Ultrastructural Localization of the Mannose 6-Phosphate Receptor in Rat Liver

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ABSTRACT  An affinity-purified rabbit antibody against rat liver mannose 6-phosphate receptor (MP-R) was prepared. The antibody was directed against a 215 kd-polypeptide and it recognized both ligand-occupied and free receptor. Anti-MP-R was used for immunofluorescence and immunoelectron microscopy of cryosections from rat liver. MP-R was demonstrated in all parenchymal liver cells, but not in endothelial lining cells. MP-R labeling was found at the entire plasma membrane, in coated pits and coated vesicles, in the compartment of uncoupling receptor and ligand, and in the Golgi complex. Lysosomes showed only scarce MP-R label.

In double-labeling immunoelectron microscopy, MP-R co-localized with albumin in the Golgi cisternae and in secretory vesicles with lipoprotein particles. Cathepsin D was associated with MP-R in the Golgi cisternae. This finding indicates that MP-R/cathepsin D complexes traverse the Golgi complex on their way to the lysosomes. The possible involvement of CURL in lysosomal enzyme targeting is discussed.

Studies on biosynthesis of lysosomal enzymes have established that these enzymes are synthesized in the rough endoplasmic reticulum where they are co-translationally directed from the ribosomes to the lumena of the endoplasmic reticulum (ER). The newly synthesized lysosomal enzymes are subjected to a series of modifications including glycosylation, phosphorylation, and other changes in the structure of the carbohydrate and protein moieties as they are transported from the ER to the Golgi complex and lysosomes (for recent review see references 1 and 2). Increasing evidence indicates that the enzymes are segregated from secretory proteins by membrane receptors that recognize mannose 6-phosphate residues, which are present uniquely on lysosomal enzymes. Biosynthesis of mannose 6-phosphate residues in the enzymes is catalyzed by N-acetylglucosamine-1-phosphotransferase and N-acetylglucosaminyl phosphodiesterase. These enzymes are associated with Golgi membrane fractions of higher or intermediate buoyant density, which probably correspond to the cis or intermediate cisternae of the Golgi complex (3, 4). These fractions are rich in α-mannosidase I (3, 4), (presumably a marker of the cis part of the Golgi complex (5)) and contain little galactosyl transferase, which is associated with Golgi membranes of lowest density (5) and with trans cisternae of the complex (6–8). This observation and the fact that lysosomal enzymes contain complex or hybrid type oligosaccharides (4, 9) that are exclusive products of trans-Golgi processes, strongly suggest that lysosomal enzymes travel in a cis to trans direction across the stack of Golgi cisternae prior to delivery to the lysosomes.

In spite of this progress there is no definite answer as to the genesis of primary lysosomes. Studies utilizing electron microscope enzyme cytochemistry indicated that lysosomes are formed from either the Golgi apparatus (10–12) or from Golgi-associated endoplasmic reticulum lysosomes (GERL) (13). GERL consists of fenestrated cisternae at the trans aspect of the Golgi complex. It was proposed to receive lysosomal enzymes directly from the rough ER (13). Thus, according to the GERL concept, lysosomal enzymes bypass the Golgi cisternae and are segregated from secretory products in GERL (for reviews see references 14 and 15).

To more precisely establish the route of lysosomal enzyme transport and sorting, we have described the subcellular distribution of mannose 6-phosphate receptor (MP-R) together with a lysosomal enzyme (cathepsin D) and secretory protein (albumin) in rat liver parenchymal cells. We used both mon-
specific antibodies against albumin, cathepsin D, and MP-R, and our double-labeling immuno electron microscope method (16, 17). The MP-R was present throughout the stack of Golgi cisternae, in secretory vesicles, and in a membrane reticulum that we have earlier called the compartment of uncoupling receptor and ligand (CURL) (18). MP-R occurred also at the entire plasma membrane and in coated pits and vesicles. Albumin and cathepsin D were present in the Golgi cisternae, the latter in an apparent association with MP-R. Our observations in general support and extend those of Willingham et al. (36) in Chinese hamster ovary cells and are most consistent with the notion that lysosomal enzymes traverse the entire Golgi complex en route to the lysosomes.

MATERIALS AND METHODS

Antibodies: Affinity-purified rabbit antibodies were used for immunocytochemistry at a concentration of 25–50 μg protein/ml. Anti-rat albumin (19) and anti-human placental cathepsin D (20) antiserum have been described previously. Preparation of anti-rat liver MP-R was as follows.

