Membrane and Secretory Proteins Are Transported from the Golgi Complex to the Sinusoidal Plasmalemma of Hepatocytes by Distinct Vesicular Carriers

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Abstract. From rat livers labeled in vivo for 30 min with [35S] cys-met, we have isolated two classes of vesicular carriers operating between the Golgi complex and the basolateral (sinusoidal) plasmalemma. The starting preparation is a Golgi light fraction (GLF) isolated by flotation in a discontinuous sucrose density gradient and processed through immunosolubilization on magnetic beads coated with an antibody against the last 11 aa. of the pIgA-R tail. GLF and the ensuing subfractions (bound vs nonbound) were lysed, and the lysates processed through immunoprecipitation with anti-pIgA-R and anti-albumin antibodies followed by radioactivity counting, SDS-PAGE, and fluorography. The recovery of newly synthesized pIgA-R was >90% and the distribution was 90% vs 10% in the bound vs nonbound subfractions, respectively. Albumin radioactivity was recovered to ~80%, with 20% and 80% in bound vs nonbound subfractions, respectively.

Other proteins studied were: (a) secretory—apolipoprotein-B, prothrombin, C3 component of the complement, and caeruloplasmin; (b) membrane—transferrin receptor, EGR-receptor, asialoglycoprotein receptor, and the glucose transporter. In all the experiments we have performed, the secretory proteins distributed up to 85% in the nonbound subfraction (large secretory vacuoles), whereas the membrane proteins were segregated up to 95% in the bound subfraction (small vesicular carriers). These results suggest that in hepatocytes, membrane and secretory proteins are transported from the Golgi to the basolateral plasmalemma by separate vesicular carriers as in glandular cells capable of constitutive and regulated secretion.

Hepatocytes, like all epithelial, polarized cells, have distinct baso-lateral (sinusoidal) and apical (biliary) plasmalemma domains, separated by tight junctions. Most of their secretion is directed towards the baso-lateral domain and reaches the blood plasma through the spaces of Disse, but direct secretion at the apical membrane does exist (24) as it has been shown for other polarized, epithelial cells (9, 20, 21). Hepatocytes are secretory cells of the constitutive type; no stimulus that can trigger their secretion has been identified. It has been assumed that in constitutive secretory cells membrane and soluble proteins destined for secretion are transported to the plasmalemma in the same vesicular carriers (5).

In previous work, it has been shown that during treatment with inhibitors of microtubule assembly (3, 24), or inhibitors of secretion (10), newly synthesized membrane proteins were transported to the cell surface with different kinetics, a finding which suggests transport of these proteins by different carriers.

Since our main interest is the identification of vesicular carriers involved in intracellular traffic, we decided to try to isolate the carriers that transport proteins at the Golgi complex-sinusoidal plasmalemma (sPM) relay.

The questions we intended to answer were: (a) Are the secretory and membrane proteins transported together in the same vesicular carriers, like in constitutive secretory cells, or are they segregated in different carriers, like in regulated secretory cells? (b) If there is segregation of soluble from membrane proteins on this relay, where does it occur? (c) Upon isolation, would these vesicles represent enough material for further work, such as biochemical characterization or reconstituted cell free systems?

In the past, we have succeeded in isolating a population of transcytotic vesicular carriers from rat hepatocytes by combining a sucrose density gradient centrifugation with an immunosolubilization procedure (30). We decided to take the

1. Abbreviations used in this paper: ApoB, apolipoprotein B; ASGP-R, asialoglycoprotein receptor; EGF-R, epidermal growth factor receptor; GLF, Golgi light fraction; pIgA-R, polymeric IgA receptor; sPM, sinusoidal plasmalemma; TM, total microsomal.
same approach (with minor modifications), but this time the target was the vesicular carriers operating at the Golgi-sPM relay. The liver remained the organ of choice because it is an abundant source of material, because fractionation procedures are well worked out (13) and the kinetics of our marker proteins, the polymeric IgA-receptor (pIgA-R) and albumin, are well established (17, 19, 22, 24, 29).

