Polarized Secretion of Lysosomal Enzymes: Co-Distribution of Cation-Independent Mannose-6-Phosphate Receptors and Lysosomal Enzymes along the Osteoclast Exocytic Pathway

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Abstract. The osteoclast is a polarized cell which secretes large amounts of newly synthesized lysosomal enzymes into an apical extracellular lacuna where bone resorption takes place. Using immunocytochemical techniques, we have localized the cation-independent mannose-6-phosphate (Man6P) receptor and lysosomal enzymes in this cell type in order to determine the expression and distribution of this receptor and its ligands. The results demonstrate that the osteoclast expresses large amounts of immunoreactive cation-independent Man6P receptors, despite the fact that most of the lysosomal enzymes it synthesizes are secreted. The lysosomal enzymes and the receptors are co-distributed along the exocytic pathway, i.e., the endoplasmic reticulum, including the perinuclear envelope, the Golgi stacks as well as numerous small transport vesicles that appear to fuse with the ruffled border membrane. Within the Golgi complex, the receptors and lysosomal enzymes were found distributed in two predominant patterns; (a) in all the cisternae, from cis to trans, or (b) predominantly in cis- and trans-Golgi cisternae, with the middle Golgi cisternae being unstained or depleted in antigen. This pattern suggests that enzymes and receptors traverse the Golgi from cis to trans and preferentially accumulate in cis- and in trans-cisternae. This study therefore suggests that, in the osteoclast, Man6P receptors are involved in the vectorial transport and targeting of newly synthesized lysosomal enzymes, presumably via a constitutive pathway, to the apical membrane where they are secreted into the bone-resorbing compartment. This mechanism could insure polarized secretion of lysosomal enzymes into the bone-resorbing lacuna.
Antibodies against the 215-kD CI Man6P receptor were as previously described in rat liver microsomes; antibodies specifically directed against the ligand-Man6P receptor system is present. We con-
ducted studies (28, 29, 41). The fact that a number of cell lines have been found in the trans-Golgi of some other cell types (9, 19, 23, 49), leading to the hypothesis that sorting was not occurring at the cis-side but in the trans-region (23). The interpretation of these data is complicated by the superimposition of intracellular sorting (Golgi region to endosomes), recycling (plasma membrane and endosome to Golgi region) and endocytic (plasma membrane to endosome) pathways, which join at the delivery site and in the Golgi region (9, 18).

Most studies, however, agree on the presence of high concentrations of Man6P receptors in late endosomes but not in lysosomes (10, 19, 23), suggesting a Golgi–endosome–Golgi cycle (10, 36).

Second, the concept that intracellular targeting to lysosomes depends upon the presence of both the Man6P recognition marker and the receptors is supported by the observation that mutants which lack either the recognition marker or the receptors secrete most of the lysosomal enzymes they synthesize (28, 29, 41). The fact that a number of cell lines deficient in cation-independent (CI) Man6P receptors can still transport and correctly target their lysosomal enzymes suggested the presence of alternative sorting mechanisms (16, 17). A second type of receptor with a relative molecular mass of 46 kD and a dependence on cations for binding has later been described, and both receptors have been cloned and sequenced (13, 25, 32, 38). The cation-dependent receptor also appears to function in the targeting of newly synthesized lysosomal enzymes although its exact role remains to be established (28).

Therefore, it seems that: (a) there is some variability in the distribution of Man6P receptors among cell types, reflecting the fact that various cells are functionally specialized (9); and (b) secretion of lysosomal enzymes occurs when a defective ligand–Man6P receptor system is present. We consequently thought it would be of interest to look at the distribution of Man6P receptors and lysosomal enzymes in the osteoclast, a cell which mostly secretes these enzymes (46).

Our goal was to determine whether the osteoclast uses the CI Man6P receptor for lysosomal enzyme transport and targeting, or lacks Man6P receptors, or positions them differently along the biosynthetic pathway. Toward this end, we have studied the distribution of Man6P receptors in the osteoclast as compared with that of lysosomal enzymes.