Purification of Rat Liver MP-R: The procedure used for preparing Dicytostelium discoideum glycoprotein secretions was that of Fischer et al. (21). This material (containing 78 mg of protein and 790 μl of β-hexosaminidase) was coupled to 30 ml of CNBr-activated Sepharose 4B (Pharmacia Inc., Freiburg, Federal Republic of Germany) according to the recommendation of the manufacturer. One unit of enzyme activity is defined as that amount of enzyme capable of cleaving 1 μmol of substrate per min at 37°C. The general procedure for isolating MP-R was that of Sahagian et al. (22), except that the affinity column described above was used instead of a phenomannan-Sepharose 4B column. Extracts from 180 g of rat liver were applied to the column and yielded 4.0 mg of protein in the final step. The protein was concentrated by ultrafiltration (final concentration 0.3–1.2 mg/ml) and dialyzed against the above buffer, concentration by ultrafiltration and centrifugation was repeated twice yielding 0.3 and 0.1 mg of protein, respectively. The precipitate was collected by addition of 25 μl of Immuno-Precipitin. The pellets were washed with 1 ml of buffer A containing 0.01% SDS, and finally with 1 ml of 0.1 M NaCl in 10 mM sodium phosphate, pH 7.4. The pellet was solubilized and solubilized material was subjected to polyacrylamide gel electrophoresis (12.5% polyacrylamide) and fluorography as described (29).

Reactivity of Anti-MP-R with Occupied Receptor: The following procedure is that of Sahagian et al. (22). 2.5 ml of β-hexosaminidase affinity purified (30) from secretions of human skin fibroblasts grown for 2 d in serum free medium supplemented with 10 mM N-acetyl-L-Cysteine (29) were used to react for 30 min at room temperature with 0.5 μg of MP-R in 20 μl of binding buffer in the presence or absence of 5 mM mannose 6-phosphate. 6 μg of anti-MP-R in 10 μl were added. Following incubation for 15 h at 4°C, 4 μg of rabbit IgG was added. Relative long incubation was necessary as compared with those in immunocytochemistry (see below). After 45 min at 4°C the mixtures were centrifuged for 2 min at 15,000 g. The supernate and the pellet (after washing twice with binding solution) were assayed for β-hexosaminidase activity (31). The conditions had not been optimized for maximal binding of β-hexosaminidase. Tissue Preparation: Male rats, fasted overnight were anesthetized with ether and received a retrograde perfusion through the abdominal aorta with phosphate buffered saline for 1 min immediately followed by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. After 5 min of perfusion, portions of two liver lobes were excised and were cut into 1 mm thin slices that were immersion-fixed in the same fixative for an additional hour. The slices were then either used immediately for immunofluorescence of semithin sections, or were placed into 2% paraformaldehyde in 1 M sucrose and stored for immunoelectron microscopy.

Immunocytochemistry: For immunofluorescence, sections (200 nm) of cryosections from prefixed livers were prepared according to the method of Tokuyasu (32). The sections were indirectly immunostained with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (33).

Immunoelectron microscopy was done with ultrathin (100 nm) cryosections (32) directly single-labeled with 5 nm colloidal gold-protein A conjugates or double-labeled with 5 and 8 nm protein A-gold particles as described before (16, 17). In double-labeling experiments the sequence of antibodies and labels was varied. Routinely the order of immunocouplings was anti-MP-R, 5 or 8 nm gold, anti-albumin or anti-cathepsin D followed by 5 or 8 nm gold. Since IgG molecules are monovalent for protein A, the second gold probe does not mark the first antibody binding site (see reference 16). Finally, the sections were stained in uranyl acetate and embedded in methacrylate (for details of the entire procedure, see 18). Background labeling as judged from sections incubated with anti-rat pancreas amylase (33) was negligible.

RESULTS

Characterization of Anti-MP-R

The specificity of the affinity purified anti-rat liver MP-R was assayed by immunochemical staining of purified receptors (lanes 1 and 2), standard proteins (lane 3), solubilized rat liver Golgi (lane 4), rat serum and total rat liver proteins (not shown) following electrophoretic separation and transfer to nitrocellulose (Fig. 1). Selective staining of the MP-R was observed. In various experiments we could detect as little as 60 ng of receptor protein. In control samples containing commercially available rabbit IgG, no reaction at the MP-R apparent molecular weight was observed.

