Distribution of Newly Synthesized Lysosomal Enzymes in the Endocytic Pathway of Normal Rat Kidney Cells

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Abstract. We have investigated the distribution of newly synthesized lysosomal enzymes in endocytic compartments of normal rat kidney (NRK) cells. The mannosyl 6-phosphate (Man6-P) containing lysosomal enzymes could be iodinated in situ after internalization of lactoperoxidase (LPO) by fluid phase endocytosis and isolated on CI-MPR affinity columns. For EM studies, the ectodomain of the CI-MPR conjugated to colloidal gold was used as a probe specific for the phosphomannosyl marker of the newly synthesized hydrolases. In NRK cells, ~20-40% of the phosphorylated hydrolases present in the entire pathway were found in early endocytic structures proximal to the 18°C temperature block, including early endosomes. These structures were characterized by a low content of endogenous CI-MPR and were accessible to fluid phase markers internalized for 5-15 min at 37°C. The bulk of the phosphorylated lysosomal enzymes was found in late endocytic structures distal to the 18°C block, rich in endogenous CI-MPR and accessible to endocytic markers internalized for 30-60 min at 37°C. The CI-MPR negative lysosomes were devoid of phosphorylated hydrolases. This distribution was unchanged in cells treated with Man6-P to block recapture of secreted lysosomal enzymes. However, lysosomal enzymes were no longer detected in the early endosomal elements of cells treated with cycloheximide. Immuno-precipitation of cathepsin D from early endosomes of pulse-labeled cells showed that this hydrolase is a transient component of this compartment. These data indicate that in NRK cells, the earliest point of convergence of the lysosomal biosynthetic and the endocytic pathways is the early endosome.

In mammalian cells, the mannosyl 6-phosphate receptors (MPRs) are responsible for diverting lysosomal enzymes from the bulk flow secretory pathway. Two receptors are implicated in this process: the cation-independent MPR (CI-MPR) which is identical to the insulin-like growth factor II receptor and the cation-dependent MPR (CD-MPR) (for review see Kornfeld and Mellman, 1989; Dahms et al., 1989). In the trans-Golgi network (TGN), the MPRs and their bound ligands are packaged into clathrin-coated vesicles and transported to the next station of the delivery pathway. From early studies showing that dense lysosomes were devoid of detectable MPRs (Sahagian and Neufeld, 1983; Willingham et al., 1983), it was proposed that the Golgi-derived vesicles would fuse with a prelysosomal compartment where lysosomal enzymes could dissociate from their receptors and be subsequently transported to lysosomes. Until now, most morphological studies have focused on the intracellular distribution of the MPRs and more specially on the CI-MPR (Willingham et al., 1983; Geuze et al., 1985; Brown et al., 1986; Griffiths et al., 1988; Geuze et al., 1988). The latter is present in low amounts in the Golgi complex, predominantly the TGN, and on the plasma membrane. Because the bulk of this receptor is found in endosomes, it was proposed that the newly synthesized hydrolases were delivered to these compartments. While morphological studies on the CD-MPR have not been as extensive, the evidence suggests that both receptors exhibit a similar qualitative distribution (Bleeke-molen et al., 1989). Biochemical studies have clearly demonstrated that the MPRs present in these different compartments are in rapid dynamic equilibrium constituting one pool of functional receptors (von Figura et al., 1984; Stein et al., 1987; Duncan and Kornfeld, 1988).

The endocytic pathway is complex, consisting of at least four distinct stations including the lysosomes (for reviews see Hubbard, 1989; Gruenberg and Howell, 1989; Kornfeld and Mellman, 1989). The first compartment of the endosomal system to which plasma membrane-derived vesicles deliver their content is the early endosome, a structure morphologically and functionally different from the more distal, acid phosphatase-rich elements of the pathway. Distal to the early endosome is a relatively large spherical structure which was proposed to be an intermediate or a "carrier vesicle" that moves along microtubules between the early endosomes and more distal, CI-MPR-enriched elements of the endocytic pathway (Gruenberg et al., 1989; Bomsel et al.,...
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1990). The early endosome and this spherical structure can be labeled with endocytic tracers internalized at 18-20°C (Griffiths et al., 1988), a condition which impairs transport to lysosomes (Dunn et al., 1980). The third station along the endocytic pathway is the late endosome that we also referred to as prelysosomal compartment. This structure is enriched in the CI-MPR, the lysosomal membrane glycoprotein 120 lgp (Griffiths et al., 1988; see also Geuze et al., 1988), and is the first station along the endocytic pathway that gives a significant cytochemical reaction for a tartrate-sensitive acid phosphatase (Griffiths et al., 1990b). This late endosomal structure, clearly distinct from the CI-MPR-negative lysosomes, does not label significantly with endocytic markers internalized at 18°C (Griffiths et al., 1988). A similar structure filled with tubulo-vesicular membranes and located close to the Golgi complex could also be detected in other cell types (Griffiths et al., 1990a; Lobel et al., 1989) and is one of the major stations along the endocytic pathway (Griffiths et al., 1989; Parton et al., 1989). Early and late endosomes have distinct protein compositions and acidification properties (Schmid et al., 1988). However, the functional relationships between these different structures are still poorly understood.

