (*small arrowheads*) in the limiting and interiorized membranes. Bar, 0.1  $\mu$ m.



**Figure 10.** Localization of endolyn-78 in the endosomal compartment of NRK cells that internalized  $\alpha$ -2-macroglobulin at 18.5°C. NRK cells incubated for 45 min (a) or 3 h (b–e) at 18.5°C with  $\alpha$ -2-macroglobulin–gold conjugates (20 nm) were processed for immunolabeling of endolyn-78 (10-nm gold particles). At 45 min (a) the endocytic marker,  $\alpha$ -2-macroglobulin (arrows), was found primarily ( $\sim$ 90% of the  $\alpha$ -2-macroglobulin labeled structures) in tubules (t) which are found near the cell surface and rarely contain endolyn-78 and, to a limited extent, in peripheral early endosomes (eE) which are usually labeled for endolyn-78 (arrowheads). By 3 h of  $\alpha$ -2-macroglobulin administration, this endocytic marker (arrows) was still most abundant ( $\sim$ 50% of the  $\alpha$ -2-macroglobulin-containing structures) in peripheral tubules (t, d), but was also present in early vacuolar endosomes (eE, b) ( $\sim$ 26% of the labeled structures) and in many MVBs (MVB, c and e) ( $\sim$ 24% of the labeled structures). Even after 3 h of administration of  $\alpha$ -2-macroglobulin at 18.5°C, this marker was not present in morphologically identifiable lysosomes (Ly, e) that contain endolyn-78 (arrowheads). At this time,  $\alpha$ -2-macroglobulin was present in negligible amounts in MVB/Ly (c and d) that, however, contain endolyn-78 (arrowheads). These bodies may represent an advanced stage in the conversion of MVBs to lysosomes since, in addition to interiorized vesicles, they also contain larger membraneous structures and membrane whorls similar to those in typical lysosomes (see Ly, e). Bar, 0.2  $\mu$ m.

and throughout the cytoplasm (Table I). Very low amounts of MPR (6%) were found in the plasma membrane, but substantial levels of the receptor (30% of the total) were present in vacuoles and MVBs that contained endolyn-78 and, on the basis of having acquired  $\alpha$ -2-macroglobulin at the low temperature (Fig. 11, Table I), could be defined as endosomes. As expected from previous observations (Geuze et al., 1984, 1985; Brown et al., 1986; Griffiths et al., 1988), the MPR was found to be absent from or present at very low amounts in most typical lysosomes which did not acquire  $\alpha$ -2-macroglobulin but did contain endolyn-78. Structures that resembled MVBs but contained some of the inclusions typical of lysosomes (MVB/Ly) and did not acquire  $\alpha$ -2-macroglobulin at 18.5°C usually lacked the MPR or had very low levels of this receptor (Fig. 11 *f*). Altogether, the lysosomal and MVB/Ly compartments contained ~8% of the MPR (Table I). In only rare instances, some of the MVB/Ly structures contained high concentrations of the MPR (Fig. 11 *g*) and, therefore, resembled the structures described by Griffiths et al. (1988), which were considered to represent a prelysosomal compartment where lysosomal enzymes are first packaged with newly synthesized membrane glycoproteins into lysosomes.

## Discussion

The lysosomal membrane proteins so far identified in a variety of cell types from different animal species (Lewis et al., 1985; Tougard et al., 1985; Chen et al., 1985*a,b*; D'Souza and August, 1986; Barriocanal et al., 1986; Lippincott-Schwartz and Fambrough, 1986, 1987; Fambrough et al., 1988) are all highly glycosylated glycoproteins bearing complex N-linked oligosaccharide chains. It has previously been noted (Barriocanal et al., 1986) that similarities in the sizes of their precursor and mature forms suggest that some of these proteins (the mouse protein LAMP-1, Chen et al., 1985*a*; the rat Igp 120, Lewis et al., 1985; LIMP-3, Barriocanal et al., 1986; and the chicken LEP-100, Lippincott-Schwartz and Fambrough, 1986), may represent equivalent gene products. However, only two of the previously described proteins of the lysosomal membrane were found to be present at significant levels in endosomes, as well as in lysosomes (Tougaard et al., 1985; Lippincott-Schwartz and Fambrough, 1986, 1987), a property which is also shared by endolyn-78, the lysosomal membrane protein described in this work.