The reactivity of anti-MP-R with unoccupied as well as with ligand-occupied receptor was examined. A single polypeptide co-migrating with purified MP-R was precipitated from extracts prepared in the presence of 5 mM mannose 6-phosphate from rat liver cells metabolically labeled with
FIGURE 1 Immunochemical detection of MP-R. Purified MP-R, standard proteins (Bio-Rad Laboratories, München, Federal Republic of Germany), and proteins extracted with 1% Triton X-100 from rat liver Golgi membranes were separated by gel electrophoresis (6% polyacrylamide) in the presence of SDS, electroblotted to nitrocellulose, and either subjected to immunochemical staining of MP-R (lanes 1-4) or staining with Amido black (lanes 5-8). Lane 1, 0.5 μg MP-R; lane 2, 0.10 μg MP-R; lane 3, myosin (200 kd), β-galactosidase (116 kd), phosphorylase B (92.5 kd), BSA (66 kd), and ovalbumin (45 kd), 0.4 μg each; lane 4, 20 μg of protein extracted from Golgi membranes; lanes 5-8, a fivefold amount of samples were applied in the same order as lanes 1-4.

[35S]methionine (Fig. 2). This indicates that anti-MP-R reacted with the unoccupied receptor. To assay the reactivity with ligand-occupied receptor, the receptor was first reacted with a high affinity β-hexosaminidase preparation known to contain enzyme forms that bind to MP-R (4). More than 40% of the enzyme was precipitated as a β-hexosaminidase-MP-R-immunoprecipitin complex. The co-precipitation of β-hexosaminidase with MP-R was abolished in the presence of 5 mM mannose 6-phosphate. Thus anti-MP-R reacts with free as well as with ligand-occupied MP-R.

Immunocytochemistry

In semithin cryosections (Fig. 3), anti-MP-R yielded substantial fluorescence along the entire plasma membrane including the bile canalicular membrane and in Golgi areas. In the peripheral cytoplasm flocculent fluorescence occurred, which most likely represented MP-R reactivity in CURL (see below). Otherwise cytoplasmic reactivity was just above background (Fig. 3). Sinusoidal lining cells were negative.

With immunogold labeling of ultrathin cryosections anti-MP-R reactivity was observed at the sinusoidal (Figs. 4 and 5), lateral, and bile canalicular (Fig. 6) plasma membrane domains. Gold particles were associated with the external surface of the top, lateral, and basal areas of the microvilli and with coated pits (Figs. 4 and 5).

Intracellularly, MP-R labeling was mainly confined to two loci. In the peripheral cytoplasm, especially at the transitions of the sinusoidal and the lateral cell surfaces, gold particles were present over a system of anastomosing tubules that extended inwards up to the Golgi areas (Fig. 5). We have previously termed this compartment CURL (compartment of uncoupling receptor and ligand, 18). Gold particles were also present in free-coated vesicles (not shown). CURL structures were only slightly labeled with anti-albumin, not with anti-cathepsin D. A detailed description of MP-R and other receptors in coated pits, coated vesicles, and CURL will be given elsewhere.

A major source of MP-R label was the Golgi complex, as was already indicated by the immunofluorescence observations (Fig. 2). The Golgi distribution of MP-R was studied in single-labeled sections (Fig. 6) as well as with double-labeling together with albumin (Fig. 7) and cathepsin D (Figs. 8 and 9). Since 5-nm protein A-gold is more sensitive than 8-nm gold, we used the smaller probe to demonstrate scarcest antigen in double-labeling experiments. As can be seen in Figs. 6-8, MP-R labeling occurred from cis to trans throughout the stack of Golgi cisternae. The trans-Golgi side was identified by developing secretory vesicles with lipoprotein particles and albumin labeling. There may be a slight increase in MP-R density from cis to trans Golgi. MP-R was also present in a trans-Golgi reticulum from which secretory vesicles seem to originate as can clearly be seen in double-labeling localization of MP-R and albumin (Fig. 7) and of MP-R and cathepsin D (Figs. 8 and 9).