**Materials and Methods**

Sprague-Dawley rats, weighing 150-200 g (Bantin & Kingman, Fremont, CA) were used in all experiments. Magnetic beads were kindly provided by Dr. J. Ugelstad (Sintef Co., Trondheim, Norway). A magnetic device was purchased from Dynal Inc. (Great Neck, NY). Chemicals and reagents were obtained from the following sources: [35S] met-cys and ovalbumin from ICN (Costa Mesa, CA); [3H]UDP-Gal, [125I] and Amplify from Amer sham (Arlington Heights, IL); electrophoresis reagents and Affigel-10 from Bio Rad Labs. (Richmond, CA); affinity purified goat anti-rabbit IgG (Fc) from Biodesign Int. (Kennebunkport, ME); protease inhibitors (Leupeptin, Pepstatin, Aprotinin) from Boehringer Mannheim Corp. (Indianapolis, IN); protein A-Sepharose from Pharmacia LKB Biotechnology (Piscataway, NJ); protein A/G Agarose from Santa Cruz Biotechnology (Santa Cruz, CA); XAR-50 film from Eastman Kodak (Rochester, NY); Ecoscint from National Diagnostics (Atlanta, GA); asialofetuin from Sigma Chem. Co. (St. Louis, MO); ketamine and xylazine (anesthetics) from Aveco Co. (Fort Dodge, IA). All chemicals used were of reagent grade.

**In Vivo Labeling and Fractionation Protocol**

Rat livers were labeled in vivo for 30 min by injection through the portal vein of 1 mCi [35S] met-cys diluted in saline. By 10 min ~95% of the radioactive counts are cleared from the blood plasma (personal unpublished results, see also reference 29), so we can assume that there is a 10-min in vivo pulse followed by a 20-min spontaneous chase. For preparing the total microsomal (TM) standard, the liver was labeled by the same procedure but for one hour and with two consecutive injections of radioactive label at time = 0 min and time = 30 min so as to label all the receptor forms (see Results). The fractionation procedure used a discontinuous sucrose density gradient as in (13). The livers were flushed with cold 0.25 M sucrose supplemented with a cocktail of protease inhibitors (Leupeptin, Pepstatin, and Aprotinin at 1 µg/ml), collected, and homogenized 1:5 (wt/vol) in the same medium. A TM fraction obtained from this homogenate by centrifugation of the postnuclear supernatant at 100,000 g for 90 min was resuspended in the same medium and then adjusted to 1.22 M sucrose and loaded at the bottom of the gradient shown in Fig. 1. The gradient was spun at 82,000 g for 3 h and the band floating at 0.25 M-0.86 M interface was collected and used as starting material for additional work. This fraction has been designated Golgi light fraction (GLF).

**Immunoisolation**

The immunoisolation protocol is based on the ability of an antibody immobilized onto a solid support (in our case magnetic beads) to specifically retrieve, from a mixture of particles or vesicles carrying different antigens on their surface, only those bearing an antigen recognized by the immobilized antibody (15). The advantages of immunosorption via magnetic beads are: speed, high specificity, bypass of numerous centrifugation steps, (beads are retrieved with the help of a magnetic device), and low contamination due to the smooth surface, size uniformity, and low hydrophobicity of the beads. The beads, which carry free-OH groups on their surface, are first activated by treatment with 1 M-ethyl chloride (EtCl, as suggested by the literature) as a catalyst (11). The activated beads are first coated with goat anti–rabbit IgG (Fc) (secondary antibody), which is expected to bind in the right orientation the specific, primary antibody introduced at the next step. In our case, the primary antibody was raised in rabbits against a synthetic peptide reproducing the last 11 amino-acids (AAQVHDQPQEA) of the COOH terminus of the rat polymeric IgA-receptor (1). For immunizing the rabbits, the peptide was coupled to a carrier protein (bovine albumin) by the glutaraldehyde method (11). The primary antibodies (anti-pIgA-R tail) were affinity purified (on a peptide-Affigel-10 column), in order to increase the surface concentration of the specific antibody on the magnetic beads. Magnetic beads, coated with ~10 µg affinity purified IgG/ml beads, were incubated for 2-12 h with gentle agitation, on an end-over-end rotator) with the starting material (SM) which was GLF in sucrose, as collected from the gradient, diluted 3:7 with 5% FCS, 2 mM EDTA in PBS (SPBS). Of the many blocking agents tried, FCS was found to be the most effective in lowering nonspecific binding. At the end of the incubation the beads with the attached vesicles were washed with SPBS (6 x 15 min). The nonbound subtraction was always saved for analysis. In this way, the SM is resolved in two subfractions: nonbound (NB) and bound (B), respectively.