To determine the earliest point of convergence of the lysosomal biosynthetic and the endocytic pathways and to define better their functional relationships, we investigated the intracellular distribution of the newly synthesized lysosomal enzymes. We have taken advantage of the phosphomannosyl residues which are one of the hallmarks of these components. In a biochemical approach using in situ iodination, we purified on CI-MPR affinity columns the hydrolases which could be labeled by lactoperoxidase (LPO) that had been endocytosed under various conditions. In parallel, we followed by quantitative EM the passage of a fluid phase marker along the endocytic pathway. We also used the ectodomain of the CI-MPR conjugated to gold particles to establish by EM the intracellular localization of the phosphorylated lysosomal enzymes. We now report that the lysosomal biosynthetic and the endocytic pathways can converge in the early endocytic structures.

Materials and Methods

Chemicals

All chemicals were of analytical grade. BSA, cycloheximide, glucose oxidase, HRP, lactoperoxidase, glucose-6-phosphate, and mannose-6-phosphate (Man6-P) were purchased from Sigma Chemical Co. (St. Louis, MO). Affi-Gel 10 and protein A-Sepharose were purchased from Bio-Rad Laboratories (München, Germany), and Pharmacia (Uppsala, Sweden), respectively. Na125I and 35S-methionine were from Amersham Buchler GmbH (Braunschweig, Germany). Hansenula holstii (NRRL Y-2448) phosphomannan was a generous gift of Dr. M. Slodki (United States Department of Agriculture, Peoria, IL).

Cell Culture

Cells were maintained at 37°C under 5% CO2. Normal rat kidney (NRK) cells were grown as monolayers in α-MEM and DME, respectively. These media were supplemented with antibiotics, nonessential amino acids, and 10% FCS. Cultures were used at 70–90% confluency.

Endocytosis of LPO and In Situ Iodination

The endosomal components were iodinated as originally described by Mellman and Galloway (1983). Briefly, cells grown in 10-cm culture dishes were washed twice with PBS. LPO (1 mg/ml in DME) was internalized either for 1 h at 4°C, 1 h at 18°C or 5, 15, 30, 60 min at 37°C. After uptake, the cells were chilled on ice and washed 5 × 5 min with ice-cold PBS/BSA (1 mg/ml). Iodination buffer (2.5 ml of PBS containing 20 mg D-glucose, 50 mM Na acetate, pH 6.6, as described by Wats, 1984) was added to each dish. The iodination was initiated by adding 100 µl PBS containing 100 ng glucose oxidase and 0.5 mCi of carrier-free Na125I and was carried out on ice for 30 min under gentle rocking. The labeling was stopped by addition of 100 µl of 50 mM KI in PBS and the cells were washed 4 × 5 min with PBS containing 5 mM KI. The cells were harvested by scraping, centrifuged, and the cell pellets were lysed in 1 ml of 10 mM Tris-HCl, pH 7, 150 mM NaCl, 5 mM Na β-glycerophosphate, 1% Triton X-100. After 30 min on ice, the Triton X-100 insoluble material was spun for 10 min at 10,000 g at 4°C. The supernatants were collected and further processed for affinity chromatography.

Chromatography on Man6-P Receptor Affinity Columns

The purification of the CI-MPR from bovine liver, its coupling of Affigel-10, and the purification of iodinated lysosomal enzymes were as previously described (Hoffack et al., 1987). Briefly, the Triton X-100 extracts of labeled cells were loaded on to 2.5-ml affinity columns (1 mg of receptor per ml of gel) and the flow was stopped for 30 min to allow efficient binding. The columns were then washed with ~100 ml of buffer A (10 mM Tris-HCl, pH 7, 150 mM NaCl, 5 mM Na β-glycerophosphate, 0.05% Triton X-100, 1 mM EDTA, 100 µg/ml BSA) and ~20 ml of the same buffer containing 5 mM glucose-6-phosphate. The bound material was eluted with 5 mM mannose-6-phosphate in buffer A and the radioactivity of the different fractions was counted.

Immunoprecipitations of Endosomal Cathepsin D

NRK cells were grown in 24-well plates as described above. The cells were washed with PBS and preincubated for 10 min in methionine-free medium. They were then incubated for 10 min in 200 µl of methionine-free medium containing 10 mM Heps, 1 mM/ml 35S-methionine, 5 mM Man6-P and 10% dialyzed FCS. The cells were chased by adding 10 µl of 0.2 M methionine. At the end of the chase, the medium was removed and the cells were washed twice with PBS/BSA (1 mg/ml). The cells were then reincubated for 5 min at 37°C with 200 µl of prewarmed medium containing 1 mg/ml of immunopurified anti-rat cathepsin D antibodies and 5 mM Man6-P. The cells were then returned to ice temperature, washed four times with cold PBS/BSA, and left on ice for 1 h to allow formation of the immunocomplex. The cells were then lysed in 500 µl of 50 mM Tris-HCl pH 7, 150 mM NaCl, 1% Triton X-100, 1 mg/ml BSA (lysis buffer) containing 5 µg of purified rat cathepsin D used as a quenching agent. The insoluble material was removed by centrifugation and the supernatant was incubated overnight at 4°C with protein A-Sepharose. The beads were washed with 10 × 1 ml of lysis buffer and twice with same buffer without BSA. The samples were then processed for SDS-PAGE. Typical pulse-chase experiments were also carried out in parallel to follow total synthesis and secretion of cathepsin D in these cells.