FIGURE 2 Gel electrophoresis of MP-R purified from rat liver and immunoprecipitated from metabolically labeled epithelial rat liver cells. The migration of the MP-R detected by staining with Coomassie Blue (lane 1, purified MP-R) or by fluorography (lane 2, immunoprecipitated MP-R) is indicated as well as the migration of the following 14C-methylated standards: phosphorylase B (92.5), BSA (69), ovalbumin (46), carbonic anhydrase (30), and cytochrome c (12.5).

2 Figs. 4-9 are ultrathin (100 nm) cryosections of paraformaldehyde perfusion-fixed livers. The sections were indirectly single-labeled with 5 nm colloidal gold for the demonstration of MP-R (Figs. 4-6) or were double-labeled with 5 and 8 nm gold for the simultaneous
of MP-R with albumin (Fig. 7). Double-labeling showed also
that there is no detectable segregation between MP-R and
albumin at the level of the Golgi complex (Fig. 7). As reported
previously (19), there was no apparent increase in the density
of albumin label across the stack of cisternae.

Cathepsin D labeling was highest in large lucent lysosomes
in the trans-Golgi area. These lysosomes essentially lacked
MP-R labeling. The cathepsin D label was seen in loose
association with the lysosomal membrane (Fig. 9). Further-
more lysosomes often contained ferritin-like particles (Fig. 6,
8, 9), thus identifying them as secondary lysosomes. Labeling
of cathepsin D in the Golgi complex was much lower than in
lysosomes. Double-labeling of MP-R and cathepsin D showed
that cathepsin D in the Golgi cisternae, generally occurred in
close association with MP-R (Fig. 8 and 9).

DISCUSSION

The present study delineates the intracellular localization of
MP-R to better define the intracellular transport route of
lysosomal enzymes, especially in regard to the role of the
Golgi complex. The MP-R was selected since this receptor
plays a key role in directing newly synthesized lysosomal
enzymes to lysosomes. Using immunofluorescence and im-
munogold labeling of cryosections from rat liver, we show the
presence of MP-R in the plasma membrane, coated pits and
vesicles, CURL, and Golgi complex of parenchymal cells.
Thus in general, the localization of MP-R is similar to that of
the receptor for asialoglycoproteins (18, 35).

Fischer et al. (34) estimated from ligand binding studies
with rat liver subcellular fractions, that ~90% of MP-R is
intracellular, and 10% occurs at the plasma membrane. The
intracellular receptors occurred mainly in the ER, and only
little in Golgi derived membranes and lysosomes. We identi-
ﬁed only scarce MP-R labeling in rough and smooth ER.
With immunofluorescence only a weak cytoplasmic MP-R
reactivity was detected. Probably the density of receptors in
ER membranes is low. Lysosomes were essentially devoid of
MP-R label.

In the present study most of the intracellular MP-R iden-
tiﬁed with antibody and colloidal gold occurred in CURL and
in the Golgi complex. These observations are in general
agreement with those of Willingham et al. (36) in CHO cells.
They showed via ferritin immunocytochemistry MP-R label-
ing of the Golgi area and receptosomes. Receptosomes most
probably correspond to portions of CURL in liver cells (18).
As a consequence of poor preservation of Golgi substructures
a more detailed description of Golgi localization could not be
given. In the present study we demonstrate MP-R from cis to trans in the cisternae of the Golgi stacks. In addition MP-R occurred in the trans-most Golgi elements from which secretory vesicles develop.

**Passage of Lysosomal Enzymes through the Golgi Complex**

By means of single-label immunocytochemistry we have previously shown that β-glucuronidase and cathepsin D occur in the Golgi cisternae of rat preputial gland cells (37) and human hepatoma cells (6), respectively. Since some label appeared to be membrane associated, the question arose as to their associations with MP-R. In addition, enzyme destined for secretion would be expected to be localized in the Golgi complex but not receptor associated. Thus, the possibility was considered that secretory enzyme traverses the Golgi complex whereas receptor mediated transport of these enzymes bypasses the Golgi complex (37). Our present observations on MP-R and cathepsin D, however, strongly argue against this hypothesis, at least in liver. Cathepsin D label in the Golgi cisternae was clearly associated with MP-R label. At other cellular sites (e.g., lysosomes, plasma membrane) association of MP-R and cathepsin D was scarce or absent. The association of MP-R and cathepsin D labels suggests that at least a portion of the cathepsin D is specifically bound to receptor in Golgi cisternae. This pool of cathepsin D is most likely en route to the lysosomes. Therefore, the present observations are not consistent with the GERL concept of lysosomal enzyme movement bypassing the Golgi cisternae (13). Passage of cathepsin D through the Golgi complex agrees with the observation that this enzyme contains complex oligosaccharides containing galactose (9), which is known to be transferred into the oligosaccharides by the trans-Golgi enzyme galactosyl transferase (7, 38). Whether cathepsin D is unique in passing through the Golgi cisternae or whether other lysosomal enzymes that lack terminal sugars transit the Golgi cisternae as well remains to be elucidated.