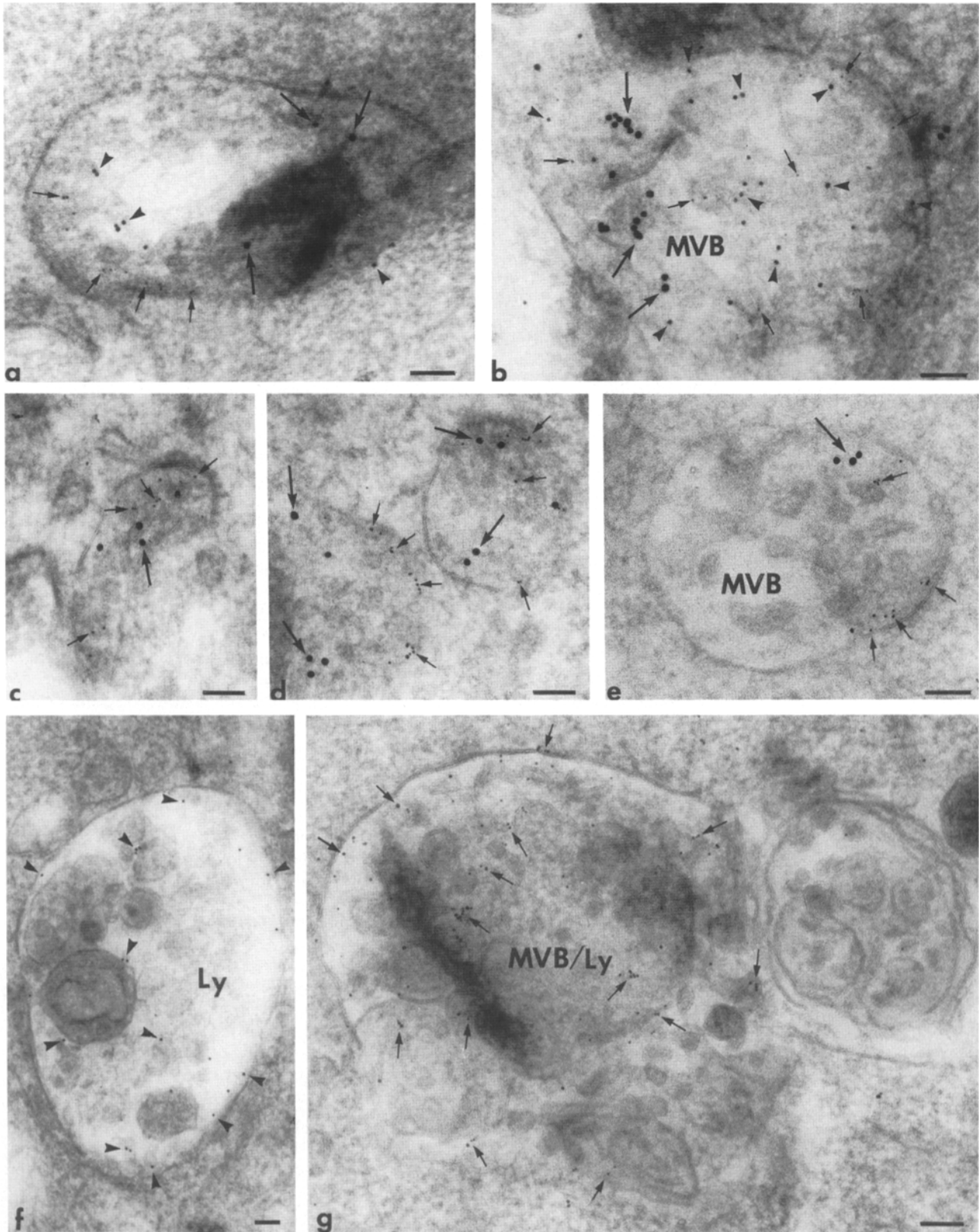
Endolyn-78 differs from other lysosomal membrane proteins not only on the basis of its size and that of its unglycosylated polypeptide backbone, but also in that it contains a large proportion of O-linked oligosaccharide chains. The polypeptide backbone in endolyn-78, identified by labeling for short times in tunicamycin-treated cells, has an *M<sub>r</sub>* of 22 kD and the addition of O-linked sugars, that are largely removable by treatment with *O*-glycanase, increased its apparent molecular mass to 46 kD. As has been suggested for the N-linked oligosaccharides of other lysosomal membrane proteins (Lewis et al., 1985; Barriocanal et al., 1986), it seems likely that the abundant O-linked moieties in endolyn-78 also play a role in protecting the protein from degradation within the lysosome.