Purification of Soluble CI-MPR and Conjugation to Gold Particles

Fragments corresponding to the luminal part of the CI-MPR were purified by affinity chromatography following the same procedure as for the purification of the CI-MPR mentioned above. Briefly, a phosphomannan column was loaded with the bovine serum, the column was washed extensively with PBS, eluted with 5 mM glucose-6-phosphate and with 5 mM Man6-P in PBS. The fractions containing the CI-MPR fragments were pooled, extensively dialyzed against PBS, and concentrated. Starting from 1 liter of serum, 10 µl of these soluble fractions was routinely obtained. The major fragment of this fraction had a molecular weight of 200,000 as judged by SDS gel (not shown) and was conjugated to gold. The conjugation to gold particles were made by binding the fragments of the CI-MPR at a concentration of 12 µg/ml to 9-nm gold (Slot and Geuze, 1985) at pH 6.

Quantitation of HRP Labeling Along the Endocytic Pathway

NRK cells were incubated in medium containing 16-nm BSA-gold (OD 520 = 0.3) for 4 h at 37°C followed by an overnight chase in medium free
of gold. The cells were then incubated in medium containing 10 mg/ml HRP for different times. The monolayers were placed on ice, removed with 25 μg/ml protease K, and fixed with 0.5% glutaraldehyde in 200 mM Hepes for 4–24 h. Cryosections were prepared and double labeling was done according to Geuze et al. (1981) with anti-HRP antibodies (a kind gift of Dr. Jean Gruenberg) and anti-CI-MPR antibodies. After labeling, micrographs were systematically selected at a primary magnification of 28,000 and the number of HRP labeling (9-nm gold particles) was estimated as described previously (Griffiths and Hoppeler, 1986). The endocytic structures were identified as follows. In addition to their content in CI-MPR, the two late endocytic structures (CI-MPR positive), and the lysosomes (CI-MPR negative) were labeled with a fluid phase marker (16 nm BSA-gold) as described earlier (Griffiths et al., 1990b). The two earlier structures, the early endosomes with their typical cisternal, tubular structure and the more spherical endosomes were identified by (a) their labeling with HRP (they are the only structures labeled with this marker internalized for up to 15 min at 37°C); (b) the absence of both significant amounts of CI-MPR and the endocytic marker of late structures; (c) their morphological appearance.

Labeling of Cryosections with CI-MPR Conjugated to Gold

Several markers were used to identify the different endocytic structures of NRK cells. The early structures of the endocytic pathway were identified by their content of 5-nm BSA-gold internalized for 1 h at 18°C. In these cells, the early endosome compartment was specifically identified by 5-nm BSA-gold and internalized for 5 min at 37°C. The late endocytic structures were characterized as above using the endogenous CI-MPR and 16-nm BSA-gold internalized for 4 h at 37°C and chased overnight. When protein synthesis was inhibited in NRK cells, cycloheximide (10 μg/ml) was directly added to the culture medium. After internalization of the markers, the cells were fixed with 8% paraformaldehyde in 200 mM Hepes for 4–24 h and cryosections were prepared as above. They were then labeled with the CI-MPR fragments conjugated to 9-nm gold used at a dilution 1:100 (final OD = 0.25). The quantitation of the labeling was performed as above.

Results

Quantitative Morphological Analysis of the Passage of a Fluid Phase Marker along the Endocytic Pathway

We first followed, by EM, the progression of a fluid phase marker through the different elements of the endocytic pathway of NRK cells. These data could then be used as a reference for the subsequent biochemical studies. In this morphological study, the late structures of the endocytic pathway were identified by their content of 16-nm gold particles conjugated to BSA internalized by fluid phase endocytosis in a pulse-chase protocol (a 4–8 h uptake followed by an overnight chase). Under these conditions, while the bulk of this marker was in CI-MPR negative lysosomes, a significant amount was always found in late endosomes as indicated by its colocalization with the endogenous CI-MPR used as a marker of these structures (Griffiths et al., 1990b). Using this protocol, the early endocytic elements were defined as structures that contained little, or no, labeling for the CI-MPR and no 16-nm BSA-gold. HRP used as a fluid phase marker was then internalized for different periods of time at 37°C before fixation. Cryosections of these preparations were double labeled with anti-CI MPR and anti-HRP antibodies followed by protein A–gold. The HRP-associated gold particles found over the different endocytic structures were then quantitated (Fig. 1).