**Antibodies**

Antibodies used were either raised in our laboratory or obtained from the following sources: (a) anti-pIgA-R ectodomain, (raised in our laboratory against the secreted fragment of the receptor isolated from bile) used mostly for immunoprecipitation studies and previously characterized (22); (b) anti-pIgA-R tail, raised in our laboratory against a synthetic peptide representing the last 11 a.a. of the carboxyterminal domain of the rat pIgA-R sequence (1); this antibody recognizes the receptor by immunoblotting, immunoprecipitation, and works very well in immunosorption; it does not recognize the biliary form of the receptor, i.e., the ectodomain, and does not cross-react with any other proteins in fraction lysates; (c) sheep antibodies against rat albumin, rat transferrin, rat C3 complement, human caeruloplasmin, rat dimeric IgA, and alkaline phosphatase-conjugated anti–sheep IgG were from The Binding Site (San Diego, CA); (d) alkaline phosphatase-conjugated anti–rabbit and anti–mouse IgG were from Sigma Chem. Co.; (e) goat conjugated anti–rabbit IgG was from Zymed (San Francisco, CA). Additional antibodies were obtained from the following laboratories: (f) anti-prothrombin from Dr. R. Horvath (University of Vienna, Austria); (g) anti-apolipoprotein B from Dr. Elovson (UCLA); (h) anti-rat asialoglycoprotein receptor from Dr. K. Drickamer (Columbia College of Physicians and Surgeons); (i) anti-human transferrin receptor endodomain, which cross-reacts with the rat receptor, from Dr. I. Trowbridge (Salk Inst.); (j) anti-rat epidermal growth factor receptor from Dr. G. Gill (UCSD); (k) anti-rat glucose transporter from East Acres Biologicals (Southbridge, MA); (l) anti-Ce-9, an antibody that recognizes a sinusoidal plasma membrane antigen from Dr. A. Hubbard (Johns Hopkins, Baltimore, MD); (m) anti-rat α-mannosidase II from Dr. M. G. Farquhar (UCSD); (n) anti-rat α-2,6 sialyltransferase from Dr. K. Colley (University of Chicago, IL). Immunoprecipitations and Western blotting were done as in (30).

**Electron Microscopy**

Electron microscopy was performed to survey the composition of SM (i.e., GLF) and NB and to visualize the elements immunosolated on the magnetic beads (B). SM, NB, and B were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in cacodylate-HCl buffer (pH = 7.2), for 45 min, and then in 1% osmium tetroxide in the same buffer for 1 h. The SM and NB, better results were obtained by omitting the first fixation step. The samples were stained in block in 2% uranyl acetate, dehydrated in ethanol and propylene oxide and embedded in Epon. Thin sections were cut, stained, and examined in a Phillips TEM. For immunocytochemical identification, the vesicles on the beads, were first reacted with anti-pIgA-R tail antibody.
jected into the portal vein; 12 min later the liver was removed and processed. The ectodomain of the pIgA-R (dilution 1:30) were carried out from the fractions collected from the gradient in Fig. 1. Equal amounts of protein/sample were used. Newly synthesized 116K is highly enriched in GLF. No mature form (120K) is yet present at the time tested, when compared with the standard control lane.

Radioiodination

Radioiodination of asialofetuin was performed by the Iodogen method (25). [125I] asialofetuin (3 x 10^6 cpm) was injected intraperitoneally at time = 0 and at time = 6 min the liver was removed and fractionated as given above. The fractions and subfractions obtained were counted in a gamma counter (5500 B, Beckman Instrs., Fullerton, CA).

