Membranes of Sorting Organelles Display Lateral Heterogeneity in Receptor Distribution

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Abstract. This study describes the distribution of an intrinsic membrane protein, the asialoglycoprotein receptor (ASGP-R) in the trans-Golgi reticulum and compartment of uncoupling receptor and ligand (CURL) of rat liver cells. Using quantitative immunogold electron microscopy and membrane length measurements, we showed lateral nonhomogeneity of receptors in the membranes of trans-Golgi reticulum and CURL, in particular in the membranes of secretory vesicles (identified by their content of albumin and very low density lipoprotein particles) and of CURL vesicles (endosomes), including multivesicular bodies. The characteristic tubulovesicular morphology of both sorting organelles defines the transition of receptor-rich tubular membrane and the receptor-poor limiting membrane of the attached vesicles. There was a direct relationship between the size of the secretory and CURL vesicles and the density of ASGP-Rs in their membranes. Receptor density in the smallest vesicles was similar to that found in adjacent continuous tubules. The larger the vesicles, the less receptor was detectable in their membranes. We propose that the receptor molecules are excluded from the vesicle membranes by dynamic lateral redistribution. Nonrandom receptor distribution in the CURL vesicle membranes was present even at the multivesicular body stage. These observations strongly suggest the existence of barriers to ASGP-R diffusion at the junctions of tubules and vesicles. In addition, our observations suggest that ASGP-Rs are transported to the plasma membrane via a mechanism other than the normal secretory pathway.

THE sorting and targeting of specific protein molecules within cells is a crucial but poorly understood process. Despite the significant interest in this area, especially in recent years, the molecular bases are still largely unknown. Two intracellular pathways in which protein segregation is essential are those of biosynthesis/secretion and endocytosis, both of which involve significant sorting of membrane proteins and solutes. For example, lysosomal enzymes and lysosomal membrane proteins, proteins destined for secretion, and plasma membrane proteins are all synthesized on the polysomes of the rough endoplasmic reticulum but ultimately reside within distinct compartments. After synthesis all these proteins are transferred to the Golgi stack of cisternae where they undergo a series of posttranslational modifications before targeting. The subcellular compartments in which sorting occurs and the mechanisms that govern this process are largely unknown. The itinerary of lysosomal enzymes and their segregation from secretory products provides one of the best examples of protein sorting during biosynthesis (30, 31, 44). Within the Golgi complex nascent lysosomal enzymes uniquely acquire phosphomannosyl residues which act as recognition markers for mannose 6-phosphate receptors present within the Golgi membranes. The binding of these modified enzymes to the mannose-6-phosphate receptors thus allows for their segregation from the bulk of proteins destined for secretion or other membrane domains. Lysosomal enzyme sorting is probably a Golgi or post-Golgi event (7, 13, 20).

In addition to sorting of proteins along the biosynthetic pathway, cells are capable of effective targeting of molecules, which is initiated at the cell surface. For example, extracellular ligands such as low density lipoproteins and asialoglycoproteins may enter cells via receptor-mediated endocytosis, after which the ligand is sorted to its appropriate intracellular destination (often the lysosome), whereas the receptor is recycled back to the cell surface. Different membrane receptor molecules may be efficiently sorted as well. For example, we have previously demonstrated that polymeric IgA receptors, which are internalized along with asialoglycoprotein receptors (ASGP-Rs)[1] via the same coated pits of rat liver cells, are segregated from one another in the tubulovesicular organelle, compartment of uncoupling receptor and ligand (CURL) (17). CURL is an acidic (1, 40) prelysosomal compartment which also functions in the segregation of ASGP-R from its ligand (4, 16).

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1. *Abbreviations used in this paper*: ASGP-R(s), asialoglycoprotein receptor(s); CURL, compartment of uncoupling receptor and ligand; TGR, trans-Golgi reticulum.

A central feature of membrane protein-mediated sorting is the requirement for lateral segregation of protein molecules within continuous membrane sheets. Thus, the generation of microdomains with different protein composition is an essential prerequisite for efficient sorting, whether via vesicular or bulk-flow mechanisms. At present, most data favor the concept of detaching coated vesicles as carriers (e.g., reference 13). Thus, a local relative enrichment of proteins destined for vesicular traffic must exit within the membrane. Lateral segregation of membrane proteins most likely underlies the maintenance of membrane specificity in general. However, the existence of membrane lateral nonhomogeneity within intracellular sorting pathways has not yet been demonstrated directly in situ.

The present study addresses this issue, and focuses on the membranes of the Golgi complex and CURL, which have been thought to be important sorting centers in the cell (13, 36). We have previously described a heterogeneous distribution of ASGP-Rs in peripheral (early) CURL structures (16). We have now used quantitative colloidal gold immunoelectron microscopy to precisely delineate the sorting sites of the intrinsic membrane protein ASGP-R with respect to both secretory vesicle and lysosome formation. We demonstrate progressive receptor retention from the membranes of developing secretory and CURL vesicles, including multivesicular bodies with concomitant receptor accumulation in trans-Golgi and CURL tubular membranes.

Materials and Methods

Tissue Processing

Livers of normally fed male Wistar rats were fixed by perfusion via the dorsal aorta with 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 10 min. Liver slices were further fixed by immersion in the same fixative for 1 h, rinsed in buffer, and frozen in liquid nitrogen.