Within the first 15 min of HRP internalization, two distinct kinds of labeled structures were evident: first, the early endosome compartment which is made up of interconnecting cisternae, tubules and vesicles and second, the distinct spherical endosome vesicles which are packed with profiles of internal membranes (see Griffiths et al., 1989; Gruenberg et al., 1989). Fig. 3 shows typical examples of these two structures. Both of these CI-MPR negative structures were classed as early endosomal elements in this study. The quantitation of gold particles associated with HRP showed that, at 37°C, the concentration of HRP in the two kinds of early structures reached a plateau within the first 15 min of internalization. As in BHK cells (Griffiths et al., 1989), we observed that the tubulo-vesicular early endosomes were more rapidly (within 5 min) accessible to HRP than the spherical vesicles. At 60 min, the CI-MPR-rich late endosomes reached the same density of HRP labeling that the two proximal endosome structures had at 15 min, suggesting that the late endosomes had filled with HRP at this time. Finally, HRP could not be significantly detected in lysosomes before a 2-h period. In these latter structures (see Fig. 4 for their morphology), the number of gold particles per unit volume reached the same levels as those found in the early endosomes only after 4 h (not shown). These data indicate that, in NRK cells, an endocytosed fluid phase marker enters sequentially early endosomes, the spherical endosome vesicles, CI-MPR-rich late endosomes and finally lysosomes. As in previous studies (Griffiths et al., 1988), HRP did not enter the CI-MPR-rich late endosomes in significant amounts at 18°C but was restricted to the early endosomal elements (not shown).

Lactoperoxidase Iodination of Endosomes

We used LPO iodination to label in situ the intraluminal components of endocytic structures. The lysosomal hydrolases leave the Golgi apparatus as phosphorylated proenzymes via clathrin-coated vesicles (Campbell and Rome, 1983; Lemansky et al., 1987). Although the late endocytic structures give a significant reaction for a tartrate-sensitive acid phosphatase (Griffiths et al., 1990b), we reasoned that the hydrolases emerging from the biosynthetic pathway
should remain phosphorylated for at least a brief period of time. With this rationale, we decided to purify on CI-MPR affinity columns the endosomal phosphorylated hydrolases which could be iodinated by the internalized LPO.

NRK cells were allowed to take up LPO by fluid phase endocytosis under various conditions. According to the morphological study described above, after a short internalization (5 min) at 37°C, the LPO should be predominantly present in the typical tubulo-vesicular early endosomes. After a longer internalization (<15 min) at 37°C or a 1-h uptake at 18°C, the LPO is present in the two sets of early endosomal elements that we defined before. Finally, LPO should fill both early endosomal elements and the CI-MPR positive late endosomes after a 1-h internalization at 37°C. The cells were then chilled to 4°C and processed for in situ iodination as described in Materials and Methods. We first measured the incorporation of 125I into the TCA insoluble material (Fig. 2 A). At 37°C, this incorporation increased linearly during the first 15 min of internalization of the probe and then leveled off when LPO was internalized for up to 60 min. The labeling was significantly reduced when the internalization was performed at 18°C. At 4°C, a condition that blocks fluid phase endocytosis, <5% of the total incorporation observed at 37°C was obtained. This indicates that, under the conditions we have used, the bulk of the labeling reflects iodination of endosomal components. The iodinated CI-MPR was then immunoprecipitated from the resulting cell extracts, analyzed by SDS-PAGE (Fig. 2 B, inset) and quantitated (Fig. 2 B). When LPO was internalized for up to 15 min at 37°C or 1 h at 18°C, only small amounts of the CI-MPR became iodinated. This was <10% of the endosomal receptor accessible to LPO. However, when NRK cells were incubated with LPO for longer periods of time (30–60 min) at 37°C, a higher proportion of the endosomal CI-MPR became iodinated. Longer incubation at 37°C, up to 2 h, did not affect the amount of CI-MPR that became iodinated (not shown). We interpret these results as indicating that the bulk of the endosomal CI-MPR is present in late structures that are filled with LPO only after 60 min of internalization at 37°C. The early endosomal elements which, by analogy to the morphological studies, should be the only structures containing LPO after an internalization of 15 min at 37°C or of 1 h at 18°C, contained only small amounts of endosomal CI-MPR. We emphasize that the use of lower concentrations of LPO during the internalization procedure did not modify the kinetics of iodination of the CI-MPR (not shown).

We then compared the kinetics of iodination of phosphorylated lysosomal enzymes with that of the endogenous CI-MPR. Fig. 2 C shows the amount of phosphorylated iodinated material that was retained by, and specifically eluted from a CI-MPR affinity column with Man6-P. Iodinated lysosomal enzymes were readily detected when LPO was internalized for 5–15 min at 37°C or for 1 h at 18°C. The amount of iodinated material retained by the affinity column increased significantly when the tracer was taken up for longer periods of time at 37°C, but was drastically reduced...
The early endosome was labeled by 5-nm BSA-gold internalized for 15 min at 37°C and proximal to the 18°C temperature block. The early endosome was labeled with 5-nm BSA-gold internalized for 5 min at 37°C accounted for 15% of the total endosomal material detected with this assay. The remainder of the phosphorylated protein species that we could detect with this assay were present in later endocytic elements distal to the 18°C temperature block which, according to our morphological and biochemical studies described above, correspond to the CI-MPR–enriched late endosomes.