Materials and Methods

Antibodies

Antibodies against the 215-kD CI Man6P receptor were as previously described (7). Briefly, rabbits were immunized with Man6P receptors purified from rat liver microsomes; antibodies specifically directed against the Man6P receptor (antibody R2) were subsequently affinity purified from the crude antiserum. These affinity-purified antibodies specifically recognize only the 215-kD CI Man6P receptor (7) by immunoprecipitation and immunoblotting. R2 was used at a concentration of 50 μg/ml for the immunocytochemical procedures.

Antibodies against cathepsin C were provided by F. Mainferme and prepared as previously described (33). Briefly, rabbits were immunized with cathepsin C purified from rat liver according to the method of McDonald et al. (34). The IgG fraction was subsequently used and specifically immunoprecipitated multiple forms of the enzymes (see reference 33 for a detailed description of the antigen). The IgG fraction was used at a concentration of 100 μg/ml for immunolocalization.

Antibodies against beta-glucuronidase were provided by M. Rosenfeld and prepared as previously described (42). Briefly, beta-glucuronidase was purified from rat preputial glands according to Himeno et al. (24) and used as antigen in rabbits. The antiserum was further purified by affinity chromatography against the enzyme. The antiserum immunoprecipitates a 72-kD polypeptide from culture medium (42). A dilution of 1:100 was used for immunolocalization.

Antibodies against the alpha-2,6-sialyltransferase were provided to us by J. Paulson and previously described in detail (43). Briefly, polyclonal rabbit antiserum against the rat liver alpha-2,6-sialyltransferase, purified according to Weinstein et al. (48), was affinity purified and recognized, by immunoblot, a 47-kD doublet both in purified antigen and rat liver Golgi preparations. The affinity-purified IgG was used at a concentration of 200 μg/ml for immunolocalization.

Controls were incubated with rabbit nonimmune serum or in the absence of the primary antibodies and processed in parallel.

Ultrastructural Localization of Antigens by Immunoperoxidase

5-d-old Wistar rat pups were perfused via the femoral artery with MEM for 1 min followed by paraformaldehyde (2%)–lysozyme (0.025 M)–sodium periodate (0.01 M), (PLP; 35) or glutaraldehyde (0.1% in 0.1 M cacodylate buffer) for 5 min. The distal femur and the proximal tibia were then quickly dissected out and slices cut out of the primary spongosia area under the growth plate. When using PLP as a fixative, the slices were fixed for an additional 4 h at 4°C and then washed in PBS containing 10% DMSO as a cryoprotectant. The tissue was subsequently frozen in liquid nitrogen, and 40-μm sections were prepared on a Bright cryostat (Huntingdon, England) equipped with tungsten carbide knives. The sections were fixed for an additional 20 min in PLP, washed in PBS, and incubated overnight with the respective primary antibodies diluted in PBS with 0.1% BSA. After washing (1–2 h) in PBS + 0.1% BSA the sections were incubated with Fab fragments of peroxidase-labeled sheep anti-mouse IgG (Biosys, Marne la Coquette, France) at a dilution of 1:100 for 2 h. After further washing, the sections were additionally fixed in 2% glutaraldehyde, and quenched in 0.1 M lysine, and washed. The sections were then reacted in DAB (1 mg/ml in 0.05 M Tris buffer, pH 7.4) in the presence of 0.01% H2O2 and postfixed in ferrocyanide-reduced OsO4. After embedding in epon (Polybed 812; Polysciences, Inc., Warrington, PA), 1-μm-thick sections were cut with a glass knife and counterstained with methylene blue for identification of areas of interest; the selected areas were then sectioned with a diamond knife, and the sections were stained with lead citrate.

Enzyme Cytochemistry

5-d-old Wistar rats were perfused via the femoral artery with MEM for 1 min followed by 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 5 min. Growth plates were dissected out and further fixed by immersion in the same fixative containing 7% sucrose for 1 h. They were washed in buffer plus 10% DMSO for 1 h, frozen in liquid nitrogen, and 40-μm cryostat sections prepared. The sections were decalcified in 4% EDTA, 5% polyvinyl-pyrrolidone, and 7% sucrose, pH 7.4, at 4°C for 15–20 h, washed in 0.1 M cacodylate for 24–48 h, and incubated in the appropriate medium.