Immunoelectron Microscopy

Cryosectioning of the livers, uranyl staining, and methyl cellulose embedding were performed as described previously (20). For indirect immunolabeling, ultrathin cryosections were first incubated with rabbit anti-rat ASGP-R or rabbit anti-rat albumin, rinsed, incubated with swine anti-rabbit serum (Nordic Immunology, Tilburg, The Netherlands) 1:1000 diluted with 0.1% BSA, rinsed, and finally labeled with 6 nm protein A-colloidal gold particles prepared according to the tannic acid–citrate method (43). In one experiment, sections were labeled with 15 nm gold for a survey view of ASGP-R distribution at low magnification (Fig. 8). The swine anti-rabbit step increased the sensitivity of the method considerably, as compared with direct protein A–gold labeling of the first antibody-binding sites. Overall, the intermediate antibody step yielded a six- to eightfold increase in labeling (details will be published elsewhere). The increased sensitivity allowed a more precise evaluation of label distribution. However, this method does not allow for the simultaneous demonstration of ASGP-R and albumin (15).

Quantitation of ASGP-R in Trans-Golgi Reticulum (TGR) and CURL

Cryosections from several tissue slices of two livers were examined for TGR and CURL profiles showing membrane continuities between the vesicular

and tubular subcompartments. CURL profiles were selected in the subsinusoidal cytoplasm, while TGR profiles occurred adjacent to the Golgi stacks (for representative areas, see Fig. 8). The TGR vesicles studied always contained lipoprotein particles and lacked internal membrane vesicles. In separate sections TGR vesicles were shown to contain albumin. Micrographs of a total of 13 TGR and 13 CURL complexes each were printed at a final magnification of 300,000×. The incidence of apparent membrane bilayer continuities between vesicles and tubules was low, probably because of the small diameter of the tubules (20–60 nm) relative to the vesicles (up to 1 μm) and section thickness (~100 nm). We presume that the larger vesicles did not arise from artifactual swelling, as the constant morphology of TGR and CURL tubulovesicle complexes in liver cells processed according to a variety of techniques, including preembedding in resin, makes this very unlikely. Therefore, a large number of sections had to be screened. Gold particles for ASGP-Rs were counted and assigned to either TGR and CURL vesicles or tubule membranes. The lengths of the vesicle and tubule membranes were measured using a curvimeter. Variations between repetitive membrane measurements of individual tubulovesicle complexes were <5%. Labeling densities of each of the four membrane domains (tubules and vesicles of TGR and CURL, respectively) were determined by calculating the ratios of the number of gold particles and the micrometers of membrane length. The total gold particles counted and membrane lengths measured are given in Table I.

Results

The hepatocyte is a secretory cell, containing small numbers of secretory vesicles at the trans face of the Golgi complex and in the subsinusoidal cytoplasm. The secretory vesicles are rich in albumin and lipoprotein particles, but contain only few ASGP-Rs (42). In rat hepatocytes, Golgi complexes possess abundant ASGP-Rs (~20% of the cell's total receptors) (16, 18). The Golgi ASGP-Rs appear to represent a mixed population of newly synthesized and recycling receptor molecules in transit to the cell surface. Since receptor-poor secretory vesicles originate from receptor-rich Golgi membranes, the Golgi complex is a likely candidate for sorting along the biosynthetic pathway. Along the endocytotic pathway, on the other hand, upon discharge of the ligand to the lysosome, the ASGP-R is retained within the recycling membrane while the vesicle transporting ligand to lysosome is receptor poor (16). Thus, the sorting of ASGP-R within the membrane is likely to occur at this stage in endocytosis.

We have used high resolution immunoelectron microscopy with colloidal gold markers to visualize the segregation and sorting sites. Fig. 1 shows an ultrathin cryosection of a rat liver Golgi complex immunolabeled with gold to localize secretory albumin. Albumin is present in the stacked cisternae of the Golgi complex and in TGR. The latter consists of tubules and attached developing secretory vesicles both containing albumin. Several stages of secretory vesicle formation can be seen in the figure. These range from small outpocketings and swellings at the TGR tubules to an almost mature secretory vesicle still attached to the TGR tubule. The secretory vesicles contain lucent very low density lipoprotein particles and lack internal bilayer vesicles characteristic of endocytic multivesicular bodies (see Figs. 6 and 7) (25). When connected to tubules they were exclusively located in close vicinity to the trans-face of the Golgi stacks. There was no apparent difference in concentration of albu-