Detection of Phosphorylated Hydrolases by Electron Microscopy

Soluble fragments of CI-MPR corresponding to its ecto-binding domain are released into the medium of cultured cells (Sahagian, 1984). They are also found in body fluids such as serum (Causin et al., 1988). We therefore purified this soluble CI-MPR from bovine serum and conjugated it to 9-nm colloidal gold particles. Unlike antibodies directed against one given hydrolase which would predominantly decorate the lysosomes where its mature form is stored, these conjugates should bind to any phosphorylated species and therefore maximize the detection of the newly synthesized lysosomal enzymes. In this morphological study, we again used different markers to identify the endocytic compartments. The early endosome was labeled by 5-nm BSA-gold internalized for 5 min at 37°C. The 5-nm BSA-gold internalized for 1 h at 18°C labels in addition to the previous compartment the spherical endosome vesicles packed with profiles of internal membranes. As described above, the late endocytic structures (late endosomes and lysosomes) were identified by their content of endogenous CI-MPR and internalized 16-nm BSA-gold. When the soluble fragments of the CI-MPR conjugated to 9-nm gold were incubated with cryosections of these cells, several structures were specifically labeled. The early endosomal structures (tubulo-vesicular early endosomes and spherical vesicles) had significant labeling of the soluble CI-MPR conjugated to 9-nm gold (Fig. 3). There was a higher labeling of the late endosomes (Fig. 4). The lysosomes (endogenous CI-MPR negative, 16-nm gold positive) were invariably devoid of labeling for phosphorylated endogenous lysosomal enzymes (Fig. 4 A). The labeling of the CI-MPR fragment–gold conjugates was then quantitated with respect to the volumes (or areas on the sections) of the compartments of interest that were identified as described above (Table I). The results indicate that 20–30% of the total phosphorylated hydrolases found within the cell were detected in early endosomal structures characterized by a low content of endogenous CI-MPR and labeled with 5-nm BSA-gold internalized for 1 h at 18°C. The early endosomes and spherical endosome vesicles contained 6–15% and <15% of the total labeling respectively. The bulk of the labeling (∼70%) was associated with the late endosomes rich in endogenous CI-MPR. Surprisingly, the Golgi stacks and putative TGN elements contained only small amounts of phosphorylated hydrolases: this was <1% of the total labeling. As expected, the labeling by the CI-MPR-gold conjugates was completely abolished in the presence of 5 mM Man6-P (not shown).

The Hydrolases of Early Endosomes Originate from an Intracellular Pathway

Most cultured cell lines secrete variable amounts of their newly synthesized and phosphorylated hydrolases (Hasilik and Neufeld, 1980). To rule out the possibility that the hydrolases we detect in early endocytic elements were secreted and then reinternalized by cell surface receptors, we compared the distribution of phosphorylated lysosomal enzymes in the endocytic pathway of NRK cells grown in the presence or in the absence of 5 mM Man6-P. Under these conditions, internalization of [3H]mannose-labeled lysosomal enzymes was drastically reduced (>95%; not shown). Therefore, NRK cells were grown for 16 h in medium containing 5 mM Man6-P, and then allowed to take up LPO for 1 h at 37°C, 18°C, or 4°C in the presence of Man6-P. After in situ ioldination, the hydrolases were purified on a CI-MPR affinity column. As shown in Table II, the amount of the phosphorylated hydrolases found in endocytic structures proximal to the 18°C block was not significantly changed when Man6-P was present. These results indicate that a re-capture mechanism cannot account for the presence of the bulk of the hydrolases we detect in endocytic structures proximal to the 18°C temperature block.

We next asked whether cycloheximide which blocks protein synthesis would affect the distribution of phosphorylated hydrolases along the endocytic pathway. Cycloheximide was added directly to the culture medium of NRK cells 4 h before their fixation. Cryosections of these treated cells were then incubated with the ectodomain of the CI-MPR-gold conjugates to identify the intracellular compartments which would still contain the phosphorylated hydrolases. Under these conditions, the phosphorylated hydrolases were no longer detected in early endocytic structures proximal to the 18°C temperature block (see Table I), strongly suggesting that they originated from the biosynthetic pathway. However, the amount of labeling over the late endosomes in the cells incubated with cycloheximide for 4 h was 45% that over late endosomes in control cells. After 14 h incubation with cycloheximide, the level of labeling was 10% that over controls (see Table I). We interpret these results as indicating that in these cells dephosphorylation of lysosomal enzymes occurs relatively slowly in late endosomes presumably because exit from late endosomes is rather slow. This would also be consistent with the slow appearance of the fluid phase marker HRP in lysosomes (>2 h of internalization).