Arylsulfatase was demonstrated according to Goldfischer (20), as modified by Bentfeld-Barker and Bainton (4), using p-nitrocathecol sulfate as a substrate. For the demonstration of acid phosphatase, the Barka and Ander- son media were used (1), with beta-glycerophosphate as substrate. Control preparations were incubated without substrate. Sections were postfixed in 1% OsO4 for 1 h at 4°C, dehydrated, and embedded in epon. Grids were stained with uranyl acetate and lead citrate.
Results

Functional Orientation of Golgi Complexes in the Osteoclast

To establish whether osteoclasts had characteristic cis- and trans-Golgi compartments and to determine their orientation in Golgi stacks, we first immunolocalized alpha-2,6-sialyltransferase, a trans-Golgi marker enzyme (43). The results confirmed that Golgi complexes are disposed in a characteristic perinuclear fashion in the osteoclast (Fig. 1 A). Furthermore, alpha-2,6-sialyltransferase was present in each stack of Golgi cisternae where it was restricted to one or two cisternae that were located away from the nuclear membrane (Fig. 1, A and B). Both in the osteoclast and in the other cell types present in our preparations, this enzyme was exclusively restricted to the membranes of the trans-Golgi cisternae. The restricted localization of alpha-2,6-sialyltransferase thus demonstrates that, in osteoclasts, Golgi complexes are polarized and functionally compartmentalized with the cis-cisternae located next to the perinuclear cisterna and the trans-cisternae facing away from the nuclei.

Localization of Lysosomal Enzymes

We then proceeded to localize lysosomal enzymes, using both cytochemical and immunocytochemical methods. As previously reported (2), arylsulfatase and beta-glycerophosphatase were found by enzyme cytochemistry all along the exocytic pathway: in the endoplasmic reticulum (ER), in all Golgi stacks, and in coated transport vesicles which appeared to fuse with the apical plasma membrane at the ruffled border (Figs. 2, A–D, and 3 A). However, it was not possible to clearly demonstrate the presence of these enzymes in the extracellular bone resorbing space.

By immunocytochemistry, cathepsin C and beta-glucuronidase were found in the lysosomes of every cell type present in these bone sections, thereby demonstrating that the antigens were appropriately recognized by the antibodies in our preparations. In the osteoclast, these enzymes were found: (a) in a few secondary lysosomes; (b) in a number of small vesicles and a network of cisternal elements belonging to the ER; (c) in Golgi cisternae including the trans-Golgi network (Fig. 2, E–H); (d) as well as in small transport vesicles located in the Golgi regions and close to or in contact with the ruffled border membrane; and (e) in coated pits interpreted as the budding sites of the transport vesicles (Fig. 3 B). Inside the cell, beta-glucuronidase was more regularly detected than cathepsin C. In contrast to the results obtained by enzyme cytochemistry, immunoreactive beta-glucuronidase and especially cathepsin C could also be detected in the extracellular space between the fingerlike projections of the ruffled border (Fig. 3, B and D).

In the Golgi complexes, whether by immunocytochemistry or by enzyme cytochemistry, we most frequently found all these enzymes either in all the cisternae or predominantly in cis- and trans-Golgi cisternae (Fig. 2, E–G). They were also localized in some more rigid elements of the trans-Golgi network, often showing coated extremities (Fig. 2 E), as well as in numerous coated vesicles.