Figures 1 and 2. Immunoelectron micrographs of ultrathin cryosections of rat liver indirectly labeled with 6 nm colloidal gold particles. (Fig. 1) Localization of albumin. Gold particles are present over the stacked cisternae of the Golgi complex (*G*) and in TGR tubules with attached developing secretory vesicles (*). The vesicles contain electron-lucent lipoprotein globules. Bar, 0.2 μm. (Fig. 2) Demonstration of ASGP-R throughout the stack of Golgi cisternae (*G*) and in the tubules and vesicles (*) of TGR. Note that the two small upper developing vesicles contain more receptor than the larger lower secretory vesicle. The transition between receptor-rich tubular membrane and receptor-poor membrane at the tubulovesicular junction of the lower profile is sharp (*arrowhead*). L, lysosome. Bar, 0.2 μm.
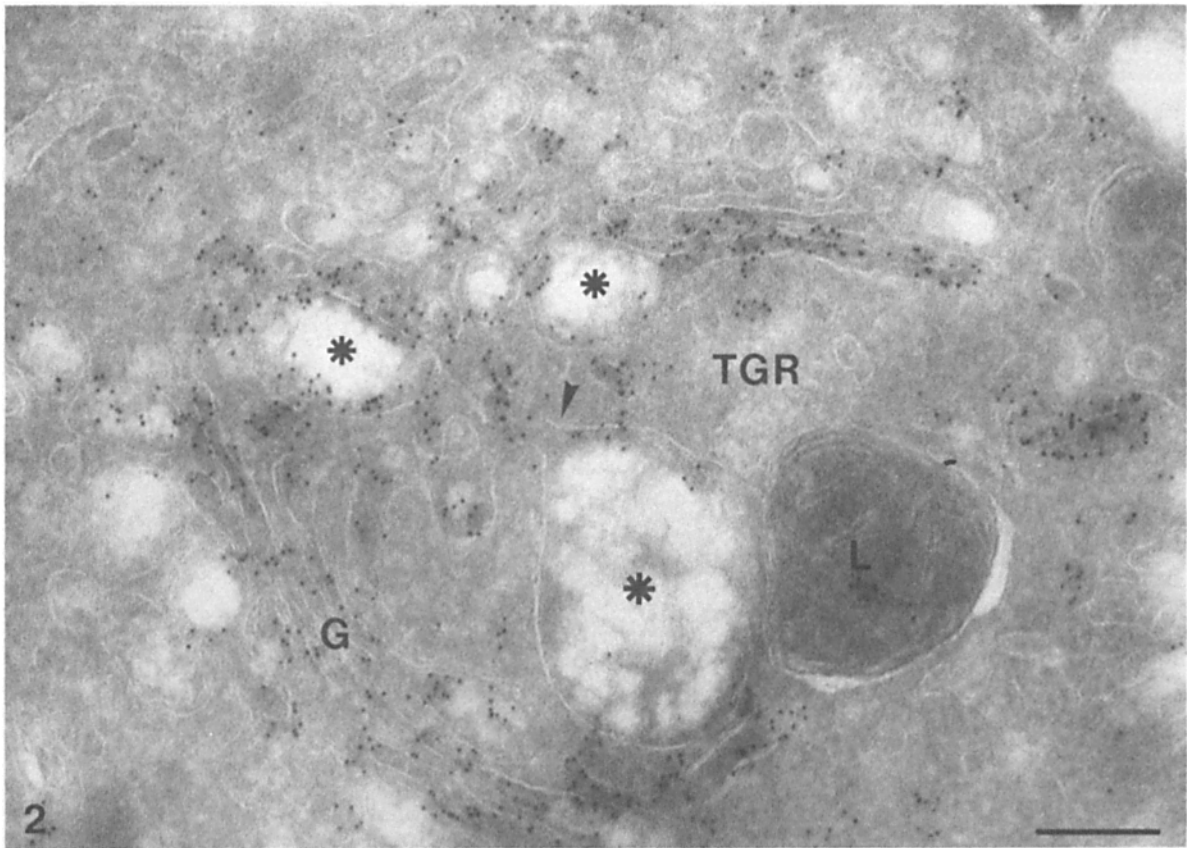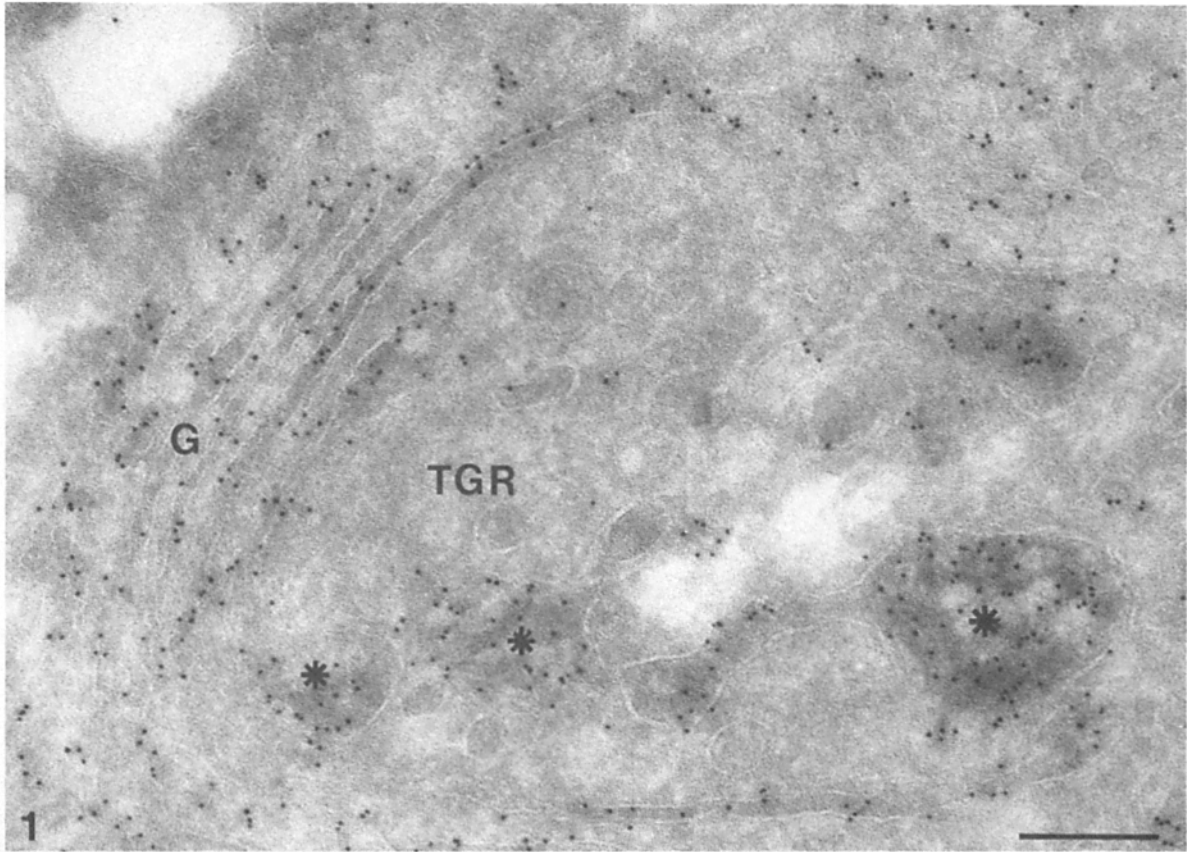
Table I. Determination of ASGP-R Densities in the Membranes of TGR and CURL Tubules with Attached Vesicles