Lysosomal Enzymes Are Transient Components of Early Endosomes

The delivery of cathepsin D to early endosomes was monitored in NRK cells metabolically labeled with radioactive tracers. For this, the cells were first labeled with methionine for 10 min and chased for various periods of time at 37°C in the presence of 5 mM Man6-P. At the end of the chase, the cells were incubated for 5 min at 37°C with an affinity-purified antibody against rat cathepsin D. The cells were then
chilled, washed, and kept on ice to allow the formation of the immunocomplex. They were finally lysed in the presence of an excess of purified rat cathepsin D used as a quenching agent and the immunocomplex was precipitated with protein A–Sepharose. In parallel, we also assayed the intracellular processing and secretion of cathepsin D. In NRK cells, cathepsin D was synthesized as a 53-kD proenzyme but rapidly converted to a 47-kD protein which was the major species detected after a 10-min pulse (Fig. 5, top). After a 1.5–2-h chase, this 47-kD intermediate was partly converted to lower molecular mass forms. A small amount (~10%) of the 47-kD form was secreted by the cells (Fig. 5, middle) and efficiently detected after a 1-h chase. After the 10-min pulse, the newly synthesized cathepsin D, which should still be in early stages of the biosynthetic pathway, could not be immunoprecipitated by the anti-cathepsin D antibody present in early endosomes following a 5-min uptake at 37°C (Fig. 5, bottom). However, the 47-kD form of cathepsin D started to meet the internalized antibody after a 20–40-min chase, a time when this form was not yet efficiently detected in the culture medium. The formation of the complex was maximum after a 60-min chase. With longer periods of chase (2 h), the extent of the meeting process decreased as evidenced by a lower amount of the 47-kD cathepsin D immunoprecipitated by the internalized antibody. We conclude from these results that lysosomal enzymes are transient components of the early endosomes. It would also appear that processing of the 53-kD precursor cathepsin D to the 47-kD intermediate form can occur in the biosynthetic pathway of NRK cells before this enzyme is diverted towards the endosomes or the extracellular medium.

Discussion

To define the earliest point of convergence of the biosynthetic and the endocytic pathways, we have combined a biochemical and a morphological approach to identify the endocytic compartments which contain newly synthesized lysosomal enzymes. Both approaches rely on the fluid phase endocytosis of different molecules. Our quantitative analysis of the internalized fluid phase marker HRP strongly suggests that, in NRK cells as described earlier in BHK cells (Griffiths et al., 1989), this marker sequentially enters early endosomes, the spherical endosome vesicles, CI-MPR-rich late endosomes (that we previously referred to as prelysosomal compartment), and finally lysosomes. Surprisingly, the bulk of HRP was efficiently detected in CI-MPR-negative dense lysosomes only after a relatively long time of internalization (>2 h). Since HRP is known to be resistant to proteolysis and can be detected in dense lysosomes a long time after its internalization (Steinman and Cohn, 1972), this suggests that exit from the late endosomes is a rate limiting step in transport to lysosomes. A similar conclusion comes from our morphological observations using the ectodomain of the CI-MPR conjugated to gold which show that phosphorylated lysosomal enzymes are still detected in the late endosomes several hours after treatment of NRK cells with cycloheximide. This is also consistent with the long kinetics of maturation of lysosomal enzymes. For example, in human fibroblasts, mature cathepsin D is first detected in dense lysosomes only after 4 h postsynthesis (Gieselmann et al., 1983).

These morphological data correlate precisely with the kinetics of iodination of the CI-MPR catalyzed by LPO internalized by fluid phase endocytosis. The bulk of the CI-MPR present in the endocytic pathway of NRK cells was efficiently labeled when LPO was internalized by fluid phase endocytosis for >30 min at 37°C. Incubation of these cells for shorter periods of time at 37°C or for 1 h at 18°C resulted in a labeling of <10% of the endosomal CI-MPR accessible to LPO. We interpret these results as indicating that most of the endosomal CI-MPR detected in our biochemical assay is located in late elements distal to the low temperature block. Even if we cannot determine with this technique whether the entire pool of the endosomal CI-MPR is fully accessible to LPO, these data would agree well with our previous EM studies showing that 90% of the intracellular CI-MPR is localized to the late endosomes in NRK cells (Griffiths et al., 1988; Griffiths et al., 1990b). The kinetics of iodination of the newly synthesized hydrolases was strikingly different from that of the CI-MPR. Iodinated lysosomal enzymes were readily detected when LPO was mostly present in early endocytic elements (early endosomes and spherical endosome vesicles) proximal to the low temperature block. This was ~40% of the phosphorylated hydrolases present in the entire endocytic pathway. The early tubulo-vesicular endosomes which, according to our morphological study, should be filled with LPO after a 5-min internalization at 37°C accounted for 15% of the total endosomal phosphorylated hydrolases. When LPO was allowed to fill the late endocytic structures and thereby to label efficiently the CI-MPR, higher proportions of phosphorylated enzymes were detected. It was deduced that these late structures contained ~60% of the phosphorylated endosomal hydrolases.