A hybridoma cell line secreting dimeric IgA was kindly provided by Dr. D. Bole (Ann Arbor, MI). The cells were biosynthetically labeled overnight with 100 μCi [35S]met-cys/ml, and the tissue culture supernatant was concentrated about 10 times using a Centriprep device (Amicon, Beverly, MA).

The cells were biosynthetically labeled overnight with 100 μCi [35S]met-cys/ml, and the tissue culture supernatant was concentrated about 10 times using a Centriprep device (Amicon, Beverly, MA). 5 x 10^6 cpm in dIgA (as determined by immunoprecipitation) were injected into the portal vein; 12 min later the liver was removed and processed as usual. Counts specific for dIgA were followed in the different fractions by scintillation counting and by specific immunoprecipitations, followed by SDS-PAGE and fluorography.

Results

A Fraction Enriched in the Elements of the Exocytic Pathway

Since our main interest was the isolation of vesicular carriers plying between the Golgi complex and sPM, we took advantage of the fact that the kinetics of the pIgA-R and albumin transport along the secretory pathway have been established. In a pulse/spontaneous chase in vivo labeling, it takes ~30 min for the receptor to reach the sPM and 30–35 min for the newly synthesized albumin to reach the blood plasma. Vesicular carriers operating between the Golgi complex and sPM (G-sPM-VC), should be characterized by maximum labeling of the newly synthesized albumin and pIgA-R at 30–35 min and by the predominant presence of the intermediary (116K) form of pIgA-R. As shown in previous kinetic studies (29), there are three different MW forms of the pIgA-R corresponding to different stages of its posttranslational processing. 105K is the ER core glycosylated form (endo H sensitive), 116K fully glycosylated, endo H resistant form is present in Golgi, and 120K the mature form (phosphorylated) is present in sPM and transcytotic vesicles. Fig. 2 shows the result of in vivo labeling for 30 min followed by fractionation of the liver and immunoprecipitation of pIgA-R from the ensuing cell fractions. We found heavy accumulation of newly synthesized 116K, the intermediary form of the pIgA-R, in GLF which was in accord with our starting premises. As shown in Fig. 2, the amount of newly synthesized 116K in the other fractions collected from the sucrose gradient is much smaller, which justifies our selection of GLF as starting material for subsequent work. By morphology (Fig. 3), GLF contains in addition to large (0.5 μm), lipoprotein loaded vacuoles and scattered unstacked Golgi cisternae, small vesicles (80–120 nm) of the size expected for vesicular carriers. These small vesicles are tethered to, or budding from, vacuolar or cisternal Golgi elements, or are scattered around apparently free (Fig. 3-gallery). Besides the 116K form of the receptor, we also immunoprecipitated the 105K form (~10% of the total number of counts in the 116K) which is the ER, core glycosylated form of the receptor. We assume that GLF also contains ER-Golgi carriers, caught in this fraction on account of recycling or present therein as contaminants. We favor the first alternative because several attempts to separate the two forms of the receptor, by running GLF on a second, continuous sucrose density gradient, were unsuccessful. As a result of these preliminary experiments, we decided to use GLF as the starting preparation for the isolation of Golgi-sPM vesicular carriers. We realize that vesicular carriers of this type may be present in lesser amounts in other fractions (GHF and 1.15 M); this remains to be established and quantitated by further work.