Another unique biochemical feature of endolyn-78 is its lack or low content of methionine. In fact, endolyn-78 could

not be labeled with [<sup>35</sup>S]methionine, but was easily labeled with [<sup>35</sup>S]cysteine, which labeled the bulk of cellular proteins and several specific proteins much less efficiently than [<sup>35</sup>S]methionine. Finally, endolyn-78 appears to easily form dimers which, in spite of the apparent abundance of cysteine, do not result from intermolecular S-S bonds. Rather, they result from interactions requiring the presence of the N-linked carbohydrate chains, since the 46-kD form produced in tunicamycin-treated cells did not dimerize. Pulse-chase experiments revealed that the O-linked sugars in endolyn-78 are incorporated into the protein with kinetics very similar to that with which the high mannose N-linked oligosaccharides are converted into endo H-resistant, complex forms. This observation is in agreement with other reports on the synthesis of glycoproteins bearing both types of sugars (Hanover et al., 1982; Johnson and Spear, 1983), and with the Golgi membrane localization of the enzyme UDP-GalNAc:polypeptide transferase that adds proximal O-linked sugars (Abeijon and Hirschberg, 1987).

The finding that O-glycosylation proceeds rapidly in tunicamycin-treated cells indicates that N-glycosylation is not a prerequisite for O-glycosylation and that the transit of endolyn from the endoplasmic reticulum to the Golgi apparatus is not dependent on the addition of the N-linked sugars. Although, like endolyn-78, lysosomal hydrolases are glycoproteins, not only do they contain a lower proportion of their mass as sugars but they display an important difference in their oligosaccharide structure. Thus, most of the oligosaccharide chains in endolyn-78 are of the complex tri- and tetraantennary types, whereas the lysosomal hydrolases contain primarily high mannose biantennary oligosaccharides (Kornfeld and Kornfeld, 1985; von Figura and Hasilik, 1986).

Immunoelectron microscopy showed that endolyn-78 is present in morphologically and immunocytochemically identifiable secondary lysosomes. In these organelles the antigen is found exposed not only on the inner face of the limiting membrane, but also on interior membranous structures that may represent either invaginations or interiorized vesicles derived from the limiting membranes. Endolyn-78, however, is also present in various components of the endosomal compartment and in cytoplasmic vesicles. A study of its distribution in cells that were allowed to ingest the endocytic marker  $\alpha$ -2-macroglobulin, at either 37°C or 18.5°C, revealed that endolyn-78 is present in vacuolar endosomes, in which the pH was not yet sufficiently low to release the gold-conjugated  $\alpha$ -2-macroglobulin ligand from its membrane receptor, as well as in late endosomes and MVBs, in which the ligand was found free in the lumen. The low but significant levels of endolyn-78 detected at the cell surface most likely reflect a dynamic equilibrium in which there is a low steady-state concentration of the protein in the plasma membrane. In fact, a significant increase (~2.6 times) in cell surface levels of endolyn-78 was observed in the cells that internalized  $\alpha$ -2-macroglobulin at 18.5°C. Another lysosomal-endosomal membrane protein, LEP-100, has been shown to be present at low concentrations in the plasma membrane, but to rapidly recycle between the lysosomal, endosomal, and plasma membrane compartments, and to appear at much higher levels at the cell surface when the cells are treated with chloroquine (Lippincott-Schwartz and Fambrough, 1987). Endolyn-78, however, does not behave in this manner since plasma membrane levels of this protein were not significantly increased