Our morphological data using the luminal domain of the CI-MPR conjugated to colloidal gold were also consistent with our biochemical observations. The evidence showed that 20–30% of the phosphorylated hydrolases detected in the endocytic pathway were in early structures proximal to the 18°C temperature block; i.e., early endosomes and spherical endosome vesicles. The early endosomes identi-
The values (means and standard error of means) express the number of gold particles per μm² determined from 25 systematically sampled micrographs. Gold doublets were scored as one positive signal. The background established by counting gold particles over the nucleus was estimated to be 0.95 ± 0.26. In the presence of 5 mM Man6-P, no gold particle could be seen on cryosections.

| Marker                          | Early endocytic elements | Late endosomes | Lysosomes |
|--------------------------------|--------------------------|----------------|-----------|
|                                 | 5 nm BSA-gold (5 min, 37°C) | 5 nm BSA-gold (1 h, 18°C) | 16 nm BSA-gold (4 h, 37°C + chase) | Golgi |
| No treatment                    | 10.4 ± 3.1               | 37.9 ± 6.6     | 130.1 ± 35 | ND*       | 1.4 ± 0.8 |
| Treatment with cycloheximide     |                          |                |           |           |          |
| 0 h                             | 12.3 ± 6                 | 26.9 ± 6       | 51.1 ± 9  | ND        |          |
| 4 h                             | ND                       | ND             | 25.2 ± 4  | ND        |          |
| 14 h                            | ND                       | ND             | 5.9 ± 1.5 | ND        |          |

The values (means and standard error of means) express the number of gold particles per μm² determined from 25 systematically sampled micrographs. Gold doublets were scored as one positive signal. The background established by counting gold particles over the nucleus was estimated to be 0.95 ± 0.26. In the presence of 5 mM Man6-P, no gold particle could be seen on cryosections.

* ND; not detectable.

fied by BSA-gold internalized for 5 min at 37°C contained 6–15% of the total labeling and, therefore, we deduced that the spherical endosome vesicles contained ~15% of the total labeling. The remainder of the labeling (~70%) was associated with the late endosomes and essentially none with lysosomes. In NRK cells, the Golgi complex exhibited a low labeling with the CI-MPR-gold conjugates (<1% of the total labeling). Because MPR-bound lysosomal enzymes usually have multiple phosphorylated oligosaccharides and are most likely detected by this probe, we would rather interpret this finding as indicating that the phosphorylated enzymes are rapidly transported from the TGN to endosomes. Finally, no other structures were labeled under these conditions. Collectively, our biochemical and morphological data indicate that early endosomal elements contain significant amounts of newly synthesized lysosomal enzymes.

The presence of phosphorylated hydrolases in early endosomes could be explained either by an intracellular transport pathway or by a constitutive secretion followed by a recapture mechanism. In the latter case, addition of Man6-P to the culture medium, which induces rapid dissociation of preexisting hydrolase-MPR complexes (Fisher et al., 1980) at the cell surface, should reduce the amount of phosphorylated hydrolases in early endosomes. On another hand, if delivery occurs from intracellular compartments, this process would not be affected even by the internalized Man6-P and, therefore, the absolute amount of hydrolases found at steady state in early endosomes should not be modified. When NRK cells were grown in the presence of Man6-P, the same amount of phosphorylated hydrolases was found in early endosomes. It is worth mentioning that phosphorylated hydrolases were also detected in early endosomes of J774 macrophages (not shown). In these cells, however, trafficking of lysosomal enzymes is mediated only by the CD-MPR, which does not function in recapture of exogenous phosphorylated ligands (Stein et al., 1987). Our data suggest, therefore, that the hydrolases found in the early endosomes first originated from an intracellular pathway. Accordingly, phosphorylated hydrolases were no longer detected by EM in early endosomes and spherical endosome vesicles when cycloheximide was directly added to the culture medium of NRK cells.

Two different intracellular pathways can be then considered, either recycling from CI-MPR-rich late endosomes or direct delivery from the Golgi complex. We believe, however, that the former pathway would account, at the most, for a minor fraction of the hydrolases detected in early endosomes. Many studies have documented the internalization of fluid phase markers along the endocytic pathway of various cell types. There is, however, no evidence in the literature for a significant recycling of fluid phase content from late to early endosomes. We have previously shown using DAMP labeling that, in NRK cells, these latter structures had an acidic pH consistent with the dissociation of the lysosomal enzymes from their receptors (Griffiths et al., 1988). It would be difficult to imagine how these soluble lysosomal enzymes, behaving as fluid phase components in late endosomes, could move backwards to the early endocytic structures. We would therefore conclude that the lysosomal biosynthetic and the endocytic pathways can converge in early endosomes. Thus, it would appear that late endosomes are not the unique site of delivery of lysosomal enzymes as suggested only by its high content of CI-MPR (Griffiths et al., 1988) which most likely accumulates in these structures before being recycled to the TGN.

The newly synthesized hydrolases are transient components of early endosomes being detected in this compartment.
The presence of Man6-P Asa control (0c: first lane), the antibody resistance of some of their oligosaccharides (Gieselmann et al., 1983). Due to this reason and also to our inability to internalize -Man6-P. The cells were then processed for iodination of endosomal proteins and lysed with detergents. Iodinated hydrolases were purified on Cl-MPR affinity columns as described under Materials and Methods. The radioactive fractions of the Man6-P eluate were pooled and counted.