Immunoisolation of a Specific Class of Vesicular Carriers

Our immunoadsorbent is the magnetic beads coated with a specific antibody which only recognizes the ectodomain of pIgA-R (see Materials and Methods). Therefore, only the vesicles that carry the receptor in the right orientation, i.e., having the endodomain exposed at the surface, are expected to bind to the antibody coated magnetic beads. After incubation of GLF with the immunobeads, the resulting subfractions, i.e., B and NB, were analyzed morphologically and biochemically. Figs. 4 and 5 show the two subfractions processed as in Materials and Methods for electron microscopy. The NB subfraction consists of lipoprotein loaded vacuoles and Golgi cisternae, generally well preserved except for some fragmentation and swelling of the vacuolar elements (Fig. 4). The immunoadsorbed vesicles in the bound subfraction are predominantly small in size and free of content (Fig. 5), but a few small vesicles containing one or a few lipoprotein particles are still present, as are occasional Golgi cisternae (Fig. 5-gallery). Large lipoprotein containing vacuoles are generally absent. To check for the specificity of the immunoadsorption, in one experiment we reacted the immunoadsorbed vesicles, with anti-pIgA-R tail antibodies, using as reporter a gold conjugated secondary antibody (see Materials and Methods). The majority of the immunoadsorbed vesicles were labeled (Fig. 5d).
Figure 3. EM morphology of GLF (SM) processed as described in Materials and Methods. The most abundant elements are large secretory vacuoles filled with lipoprotein particles; also present are scattered, unstacked cisternae as well as small vesicular carriers. The latter appear to be tethered to the large elements, but a few of them are apparently free. The gallery on top shows different small vesicular carriers again most of them tethered to or budding from the larger elements. In general, these budding vesicles are free of dense content. Arrow in b points to an apparently free vesicle, arrowheads point to tethered vesicles. Bar, 0.1 μm.

Figure 4. NB subfraction examined by EM, after incubation of the SM for 12 h with the beads. Large vacuoles containing lipoprotein particles are the predominant component of the preparation which also contains a few Golgi cisternae and a few small vesicles. The large vacuoles have intact membranes but their content is of low density suggesting swelling and partial extraction. Bar, 0.1 μm.

Segregation of Albumin from pIgA-R Along the Secretory Pathway

After a series of immunoisolation experiments, SM (GLF), and the subfractions derived therefrom (NB and B) were lysed and lysate immunoprecipitations were carried out using antibodies against the pIgA-R and albumin, followed by either scintillation counting of the precipitates and/or SDS-PAGE and fluorography. The distribution as well as the recovery of the two proteins in the SM, was followed in the subfractions obtained (NB and B). The data were normalized to SM volume, taking into account dilution factors and differences in sample volumes. Normalization to protein amount (enrichment) was not possible because of the presence of FCS and extraneous antibodies present in NB and B. Fig. 6 shows a bar graph representing the SM pIgA-R distribution between the NB and B subfractions. The recovery of pIgA-R specific counts in NB and B approached 100% of the counts in SM. Above the graph, the results of an immunoprecipitation experiment, followed by SDS-PAGE and fluorog-
Figure 5. Composite showing partial view of sectioned magnetic beads. *f* shows the control sample in which the primary antibodies have been omitted. *e* represents the B subfraction in which most of the vesicles immunoadsorbed on the beads are small in size and free of content as compared to the SM (Fig. 3) or NB (Fig. 4). A gallery of vesicles immunoadsorbed on the beads illustrates the occasional presence of a few lipoprotein particles in bound vesicles (*a*), the occasional binding of Golgi cisternae (*c*), and the positive identification of immunoadsorbed vesicles by immunogold labeling (*d*). Arrows point at small vesicular carriers (*b*) and arrowheads indicate other elements occasionally bound to the beads (*a* and *c*). In *d*, the arrowhead points at a vesicle heavily labeled by immunogold. Bar, 0.5 μm.

Figure 7 shows the distribution and recovery of newly synthesized albumin in the two subfractions. The data were obtained by quantitative immunoprecipitation followed by scintillation counting (bar graph) or SDS-PAGE and fluorography (above the graph). Albumin was distributed ~80% vs ~20%, in the NB vs B, respectively, with recovery rates between 80–100% of the amount present in SM. One concern was that, during the incubation, albumin may leak out of the vesicular carriers into the reaction medium. This possibility was checked by pelleting in one replicate experiment the nonbound subfraction at 100,000 g for 1 h, and measuring the distribution of radioactivity in the pellet and the supernatant. Over 86% of the radioactivity counts (most of them in newly synthesized albumin) were present in the pellet, whereas less than 14% were left in the supernatant.