	ASGP-R density*	No. of gold particles counted	Membrane measured
			μm
TGR vesicles	4.5–36.8‡	164	9.2
TGR tubules	38.1 (± 2.33)‖	374	10.1
CURL vesicles	1.7–30.7§	139	13.2
CURL tubules	45.8 (± 3.72)‖	463	10.8

* Densities are expressed as the number of immunogold particles per micrometer membrane.
‡ Range (mean, 19.6).
§ Range (mean, 11.4).
‖ Mean (± SEM).

min between the TGR tubules and attached secretory vesicles (Fig. 1). In contrast, the ASGP-R densities in TGR tubule and secretory vesicle membranes were remarkably different (Fig. 2). Like albumin, ASGP-R was found in the Golgi cisternae and TGR tubules. However, in the secretory vesicles connected with TGR, ASGP-R labeling varied considerably. Examination of many sections similar to Fig. 2 indicated that less receptors were present in the larger tubule-attached vesicles than in the smaller ones. To substantiate this we quantified ASGP-R immunogold particle distribution and measured membrane lengths of TGR tubulovesicle complexes sampled from trans-Golgi areas as shown in Fig. 8. From these data receptor densities in the membranes of tubules and vesicles were determined. Of note, only complexes with apparent membrane continuity between tubules and lipoprotein-containing vesicles were taken into account. The ratios of receptor gold to membrane length were used as a measure of receptor density.

Table I shows that the mean ASGP-R density of TGR tubules was 38 (immunogold particles/μm membrane length). The SEM was <6%. The densities of secretory vesicles, however, ranged from 4.5 to almost 37, with a mean of 19.6. The highest ASGP-R densities occurred in the smallest vesicles. In Fig. 3, ASGP-R densities of individual vesicles are plotted against the respective membrane lengths. When the mean diameters of the vesicles were used instead of membrane circumference, the same results were obtained (not shown). Fig. 3 shows a direct relationship between ASGP-R density and vesicle size, in which the labeling density decreased almost linearly with increasing vesicle membrane surface. The smallest secretory vesicles had a receptor density essentially the same as the tubules. The larger vesicles showed a sharp transition between the poorly labeled vesicle membranes and the heavily labeled membranes of the TGR tubules at the nexus of the complexes. In extreme cases the overall densities of a vesicle and tubules differed by 700%. Free secretory vesicles in the cell periphery contained only sparce receptors (not shown).
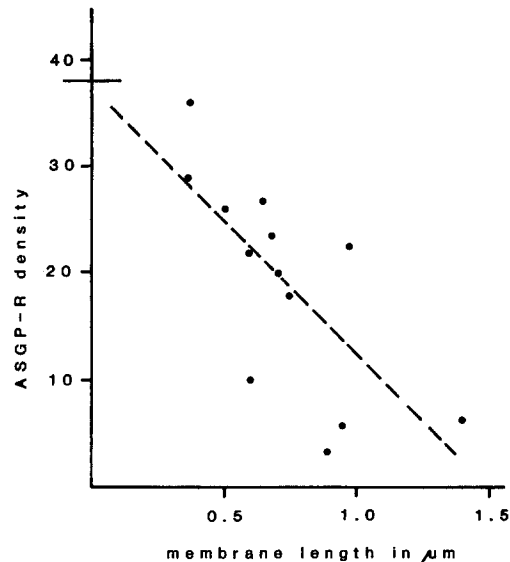

Figure 3. ASGP-R labeling densities of developing secretory vesicles as a function of the membrane length of the individual vesicles. Each point represents the value of one TGR vesicle. The line is the best fit by linear regression with a correlation coefficient of 0.71. The horizontal bar at the vertical axes represents the mean level of ASGP-R density in TGR tubules.

A corresponding but even more pronounced situation was found in CURL vesicles when compared with their connected tubules. As these observations are not dynamic, it is not possible to precisely define the maturation of these organelles. However, it is not unreasonable to consider that the smaller TGR and CURL vesicles are precursors of the larger ones. Indeed, the larger albumin- and lipoprotein-containing TGR vesicles had dimensions equal to or somewhat larger than mature free secretory vesicles within the sinusoidal cell periphery (Figs. 1 and 2). Furthermore, vesicles of various sizes containing secretory proteins, yet smaller than mature secretory vesicles, were readily identified. For CURL vesicles this was less evident, perhaps because their surface area is reduced during maturation by the internalization of portions of membrane that give rise to multivesicular CURL vesicles (Figs. 4, 5, 6, and 7). However, it appears that the small outcroppings of CURL tubules, which contain few if any internal vesicles, are developmental precursors of the larger multivesicular bodies. This notion is supported by many morphological observations of the endocytotic compartments (2, 11, 25, 29, 37, 49). CURL tubulovesicle complexes were especially rich in the basolateral corners of the cells (Fig. 8). Areas as outlined in Fig. 8 were selected for ASGP-R measurements. As we have shown previously (16), CURL tubules were generally rich in ASGP-R in contrast to CURL vesicles. We now demonstrate that relatively small, probably young stages of CURL vesicles were as densely labeled for ASGP-R as CURL tubules were (Fig. 4). Large,