NRK cells were grown for 16 h with or without 5 mM Man6-P in the culture medium. LPO was internalized for 1 h at 4, 18, and 37°C in the presence or the absence of 5 mM Man6-P. The cells were then processed for iodination of lysosomal enzymes or whether this pathway reflects some redistribution of lysosomal enzymes otherwise destined to late endosomal elements. In vitro reconstitution of this pathway may help to answer this question.

Our data would imply that, at steady state, low concentrations of lysosomal enzymes are maintained in early endosomes. They would be the first compartment along the endocytic pathway in which an internalized molecule meets degradative enzymes. Others have also reported that early elements of the endocytic pathway in other cell types may contain proteases. In none of these studies, however, was the origin of the hydrolases determined. Thus, Diment and Stahl (1985) observed that rabbit alveolar macrophages can degrade mannose-BSA as early as 6 min after its uptake at 37°C. They identified a 46-kD molecular form of a Cathepsin D-like protease which is, in part, membrane associated (Diment et al., 1988). Similarly in 3T3 cells, the degradation of a fluorogenic substrate of Cathepsin B occurred after 3 min of internalization at 37°C and was still detected at 17°C (Röderer et al., 1987). It appears, therefore, that hydrolases are potentially active in these early compartments. Identical observations were reported for rat hepatocytes (Casciola-Rosen and Hubbard, 1991). This could be of importance for rapid inactivation of some biologically active molecules. Liver cells for example can degrade insulin with kinetics which are consistent with degradation occurring in early endocytic elements (Doherty et al., 1990). It is likely that for many substrates, this degradation process does not go to completion since only low concentrations of lysosomal enzymes (our study) and mild acidic conditions are found in these compartments (Fuchs et al., 1989; Cain et al., 1989). Limited degradation occurring in early endosomes could also be important for the processing of antigens whose peptides have to be presented at the cell surface of specialized cells by the class II histocompatibility molecules (for review see Yewdell and Bennik, 1990). However, the precise compartment(s) where antigen processing and binding to the MHCII complex occurs as well as its route(s) of recycling to the plasma membrane, have still to be defined.

We would expect that most of the phosphorylated hydrolases detected in early endosomes are diverted towards later endocytic structures, either as bound ligand or as part of the fluid. We found by EM a significant fraction of phosphorylated lysosomal enzymes in spherical endosome vesicles proximal to the 18°C block, with a morphological aspect very reminiscent of the "carrier vesicles" which are thought to transport endocytosed material to late endosomes (Gruenberg et al., 1989) or to mature into these late structures. In early endosomes of NRK cells, 25% of the fluid is recycled to the cell surface (our unpublished observations). Soluble hydrolases recycling to the cell surface as part of the fluid could contribute to secretion. It remains however to establish what fraction of hydrolases is still receptor-bound in early endosomes since their mild acidity would be compatible with a tight interaction between the hydrolases and their receptors. It is likely that some MPRs and their bound ligand are recycled to the cell surface. If the presence of the MPRs on the plasma membrane could be explained by missorting in the TGN, a pathway connecting the TGN and the early endocytic compartment could provide an alternative explanation. This could appear as a futile pathway for the newly synthesized hydrolases bound to the Cl-MPR. The interaction of ligand with this receptor is stable at the pH found at the cell surface and therefore the bound hydrolase would be reinternalized along with exogenous phosphorylated ligands. This would be compatible with the function of the Cl-MPR in intracellular targeting of lysosomal enzymes: expression of the Cl-MPR in cells lacking this protein results in a reduction of secretion of hydrolases (Kyle et al., 1988; Lobel et al., 1989).

Table II. Effect of Man6-P on the Distribution of Hydrolases in the Endocytic Pathway of NRK Cells

| Temperature of internalization | −Man6-P | +Man6-P |
|--------------------------------|---------|---------|
| cpm                           | %       | cpm     | %       |
| 4°C                            | 2,100   | 8       | 2,345   | 11      |
| 18°C                           | 9,520   | 39      | 10,263  | 50      |
| 37°C                           | 24,406  | 100     | 20,674  | 100     |

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al., 1989). The situation is however different for the hydro-
lases bound to the CD-MPR. As shown earlier (Hoflack et al., 1987; Tong and Kornfeld, 1989), the affinity of this
receptor for ligand is low at the neutral pH of the extracellu-
lar medium, and accordingly, CD-MPR does not bind phos-
phorylated ligands at the cell surface (Stein et al., 1987). It
is likely therefore that the phosphorylated hydrolyses bound to
this receptor would dissociate upon their arrival at the plasma
membrane and be released into the culture medium.
Subsequent to the TGN-early endosome pathway, the CD-
MPR could function both in secretion of newly synthesized
lysosomal enzymes and in their intracellular targeting to
lysosomes. A recent report showed, in fact, that overexprer-
ession of the CD-MPR in BHK cells could induce elevated
secretion of lysosomal enzymes (Chao et al., 1990). The at-
tractive possibility that the CD-MPR could regulate secre-
tion of lysosomal enzymes could explain why two Man6-P
receptors with different binding properties are found in
mammalian cells.

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