**Segregation of Other Soluble and Membrane Proteins**

We decided to widen our survey by analyzing the distribution of other soluble and membrane proteins between the NB and B subfractions. Besides albumin and pIgA-R we followed the apolipoprotein B (ApoB), known to be present in GLF from previous work (12), prothrombin, C₃ component of complement and caeruloplasmin—as representative soluble proteins—and transferrin receptor, epidermal growth factor receptor (EGF-R), asialoglycoprotein receptor (ASGP-R), and glucose transporter—as membrane proteins. Fig. 8 shows the distribution of SM secretory proteins between NB and B. The upper panel shows newly synthesized ApoB detected by immunoprecipitation and fluorography and prothrombin detected by immunoprecipitation followed by SDS-PAGE, Western blotting, and detection by overlay with [¹²⁵I] goat anti–rabbit IgG, followed by autoradiography. The lower panel shows the C₃ component of complement, and caeruloplasmin detected by Western blotting with specific antibodies.
followed by donkey anti-sheep IgG conjugated to alkaline phosphatase. ApoB distribution, like albumin was quantitated by following the biosynthetically labeled (newly synthesized) proteins, whereas the others were detected at steady state using radioactive or alkaline phosphatase-tagged reporter antibodies. In all cases, the distribution followed the albumin pattern.

The only exception among secretory proteins was transferrin which distributed equally between NB and B in pulse/spontaneous chase experiments as well as at steady state (not shown). Transferrin may represent part of the cargo of the small vesicles in the B subfraction.

Fig. 9 shows the membrane proteins surveyed in the steady state condition. The corresponding transfers were overlaid with specific antibodies and the latter were detected by using alkaline phosphatase- or 125I-tagged reporter antibodies. In the upper panel, transferrin-R and EGF-R overlays were detected by radiiodinated secondary antibodies and their quantitation showed that ~90% of the activity present in the SM was recovered in the B subfraction. In the lower panel, ASGP-R and the glucose transporter overlays were detected by alkaline phosphatase-tagged secondary antibodies. We have also surveyed the distribution of Ce-9, a membrane protein restricted to the sinusoidal plasmalemma (2), and found...
The membrane proteins surveyed were detected by Western blotting with specific antibodies followed by radioiodinated (upper panel) or alkaline phosphatase-conjugated (lower panel) reporter antibodies. None of them was detected in the NB subfraction.

It to be distributed >90% in the B subfraction (data not shown).

The Immunoadsorbed Fraction Does Not Contain Elements from the Endocytic or Transcytotic Pathways

Since the integral membrane proteins surveyed are, with the exception of Ce-9, either endocytic or transcytotic receptors, we had to exclude the presence of endosomes and endocytic and transcytotic vesicles in the bound subfraction and thereby ensure that the small vesicular carriers immunoisolated in our protocol are true, exocytic vesicles. To this intent, we followed the uptake and distribution of an endosomal-lysosomal marker (asialofetuin), and a transcytotic marker (dimeric IgA), in the fractions collected from the gradient (see Fig. 1), as well as in the GLF subfractions obtained by immunoadsorption. Table I shows the distribution of 

\[ ^{125}\text{I} \text{ASF} \]

in the fractions initially collected from the sucrose gradient, while Fig. 10 shows the distribution of SM (GLF) counts, between the NB and B subfractions. Out of the total number of counts present in TM, only 9.45% are present in the GLF, and considering that figure as 100% for the immunoadsorption step, only 4.5% are adsorbed on the beads. By extrapolation from the initial TM number of counts, only 0.4% are present in the B subfraction. The dimeric IgA uptake and distribution were checked at 12 min after its infusion in the portal vein. Table I also shows the distribution of the TCA precipitable dIgA counts in the fractions collected from the sucrose gradient. By immunoprecipitation followed by SDS-PAGE and fluorography, no detectable dIgA was found in GLF (not shown). We also checked—by Western blotting—the steady state distribution of endogenous dIgA in the liver fractions collected from the sucrose gradient, but could not detect a signal in GLF (not shown).