Figures 4 and 5. Immunoelectron micrographs of ultrathin cryosections of rat liver indirectly labeled with 6 nm colloidal gold particles. (Fig. 4) Developing CURL (C) vesicle in a CURL tubule just beneath the sinusoidal plasma membrane that also reveals a coated pit (CP). There is no obvious difference in ASGP-R labeling density between the vesicle and the tubule. M, mitochondria. Bar, 0.2 μm. (Fig. 5) Tubulovesicular CURL complex (C) in the peripheral cytoplasm. ASGP-R labeling is present at the microvilli projecting into the space of Disse (upper part) and in the CURL tubules. Note the dramatic lateral heterogeneity in receptor density between the continuous (arrowhead) membranes of the CURL tubules and CURL vesicle. The lumen of the vesicle contains some profiles of internal vesicles. Bar, 0.2 μm.
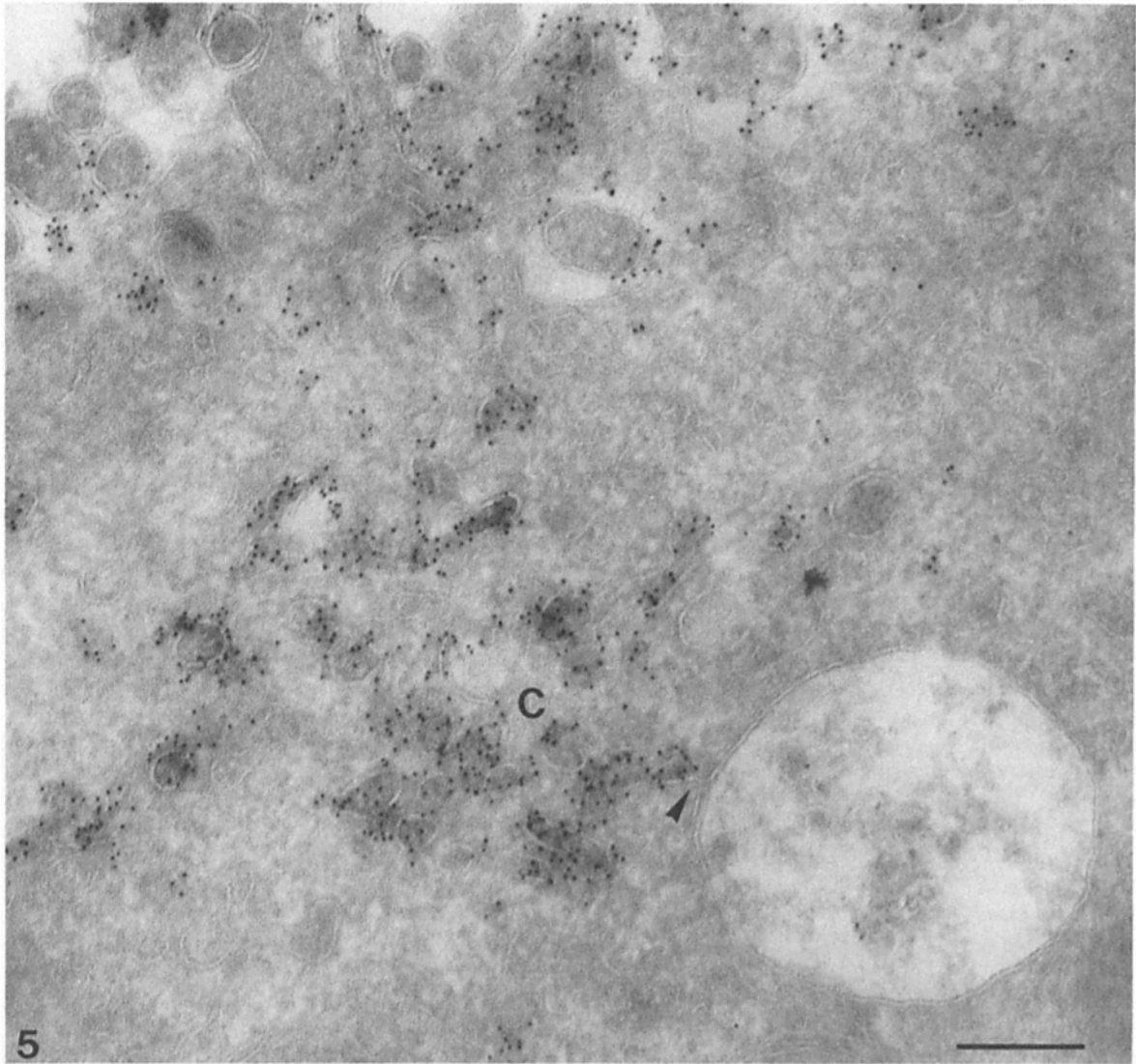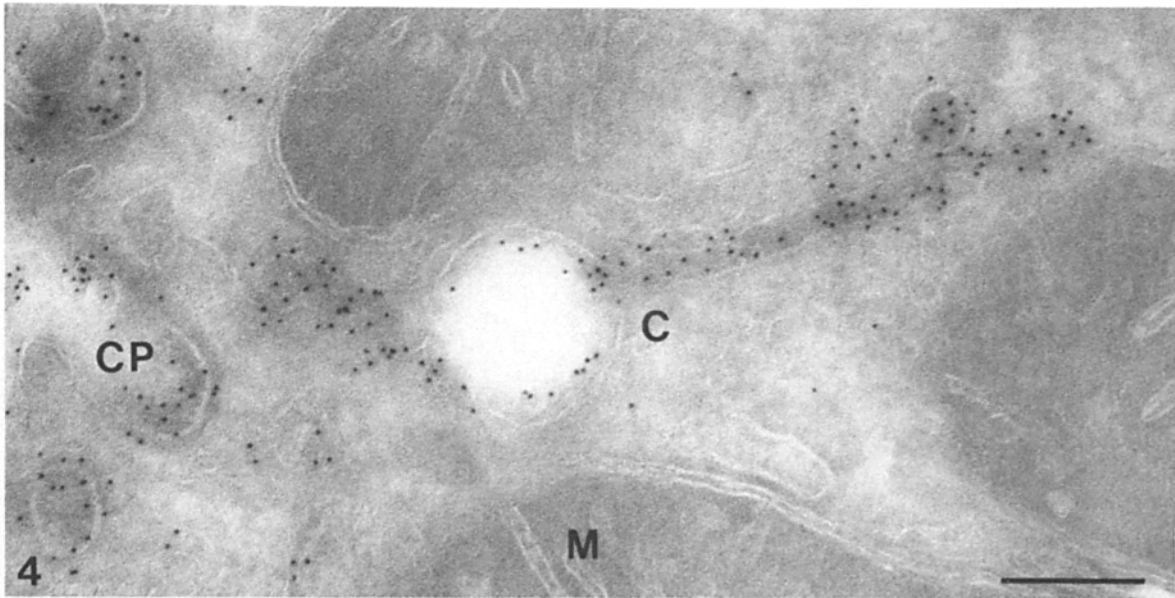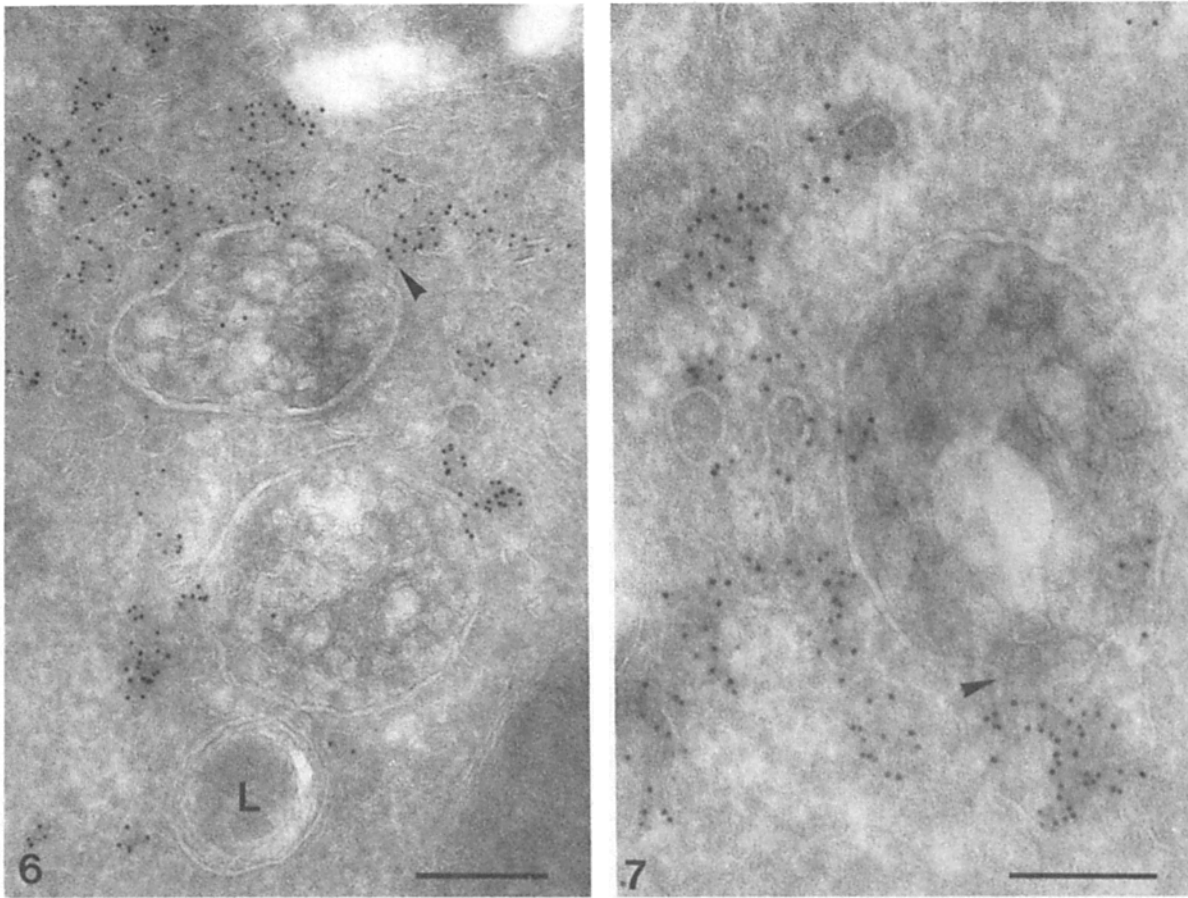
Figures 6 and 7. Immunoelectron micrographs of ultrathin cryosections of rat liver indirectly labeled with 6 nm colloidal gold. Dense ASGP-R labeling of tubules connected to typical multivesicular body-type CURL vesicles. There is an apparent lateral heterogeneity with respect to ASGP-R labeling in the continuous membrane at the tubulovesicular junctions (*arrowheads*). The multivesicular body profiles were collected from the Golgi area in the apical (bile) pole of the cells. L, lysosome. Bar, 0.2 μm.