The majority of MP-R labeling sites in the Golgi cisternae were free of cathepsin D label. Several factors may have contributed to the paucity of MP-R/cathepsin D associations relative to free MP-R label. (a) Many receptor molecules can be expected to have bound other lysosomal enzymes. (b) Only half of the receptors in the Golgi complex of rat liver have been shown to be occupied by enzyme (34). (c) A larger proportion of cathepsin D than of MP-R may have been undetected as a result of fixation damage and loss from the tissue. (d) The anti-human cathepsin D may exhibit limited cross-reactivity with rat enzyme. However, observations with this antibody showed a similar low Golgi reaction in human liver tissue. We feel that the first two reasons most probably account for the relatively few MP-R/cathepsin D associations observed.

**MP-R in trans-Golgi Structures and CURL**

The present observations do not allow firm conclusions as to the site at which albumin and cathepsin D separate. To define such a site one must identify structures lacking albumin but containing cathepsin D and MP-R either in association or separate. As a result of the paucity of label of cathepsin D relative to albumin this was not possible. However, we were able to identify both present in the cisternae of the Golgi complex and in the trans-most Golgi elements, which also contained detectable lipoprotein particles. Thus, at the level of the whole Golgi cisternae and the trans-Golgi elements no definite segregation of albumin and MP-R was noticed. On the other hand, the possibility remains that microdomains exist in these organelles. In addition, small vesicles with either of the antigen may easily have been missed.

CURL was the only compartment that contained significant labeling of receptor and simultaneous paucity of albumin. In previous studies on rat liver, we have suggested that CURL plays an important role in transport and dissociation
FIGURES 6 and 7  Fig. 6: Anti-MP-R-5 nm gold. Gold particles are present throughout the stack of cisternae of a small Golgi complex (G) and in trans-Golgi elements (see also next figure), but are absent from a lysosome (L) containing some ferritin particles. The membranes of the bile caniculus (B) show abundant label. x 140,000. Fig. 7: Anti-MP-R-5 nm gold, anti-albumin-8 nm gold. Both albumin and MP-R are present in all the cisternae of the Golgi complex. The trans-Golgi side can be identified by the presence of developing secretory granules (SG) showing lipoprotein particles (lucent globules), albumin, and MP-R. Lysosomes (L) show only little MP-R labeling at the limiting membranes. x 125,000.
Figures 8 and 9: Fig. 8: Anti-MP-R-8 nm gold, anticathepsin D-5 nm gold. MP-R and cathepsin D can be seen throughout the stack of Golgi cisternae (G) and in the trans-Golgi area. Cathepsin D label occurs freely, i.e., not in association with detectable MP-R (small arrowheads) as well as receptor-associated (large arrowheads). Most of the cathepsin D can be seen in the large lysosome (L), which also contains moderately dense ferritin-like particles. × 75,000. Fig. 9: Anti-MP-R-8 nm gold, anticathepsin D-5 nm gold. Similar to previous figure. Note the loose association of cathepsin D label with the membrane of the lower lysosome and the cathepsin D in the lumen of the autophagic vacuole (A) containing a mitochondrion. The lysosomes lack detectable MP-R labeling. × 90,000.
of receptor–ligand complexes (18). Receptor-mediated endocytosis of asialoglycoproteins is followed by delivery of the receptor–ligand complexes to CURL where the ligand and the receptor are separated (18, 39). This separation requires an acidic environment (40) probably provided for by the CURL interior. Subsequently, receptor molecules are transferred back to the cell surface (41), whereas the ligand is directed to the lysosomes for final degradation. MP-R-lysosomal enzyme uncoupling depends on a prelysosomal acidic compartment as well (42). Whether CURL plays a role in MP-R-lysosomal enzyme directional transport remains to be elucidated.

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