**Figure 11.** The MPR is present in endosomes that contain endolyn-78 and receive internalized  $\alpha$ -2-macroglobulin in NRK cells incubated with the endocytic marker at 18.5°C: evidence for the conversion of endosomes and MVBs into lysosomes. NRK cells incubated at 18.5°C for 3 h with the endocytic tracer  $\alpha$ -2-macroglobulin (20-nm gold particles, *large arrows*) were processed for double immunolabeling (*a*, *b*, and *f*) with anti-endolyn-78 (10-nm gold particles, *arrowheads*) and anti-MPR (5-nm gold particles, *small arrows*) or only for labeling with anti-MPR (*c*, *d*, *e*, and *g*). Endosomes, defined by the presence of  $\alpha$ -2-macroglobulin (large arrows) at 18.5°C with the morphological appearance of vacuoles (*a*) or MVBs (*b*) contain endolyn-78 (*arrowheads*), as well as the MPR (*small arrows*). On the other hand, morphologically identifiable lysosomes (*Ly*, *f*) do contain endolyn-78 (*arrowheads*) but not  $\alpha$ -2-macroglobulin or the MPR. The MPR (*small ar-*

after treatment with chloroquine (our unpublished observations).

Our observations add further support to that provided by Brown et al. (1986) to a maturation model for lysosome biogenesis (see Helenius et al., 1983; Hopkins et al., 1986; Brown et al., 1986) in which there is no sharp transition between endosomes and lysosomes but in which endosomes (MVBs) are gradually converted into lysosomes. Thus, endolyn-78 was present at relatively high concentrations in MVBs that acquired  $\alpha$ -2-macroglobulin at 18.5°C and contained the MPR as well as variable amounts of the lysosomal hydrolase  $\beta$ -glucuronidase. These multivesicular endosomes varied considerably in their size and content of internalized vesicles, which presumably reflects their different degrees of maturation. The presence of the lysosomal enzyme in these endosomal structures suggests that they, like lysosomes, may participate in intracellular digestion, as has been shown for the endosomes of macrophages (Diment and Stahl, 1985). Other large MVBs showed some of the properties of lysosomes, such as the absence of  $\alpha$ -2-macroglobulin after prolonged incubation at 18.5°C and the presence of more extensive membranous inclusions that may result from autophagy. Since structures of this morphological appearance were found to contain  $\beta$ -glucuronidase and generally lacked the MPR, it is likely that they represent an even more advanced stage in the conversion of multivesicular endosomes to lysosomes and, therefore, have been designated MVB/Ly.

In the very rare instances, MVB/Ly-type structures were observed (i.e., structures that did not acquire  $\alpha$ -2-macroglobulin at 18.5°C) that contained high concentrations of the MPR. These structures may be equivalent to a recently described MPR-enriched "prelysosomal intermediate" that was found to also contain substantial levels of the lysosomal membrane protein Igp 120 (Griffiths et al., 1988). However, in the specimens we studied, such structures could not represent an obligatory intermediate in the biogenesis of the lysosome, i.e., one in which lysosomal membrane proteins and hydrolases would first meet each other, since we found the MPR to be abundant and lysosomal hydrolases to be present in many other types of endosomes and MVBs to which  $\alpha$ -2-macroglobulin was delivered at 18.5°C. The putative "prelysosomal intermediate" observed by Griffiths et al. (1988) contained an extensive system of "thin, wormlike tubules" packed to high density inside its lumen. We have not observed a structure with the same characteristics in our studies with the same cell line (NRK). It seems therefore likely that some of the MVBs and MVB/Ly described here may correspond to the "prelysosomal intermediate" of Griffiths et al. (1988), and that the differences in morphological appearance simply result from differences in sample preparation during fixation, cryomicrotomy, immunolabeling, or staining. Nevertheless, it remains to be determined why the putative intermediate described by Griffiths et al. (1988), contained the

bulk of the cellular complement of MPR, whereas in our study MVBs containing the MPR but no  $\alpha$ -2-macroglobulin at 18.5°C were only rarely observed. In fact, the bulk of the MPR was contained in structures that received  $\alpha$ -2-macroglobulin at 18.5°C and, therefore, were bona fide endosomes, as well as in tubulovesicular elements near the Golgi apparatus and throughout the cytoplasm. This distribution is consistent with a gradual conversion model in which endosomes acquire lysosomal enzymes brought by the MPR in Golgi-derived vesicles, while undergoing a remodeling of their membranes that ultimately leads to the complete removal of the MPR.