Although arylsulfatase and beta-glycerophosphatase could be detected by enzyme cytochemistry in a large majority of the numerous coated vesicles found in the vicinity of each Golgi stack and presumed to be transport vesicles, it was not possible to regularly detect them with the immunocytochemical methods used here (See Fig. 2). Since labeling for beta-glucuronidase was very strong in the ER and Golgi cisternae (Fig. 2), the difference between the results obtained with these two methods at the level of coated vesicles cannot be explained on the basis of the concentration of antigen but more likely is due to limited diffusional access of the antibodies to the inside of these small structures (50–75 nm diam). However, based upon the results obtained by enzyme
Figure 2. Distribution of arylsulfatase (A-D) and beta-glucuronidase (E-H) in the ER and the Golgi stacks of the osteoclast. (A-D) Glutaraldehyde fixation; (E-H) PLP fixation. The ER is consistently labeled for both enzymes (D and H). Although the distribution of the two enzymes in the Golgi cisternae is very similar, more consistent labeling of coated transport vesicles is achieved with enzyme cytochemistry (A-C) than with immunocytochemistry (E-G). The enzymes are often found in the perinuclear envelope (A, E-G) and in
Figure 3. Localization of lysosomal enzymes and Man6P receptors along the ruffled border membrane of the osteoclast and in the extracellular bone-resorbing compartment. (A and C) Glutaraldehyde fixation; (B and D) PLP fixation. Beta-glycerophosphatase (A) and betaglucuronidase (B) are both found associated with coated pits (B, arrows) and in the extracellular areas (e) immediately adjacent to these pits; this distribution is exactly similar to the localization of Man6P receptors (C). In addition, cathepsin C is also found to fill most of the extracellular spaces (e) between the fingerlike projections of the ruffled border (D) in the bone-resorbing compartment. Upon reaching the low pH bone-resorbing compartment, the lysosomal enzymes are released from the Man6P receptors which carried them intracellularly. Bars, 0.15 μm.

Cytochemistry, we can safely conclude that lysosomal enzymes are present in a large majority of these transport vesicles (Fig. 2 and 3). Vesicles containing lysosomal enzymes were also found between the Golgi cisternae and numerous vacuoles associated with the ruffled border where they probably fuse at various levels with the deep membrane infoldings (Fig. 3 A).

These observations demonstrate that lysosomal enzymes can be detected along the entire exocytic pathway of the osteoclast (ER, Golgi cisternae, presumptve transport vesicles), including the apical extracellular space. We interpret these data as further demonstration (2) that the osteoclast synthesizes and vectorially transports lysosomal enzymes packaged into small coated vesicles which are secreted into

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all cisternae of the Golgi stacks (A, B, E, and F); the middle region of the Golgi was however often depleted in enzymes (B and F) leading to a frequent pattern of cis- and trans-most cisternae labeling (C and G). In the trans-region of the Golgi, cisternal elements often showed coated extremities (E, arrow) and a morphology reminiscent of the trans-Golgi network (E and F). These findings suggest that, from the endoplastic reticulum, the newly synthesized enzymes traverse the Golgi from cis to trans and accumulate in cis- and trans-cisternae. n, nucleus. Bars: (A-C, E-G) 0.2 μm; (D and H) 0.5 μm.
the sealed off extracellular bone-resorbing compartment. It is noteworthy that, in most cases, the enzymes were detected throughout the Golgi cisternae, although often with a higher signal in cis- and trans-cisternae.

**Distribution of the CI Man6P Receptor**

Having established the functional orientation of the Golgi stacks and the distribution of lysosomal enzymes in osteoclasts, we then immunolocalized the CI Man6P receptor in order to compare its distribution to that of lysosomal enzymes.

Although most cells present in our tissue preparations demonstrated some degree of staining, the osteoclast was intensely labeled after incubation with the anti-Man6P receptor IgG. At the ultrastructural level, reaction product was present all along the secretory pathway (rough ER, Golgi, and transport vesicles). The antigen was strongly labeled in the endoplasmic reticulum where heavy deposits of reaction product were seen in virtually every osteoclast (Fig. 5 A). Man6P receptors were also detected in all the perinuclear stacks of Golgi cisternae where it assumed various patterns of distribution (Fig. 4). The most frequent pattern consisted of receptors in all the cisternae of the stacks from cis to trans (Figs. 4, B and C), including some elements of the trans-Golgi network (Fig. 4, A and B), as well as in a number of small coated transport vesicles in the Golgi area (Figs. 4 A, and 5, B and C). The second predominant labeling pattern consisted of Man6P receptors in only the cis- and the trans-cisternae but not the middle one or two cisternae (Fig. 4, A and C). As for lysosomal enzymes, the middle Golgi cisternae often showed traces of the antigen, more frequently toward their dilated extremities. Finally, more rarely, we also observed Golgi stacks with only the cis-most (Fig. 4 D), the middle, or the trans-most cisternae labeled (Fig. 5 B). Frequently, the extremities of the trans-most labeled cisternae showed coated membranes, suggestive of budding or fusion of coated transport vesicles (Fig. 5, A-C).