Presence of Golgi Markers in the Bound Subfraction

By immunoblotting followed by \[^{125}\text{I} \text{-tagged reporter antibody},

we found that 30–35% of the Golgi proteins, \(\alpha\)-mannosidase II and \(\alpha\)-2,6 sialyltransferase present in the TM fraction, distribute in GLF (Table II). After immunosoliation from GLF (SM), the distribution of these proteins was followed by the same procedure in the ensuing subfractions and found to be 40% vs 60% in the NB vs B, respectively. By extrapolation to the initial TM fraction, the amount of these two Golgi proteins present in the B subfraction represents between 16–23% of the TM content (Table III). We have also surveyed in a few preliminary experiments, another Golgi protein namely galacosyltransferase, by a specific enzymatic assay (14). Its distribution closely followed the same pattern as for the other

Table I. Percent Distribution of TCA Precipitable ASF and dIgA Counts in Liver Fractions

| Marker* | TM | GLF | GH | 1.15 M | RMF |
|---------|----|-----|----|--------|-----|
|         | %  | %   | %  | %      | %   |
| \(^{125}\text{I-ASF}\) | 100 | 9.45 | 33.9 | 26.8 | 27.5 |
| \(^{35}\text{S-dIgA}\) | 100 | 3.06 | 18.4 | 20.4 | 48.9 |

Labeled ASF and dIgA were injected in the portal vein as described in Materials and Methods. The table shows the TCA precipitable counts in the different fractions collected from the gradient. Both ASF and dIgA are present in very low amounts in GLF (average of two experiments).

* An endocytic and a transcytotic marker
Golgi proteins studied (data not shown). The B subfraction appears to be characterized by its high relative content of membrane proteins destined for the sinusoidal plasmalemma and also by a lower, yet significant content of typical Golgi complex proteins or activities. The implications of these findings are considered under Discussion.

Discussion

By flotation of a TM fraction in a discontinuous sucrose density gradient (see Fig. 1), we have obtained a Golgi light fraction with a density of 1.06 g/ml (determined by refractometry of the collected material). GLF was used as starting material for the immunoisolation step, and thereby resolved in a population of small vesicles (B subfraction), and a population of large secretory vacuoles (NB subfraction). All the membrane proteins surveyed were found segregated in the B subfraction (>90%), whereas nearly all soluble (secretory) proteins tested were left behind in the NB subfraction (<80%). The only exception was transferrin which has slower transport kinetics than other secretory proteins (8) and—due to its recycling pattern (7, 26)—could represent part of the cargo of the small vesicular carriers. The presence of transferrin in the B subfraction is in accordance with another study in which transferrin was colocalized with plgA-R in vesicular carriers budding in vitro from a stacked Golgi fraction (23). The segregation of secretory from membrane proteins on their way to the sPM is advanced (up to 80–90%), but never complete in our 30-min pulse/spontaneous chase, in vivo experiments. In trying to improve the segregation, we have used colchicine (a microtubule inhibitor), which is known to abolish almost completely albumin and lipoprotein secretion from hepatocytes (22, 27), but to affect less extensively the transport of membrane proteins to the sPM (24). In the case of colchicine, we increased the pulse/spontaneous chase to 55 min, which led to >95% segregation of the newly synthesized plgA-R and albumin, between the B vs NB subfractions, respectively. Taken together, these findings suggest that the segregation is a progressive event, less advanced at 30 min (controls), than at 55 min (colchicine experiments).

The segregation of secretory from membrane proteins on their way to the sPM is reminiscent of constitutive vs regulated secretion found in other secretory cells (5). The regulated pathway involves a concentration step in secretion granules or vacuoles, intracellular storage, and discharge triggered by a primary messenger (secretagogue). The hepatocytes concentrate and apparently store their secretory products, but so far no trigger for their discharge has been identified. These cells appear to represent a mixed secretory cell type: they concentrate and store their secretory products like cells involved in regulated secretion, but discharge these products without the involvement of a secretagogue, like cells capable of constitutive secretion. All the membrane proteins we have surveyed apparently follow, as in other cells, a constitutive pathway.