The oligosaccharide chains of lysosomal hydrolases are essential for the targeting of the enzymes to lysosomes since they provide the sites for formation of the mannose-6-phosphate recognition marker (see Kornfeld, 1987). In contrast, endolyn-78, like other lysosomal membrane proteins previously described (Barriocanal et al., 1986), is efficiently targeted to lysosomes even in tunicamycin-treated cells, in which the mannose-6-phosphate recognition marker cannot be formed. The fact that lysosomal membrane components can reach the lysosome independently of the mannose-6-phosphate recognition mechanism has been apparent from the observation that some membrane-associated lysosomal hydrolases, such as  $\beta$ -glucocerebrosidase (Erickson et al., 1985) and acetyl-CoA ( $\alpha$ -D-glucosamine *N*-acetyl transferase) (Kornfeld, 1987) are still present in lysosomes of fibroblasts from I-cell disease patients. The finding that lysosomal membrane proteins are incorporated into the organelle in the absence of the concomitant delivery of newly synthesized hydrolases, suggests that the conversion of an endosome into a lysosome could be effected by a membrane remodeling that does not require the constant delivery of packets of hydrolases from the TGN. Alternatively, endosomes containing the newly synthesized membrane proteins could fuse with preexisting lysosomes.

The maturation model of lysosomal biogenesis implies that at least some endosomes, such as MVBs, are not permanent cellular structures and that the endocytosed material present in them is not simply transferred by shuttling vesicles to a lysosome. On the other hand, because of the high concentrations of ligands that are observed in lysosomes, it is unlikely that during the maturation of endosomes to lysosomes each individual endosome generates a single lysosome. Rather, it is likely that vacuolar endosomes and later stage MVB-like endosomes undergo fusion with each other, followed by removal of excess membranes (probably by the formation of interiorized vesicles). The capacity of endosomes to fuse with each other (Braell, 1987; Gruenberg and Howell, 1987; Salzman and Maxfield, 1988) and of lysosomes to fuse with other lysosomes has been demonstrated (Ferris et al., 1987; Deng and Storrie, 1988) and, in at least one case, evidence for endosome-lysosome fusion has been

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rows) is found throughout the endosomal compartment (defined by the presence of  $\alpha$ -2-macroglobulin) (i.e., in peripheral tubular/vacuolar structures that contain  $\alpha$ -2-macroglobulin [*large arrows*, c], some of which are partially covered by a fuzzy coat similar to that in coated vesicles; in vacuolar endosomes with few internalized vesicles [*d*]; and in well-developed MVBs [*e*]). Although most MVB/Ly (i.e., bodies that contain interiorized vesicles and membrane whorls but do not acquire  $\alpha$ -2-macroglobulin at 18.5°C and presumably represent an advanced stage in the conversion of an MVB to a lysosome) lack the MPR, in very rare instances bodies with the features of MVB/Lys (*g*) contain substantial amounts of MPR (*small arrows*). Bar, 0.1  $\mu$ m.

presented (Harding et al., 1985), a process which could account for the concentration of endocytosed markers in individual lysosomes.

Whereas our studies did not directly address the question of the pathway taken by lysosomal membrane proteins to lysosomes, it is clear that some lysosomal membrane proteins, such as endolyn-78, are already present in endosomes and that others, such as lgp 120, which are not present at significant levels in endosomes (Lewis et al., 1985), must be added to them during the late stages of maturation, presumably by direct transfer from the Golgi apparatus or the TGN (Green et al., 1987). The fact that some membrane proteins are present in endosomes and not in lysosomes (Schmid et al., 1988) implies that proteins are also removed from the endosomal membrane during the maturation process. This is, of course, the case for recycling receptors that are returned to the plasma membrane after releasing their ligands in the endosomal compartment (Geuze et al., 1984, 1985), as well as for the MPR that brings the newly synthesized lysosomal enzymes. It remains to be established whether there is any "permanent" endosomal subcompartment that maintains a distinct composition of its own limiting membranes while it receives material endocytosed at the cell surface and transfers it to other endosomes that undergo conversion to lysosomes.

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