At the level of the ruffled border membrane, Man6P receptors (Fig. 5, D and E) were found only in coated pits on the plasmalemma or coated vesicles close to the membrane (Fig. 5, D and E, Fig. 3 C). Similar structures were also found at the basolateral membrane, albeit much less frequently. Finally, in all cells the CI Man6P receptor was found in multivesicular bodies and vacuoles apparently belonging to the endocytic pathway.

As mentioned earlier for the immunolocalization of lysosomal enzymes, the limited accessibility of the lumen of the small transport vesicles in the Golgi regions made it difficult to firmly establish the presence of the receptors in these structures. However, experiments in which the permeability of the tissue was either intentionally increased with saponin (0.01%) or unintentionally disrupted, as in areas close to the cut surface of the frozen sections, many vesicles containing immunoreactive receptors were detected (Figs. 4 A, and 5, B and C). This convinced us that most, if not all, of the coated vesicles in the Golgi region contained Man6P receptors. The distribution of the CI Man6P receptor in the osteoclast, therefore, essentially paralleled that of lysosomal enzymes as described above.

We interpret these data to show that in the osteoclast, the CI Man6P receptor co-distributes with lysosomal enzymes from their site of biosynthesis to their site of delivery. In the ER this probably reflects the high level of synthesis of the receptor protein itself, whereas its presence throughout the Golgi cisternae and in transport vesicles from the Golgi area to the ruffled border membrane is consistent with the idea that Man6P receptors are involved in the transport and targeting of lysosomal enzymes to their delivery site, be it the endosome for endocytosis in other cells or the apical plasma membrane for polarized secretion in the osteoclast.

**Discussion**

By using immunocytochemical techniques, we have localized the cation-independent mannose-6-phosphate receptor and lysosomal enzymes in the osteoclast in order to compare the expression and distribution of the receptor with that of its ligands in a polarized cell which secretes large amounts of newly synthesized lysosomal enzymes. We were particularly interested in knowing: (a) whether a cell in which lysosomal enzymes are largely secreted would express the CI Man6P receptor at detectable levels, and (b) if so, how the distribution of this receptor along the exocytic and/or the endocytic pathways would compare with that of other cell types reported so far.

The results demonstrate that the osteoclast expresses high amounts of the CI Man6P receptor. Moreover, the receptor appears to co-distribute with the four lysosomal enzymes we have localized, namely beta-glucuronidase and cathepsin C by immunocytochemistry and arylsulfatase and beta-gluceronphosphatase by enzyme cytochemistry. Both the receptors and their ligands are found most prominently along the exocytic pathway: the membranes of the endoplasmic reticulum (including the nuclear envelope), of Golgi cisternae and coated transport vesicles all contained the Man6P receptor. Immunoreactive Man6 receptors were also found along the ruffled border membrane, the apical delivery pole of the cell, but only in small coated pits. We conclude that the CI Man6P receptor is actively synthesized and widely distributed along the exocytic pathway in the osteoclast, even though this cell vectorially secretes most of the lysosomal enzymes that it synthesizes.

We interpret this data as follows: (a) newly synthesized lysosomal enzymes bind to Man6P receptors in the Golgi cisternae, are carried through the Golgi cisternae up to the trans-most cisternae where coated transport vesicles containing these enzymes are formed; (b) the coated vesicles transport the lysosomal enzymes to the apical pole of the cell (towards the bone matrix) where they fuse with the ruffled border membrane, exposing their content to the low pH of the bone resorbing compartment; (c) in this acidic environment, lysosomal enzymes dissociate from the Man6P receptors and are released in the extracellular space where they act upon their substrate (i.e., the bone matrix proteins); and (d) by analogy with other systems, free receptors may then be recycled back to the Golgi complex for additional rounds of transport. It is interesting, however, that this cell seems to synthesize more new Man6P receptors than most other cell types, as indicated by the strong labeling of its endoplasmic reticulum. This study therefore suggests that in the osteoclast, Man6P receptors are involved in the vectorial transport and targeting of newly synthesized lysosomal enzymes to the apical membrane for extracellular bone resorption.
Figure 4. Immunolocalization of the 215-kD cation-independent Man6P receptor in the Golgi stacks of the osteoclast. (A–C) Glutaraldehyde fixation; (D) PLP fixation. The distribution of Man6P receptors in the Golgi stacks of the osteoclast parallels that of the lysosomal enzymes (compare with Fig. 2). The receptors were found either in all cisternae of the Golgi stacks (B and C) or in both cis- and trans-most cisternae (A); exclusive cis-most labeling was also encountered (D, arrowheads). Coated transport vesicles (A, small arrows) and coated cisternal ends (open arrows) were frequently observed, often in elements resembling the trans-Golgi network (A, B, and D). Newly synthesized lysosomal enzymes and Man6P receptors, therefore, have similar distributions in the osteoclast Golgi regions. n, nucleus. Bars, 0.1 μm.