The protocol followed and the data obtained indicate that the vesicular carriers isolated in the B subfraction are true exocytic carriers free of contamination by endocytic elements and transcytotic vesicular carriers. While the exocytic nature of these carriers appears to be well documented, we still have problems posed by the apparent inhomogeneity of the B subfraction: the first is the detection of the ER (105K) form of plgA-R and the second is the presence of Golgi antigens and enzyme activities therein. Accordingly, we have to decide if these problems reflect cell fractionation artifacts (contamination) or represent physiological conditions prevailing in situ.

Flotation of GLF in the low density sucrose layer (density = 1.06 g/ml), explained by the presence of VLDL (12), renders contamination by microsomes (density = 1.18 g/ml), highly unlikely, an assumption confirmed by electron microscopy. Also unlikely is the contamination by detached vesicular carriers (density = 1.14–1.15 g/ml). On this account we assume that all vesicles apparently free in GLF sections (Fig. 3, b and g) are in fact tethered to large Golgi elements at other levels in the pellet. The ER form of the plgA-R (105K) represents ~10% of the total receptor counts in the B subfraction. We assume that its presence in GLF is due to vesicular carrier membranes caught in transient residence in Golgi elements before recycling back to the ER. On morphological grounds and on account of our fractionation protocol, we believe that contamination by microsomes can be ruled out.

The presence of Golgi antigens and enzyme activities in the B subfraction (8–20% of the TM content) could indicate that the proteins in case are in transit for residence in the plasmalemma as documented in other cell types (28, 31) or for secretion in the blood plasma (4, 18, 28). Another possible explanation, which we consider more likely, is that these proteins recycle back from the plasmalemma to the Golgi complex (16). Finally, in principle the membrane of a vesicular carrier could be the membrane of one of the termini serviced by that carrier, in this case the trans elements of the Golgi complex. Substantial amount of galactosyl- and sialyltransferase (25% of the postnuclear supernate content) was detected in a comparable vesicular fraction in (6).

Despite the presence of these Golgi proteins in the B subfraction, we do not believe that the small vesicular carrier

| Enzyme         | TM  | GLF | GH | 1.15 M | RMf |
|----------------|-----|-----|----|--------|-----|
| α-Mann II      | 100 | 30.0| 18.6| 19.6   | 31.5|
| α2,6-Sialytr   | 100 | 35.0| 18.3| 18.2   | 28.5|

α-mannosidase II and α2,6-sialyltransferase antigens present in the fractions collected from the gradient, were detected by Western blotting followed by ["β"] reporter antibody and exposure to a phosphor-screen. Quantitation was done with the help of a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA), by counting the specific bands areas after subtracting the background.

| Enzyme         | TM  | SM  | NB  | B    |
|----------------|-----|-----|-----|------|
| α-Mann II      | 100 | 30.1| 13.4| 16.6 |
| α2,6-Sialytr   | 100 | 35.0| 11.6| 23.4 |

Golgi antigens were detected immunologically in SM as well as in the ensuing NB and B subfractions (same procedure as for Table II). Comparison with the TM fraction shows that a significant amount of these Golgi antigens is present in the B subfraction.
population isolated in our protocol represents vesiculation of Golgi elements. The high degree of segregation between soluble and membrane proteins is not compatible with mechanical fragmentation during the process of homogenization.

It appears that the vesicular carriers in the B subfraction are formed by progressive segregation of membrane proteins followed by continuous budding of the segregated domains and final separation during the incubation (in this case in the absence of ATP). We assume that the segregation of membrane proteins is progressive and selective (90% pIgA-R vs 50–60% Golgi antigens) and that our immunoisolated subfraction, contains mainly small vesicular carriers enriched in membrane cargo, still carrying Golgi proteins (to be recovered by recycling), in addition to other specific proteins that remain to be identified and characterized.

While our study presents clear evidence that in hepatocytes (which are considered to be constitutive secretory cells), secretary and membrane proteins segregate in different vesicular carriers plying between the Golgi complex and sPM, the work so far done represents only the first step towards analyzing the composition of these vesicular carriers membranes and identifying the critical components involved in sorting the cargo as well as in directing and docking these vesicles at their right destinations.

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