probably later stages of CURL vesicles lacked ASGP-R almost entirely. An extreme example is shown in Fig. 5. Quantitation of receptor density within CURL tubules yielded 46 particles/μm with a SEM of 8% (Table I). Attached CURL vesicles displayed a wide range from 2 to 31 with a mean of 11. In the largest vesicles, which occurred predominantly in the bile region of the cell, there was an even greater increase in receptor density from vesicle to tubule membrane than seen in TGR (Figs. 5–7). These larger CURL vesicles contained internal bilayer vesicles and could easily be distinguished from the lipoprotein-containing secretory vesicles, which lack internal membrane vesicles. In albumin-labeled sections CURL vesicles of any type show scarce label if any. Since the internal membrane of CURL vesicles presumably derives from the limiting membranes, the morphometry of CURL vesicles (in order to compare vesicle density with vesicle circumference) was not performed. TGR and CURL tubules have diameters slightly larger than the section thickness. This may have caused some overestimation of receptor density in tubules, but can not explain the observed labeling differences between tubules and vesicles.

Discussion

The present immunoelectron microscopic observations demonstrate for the first time the considerable lateral heterogene-

ity of an intrinsic cellular membrane protein, the ASGP-R, within intracellular sorting organelles. ASGP-Rs are long-lived ($t_{1/2}$ 20 h) intrinsic (5) membrane proteins of the hepatocyte that perform endocytosis of multiple rounds of ligand internalization, sorting, and receptor recycling (e.g., reference 4). Along the biosynthetic pathway, the ASGP-Rs must be segregated and sorted away from secretory vesicles, which contain little ASGP-R. Similar findings pertain to other receptors (20, 42), including those involved in trafficking of enzymes to the lysosomes via mannose-6-phosphate receptors (19, 20).

Our present data demonstrate that ASGP-Rs are sorted from the secretory pathway at the TGR secretory vesicles. Significant differences in ASGP-R concentrations were found between TGR tubules and attached secretory vesicles containing albumin and very low density lipoprotein particles. There was an inverse relationship between secretory vesicle size and membrane receptor content. Similarly, CURL tubules displayed a higher receptor density than did CURL vesicles (equivalent to endosomes), consistent with our earlier qualitative observations (16). As with TGR, CURL displayed a striking increase in ASGP-R density from the vesicular to the tubular membranes. In perfused rat liver Mueller and Hubbard (34) have recently defined movement of ligand from ASGP-R–containing endocytotic sorting structures to vesicles without ASGP-R and present in the
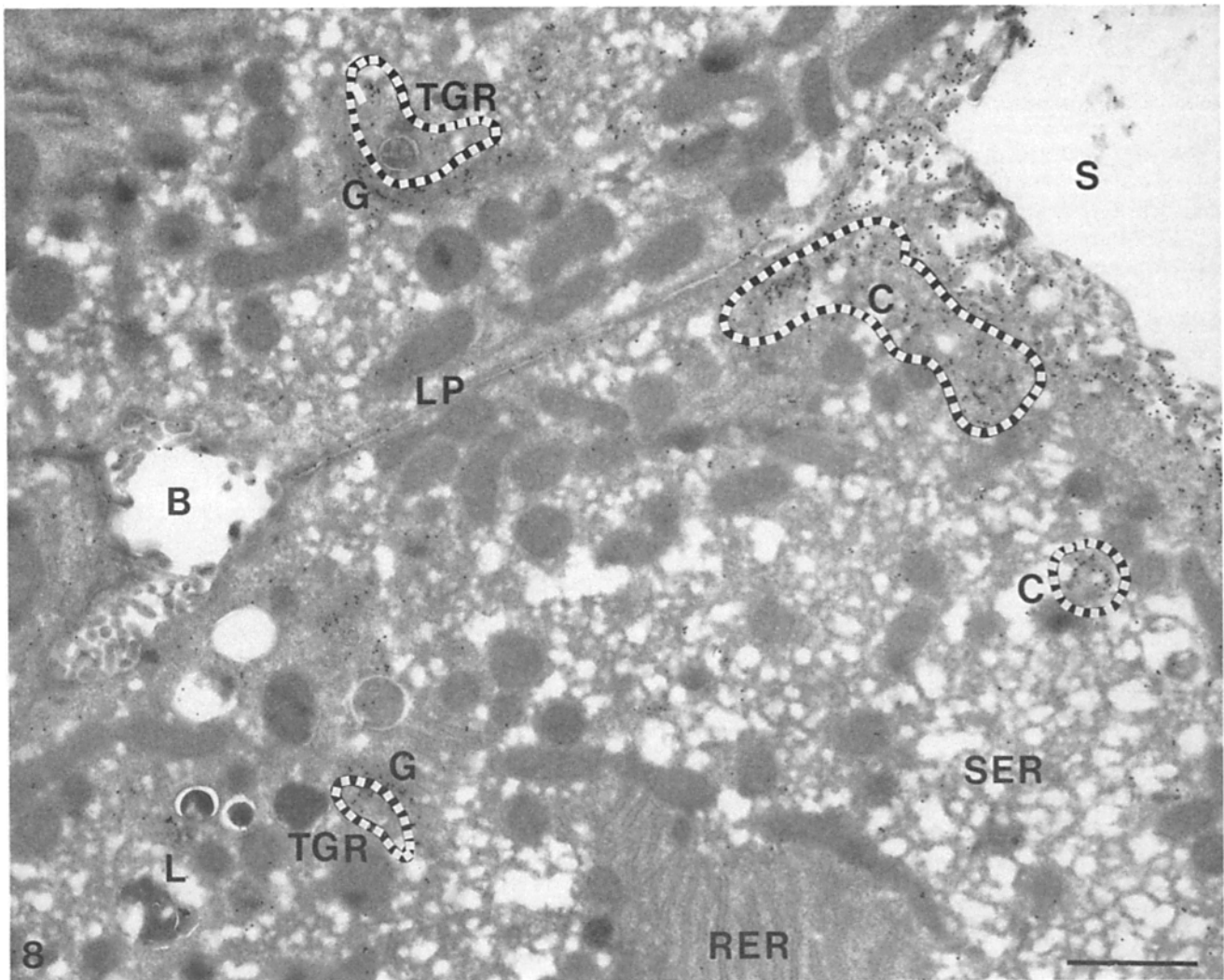
Figure 8. Low power electron micrograph of cryosection labeled with 15 nm gold particles for the demonstration of ASGP-R. At the cell surface, the receptor is present at the microvilli facing the blood sinus (*S*) and along the lateral border (*LP*), but is almost absent from the bile canalicular (*B*) plasma membrane. Intracellularly, ASGP-R can be detected in CURL (*C*), which is most prominent in the basolateral cell corners, and in Golgi stacks (*G*) and TGR. The tubulovesicular profiles which were immunoquantified for ASGP-R density measurements were collected from areas as outlined. L, lysosomes; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum. Bar, 1 μm.