These observations can be contrasted with other situations in which lysosomal enzymes are secreted because they are deficient in Man6P receptors or the Man6P recognition marker (29, 41), implying that secretion of lysosomal enzymes occurs primarily as a consequence of defective receptor or ligand. The present observations would indicate that (a) Man6P receptors can be used not only for targeting lysosomal enzymes to intracellular compartments, but also for targeting them for secretion and, (b) the secretion of newly synthesized lysosomal enzymes does not necessarily imply Man6P-independent mechanisms. These data strongly suggest that constitutive secretion can also be signal mediated,
zyme-receptor dissociation at the cell surface. In most other delivery of secretory proteins to at least one pole of the cell as in other polarized cells active sorting may be required for and need not occur by a bulk flow mechanism (27, 44): here as in other polarized cells active sorting may be required for targeting to intracellular compartments.

The fact that the bone-resorbing compartment has a low pH (2) renders it highly improbable that the coated pits and coated vesicles located along the ruffled border membrane which carry Man6P receptors and lysosomal enzymes represent elements of the endocytic pathway because binding of lysosomal enzymes to the Man6P receptor could not occur in this acidified microenvironment (26). Both the ruffled border and late endosomal membranes, are therefore the targets for Man6P-positive coated vesicles transporting lysosomal enzymes. This is noteworthy because the 100-kD membrane proteins that we have found to be present at the ruffled border are also present in endosomes (31, 40), whereas strictly lysosomal membrane glycoproteins (lgp 120 and 110; 30) were absent from the ruffled border membrane (3).

In terms of traffic pathways for lysosomal enzymes, the distribution of the latter and of Man6P receptors in the multiple Golgi stacks of the osteoclast is potentially of interest. Despite the fact that, as established by immunolocalization of alpha-2,6-sialyltransferase, all the Golgi stacks in the osteoclast are polarized and oriented in the same way, with the cis-cisternae close to the nuclei and the trans-cisternae away from the nuclei, we found a certain variability in the distribution of the Man6P receptors in the Golgi complex within the same cell type and even among the various Golgi stacks in the very same cell. The distribution of both the lysosomal enzymes and the Man6P receptors were essentially of two predominant types at the Golgi level. The most frequent was the presence of the receptors throughout all cisternae of the Golgi stacks. The other frequent pattern was represented by Golgi stacks in which only one or two cis-cisternae and one or two trans-cisternae were labeled, leaving one or two middle Golgi cisternae either unstained or much less intensely labeled for both the receptors and their ligands. In addition, some Golgi stacks showed Man6P receptors in only one or two cisternae, the labeled cisternae being equally often in the cis, trans, or middle region of the Golgi. Therefore, the Golgi stacks within the same cell and even around the same nucleus exhibited the various types of distribution previously encountered in different cell types (7, 9, 19, 23, 49). This raises the interesting possibility that the variability in CI Man6P receptor distribution in the Golgi cisternae reflects various functional states rather than essentially different pathways.

In conclusion, this immunocytochemical study demonstrates that a cell actively engaged in the synthesis of lysosomal enzymes destined for polarized secretion also highly expresses immunoreactive CI Man6P receptors. Whether or not the osteoclast secretes other proteins apically or basolaterally, the secretory lysosomal enzymes still have to be targeted to the apical membrane. Our observations thus raise the possibility that Man6P receptors are not only required for targeting to intracellular compartments, but also are used in the active sorting and targeting of newly synthesized lysosomal enzymes to the apical pole of the osteoclast where they are secreted into an acidic extracellular compartment.

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