Golgi-lysosome region of the cell. They suggest that ASGP-R sorting within the endocytotic compartments occurs before arrival of the ligand at the Golgi lysosome area. A more likely possibility is that this sorting occurs as a continuous process rather than at discrete stages.

The interpretation of our data, which detail the lateral heterogeneity of ASGP-R within the intracellular sorting compartments, needs some presuppositions. For example, the progressive loss of ASGP-Rs from TGR and CURL vesicles is not a result of receptor degradation, but rather reflects receptor redistribution. Thus, our data imply a dynamic process for ASGP-R distribution within TGR and CURL vesicles. That is, the ASGP-Rs appear to be excluded from the vesicles by lateral concentration gradients, with resultant accumulation in TGR and CURL tubules. Therefore, while moving from the cell periphery to the bile region of the cells, CURL vesicles probably fuse with each other and concurrently form internal vesicles. Many of the multivesicular CURL vesicle profiles show tubular extensions. Importantly,

our present data show that ASGP-R sorting proceeds up to the multivesicular body stage. The small amount of ASGP-R in the membranes of the internal vesicles is most likely degraded and may provide for the normal turnover of the receptors. Thus, sorting by exclusion appears to proceed predominantly in the limiting membranes of TGR and CURL vesicles. It is of note that the observed sorting does not appear to be mediated by coated membrane vesicles.

Recently Rome (38) proposed that the asymmetrical distribution of membrane over the two subcompartments of CURL (i.e., the vesicular and the tubular parts) would automatically sort receptors from the lysosome-directed degradative pathway. Even with equal receptor density within the tubule and vesicle membranes, the characteristic geometry of CURL would lead to proportionally less membrane protein and more content solutes in the degradative detaching vesicles than in the tubules (which have the greater surface/volume ratio). Morphological studies of the peripheral endocytotic compartments of various cell types have indeed

illustrated the extensiveness of the tubular system relative to the vesicles (20–23, 27, 49). Preliminary morphometrical data on CURL in hepatoma cells reveal a three times higher surface density of tubules than of vesicles (our unpublished observations). However, in addition to this type of passive sorting, our present data demonstrate the presence of dynamic lateral ASGP-R redistribution in secretory and CURL vesicles, which is most consistent with a progressive retention of ASGP-R from growing secretory and CURL vesicles. The persistance of tubulovesicular connections even in large multivesicular body-type CURL vesicles of the degradative pathway (Figs. 6 and 7) suggests that escape of receptor from the vesicles can potentially occur even as late as the early lysosome stage.

It is generally assumed that newly synthesized and recycling receptors, along with other membrane proteins, are transported to the plasma membrane by small transport vesicles (3, 13, 23, 33, 35, 45, 47). Despite the presumed abundance of these vesicles, they have yet to be identified in situ with certainty. TGR and CURL tubules are likely candidates to give rise to such small membrane protein-rich vesicles (23, 47).

While the precise mechanism(s) responsible for the lateral segregation of receptor molecules into the tubular elements of TGR and CURL remain unknown, several possibilities exist. For example, segregation may involve direct interaction of the cytoplasmic receptor tail with cytoskeletal elements or alterations in the intramembranous mobility of oligomers of the receptor (i.e., state of receptor aggregation) or other receptor-protein complexes (see references 33 and 41). Another possibility would be a reversible, highly ordered packing of receptor (and other protein) molecules, with the exclusion of solvent and other membrane constituents. This would then allow the membrane to sort away from the receptor molecules. In addition to these possibilities, alterations in the (phospholipid) composition, enhanced lipid insertion in the vesicle membrane, or changes in the physical state of the membrane as a result of interaction with the vesicular contents may promote active lateral sorting within the continuous membrane. For example, acidification of vesicular contents may provide one potential mechanism, as it is essential for ASGP-R ligand dissociation and recycling (e.g., references 4, 8, and 39). Furthermore, the acidic (pH 5–5.5) nature of CURL vesicles has been directly demonstrated (1, 40, 46). Indeed, conformational changes in the ASGP-R molecule occur at this mildly acidic pH (10). In addition to the acidic nature of CURL vesicles, Yamashiro et al. (50) showed a somewhat milder average acidity (pH 6–6.5) present in a tubulovesicular compartment, possibly TGR (26, 27). There is, however, as yet no evidence that the generation of a low pH in CURL and TGR has any direct relationship to the membrane sorting within these compartments.

The observation of lateral sorting and exclusion of ASGP-Rs from vesicles must be accompanied by a countercurrent movement of proteins into the vesicle membranes, since secretory vesicles (e.g., reference 32) and CURL vesicles (9, 24, 48) possess their own characteristic membrane proteins. We and others have described a special class of CURL vesicles derived from tubules in liver cells that are involved in receptor-mediated transport of polymeric IgA (6, 17, 28) but contain little ASGP-R (17). Additional high resolution im-

munocytochemistry will aid in elucidating possible sorting pathways of specific vesicular membrane proteins.

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