Hepatocytes must transport newly synthesized apical membrane proteins from the basolateral to the apical plasma membrane. Our earlier morphological study showed that the apical proteins share a late (subapical) part of the transcytotic pathway with the well characterized polymeric immunoglobulin A receptor (Barr, V. A., and Hubbard, A. L. (1993) Gastroenterology 105, 554-571). Starting with crude microsomes from the livers of (35S)methionine-labeled rats, we sequentially immunoadsorbed first vesicles containing the endocytic asialoglycoprotein receptor and then (from the depleted supernatant) vesicles containing the polymeric IgA receptor. Biochemical characterization indicated that early basolateral and late endosomes were present in the first population but not in the second. Neither Golgi-, apical plasma membrane (PM)-, nor basolateral PM-derived vesicles were significant contaminants of Golgi-, apical plasma membrane (PM)-, nor basolateral PM-derived vesicles were significant contaminants of early basolateral and late endosomes were present in the first population but not in the second. Neither Golgi-, apical plasma membrane (PM)-, nor basolateral PM-derived vesicles were significant contaminants of polymeric immunoglobulin A receptor. Biochemical characterization indicated that early basolateral and late endosomes were present in the first population but not in the second. Neither Golgi-, apical plasma membrane (PM)-, nor basolateral PM-derived vesicles were significant contaminants of either population. Both vesicle populations contained 35S-labeled receptor and 35S-labeled-dipeptidyl peptidase IV. Importantly, the elevated relative specific activity of the dipeptidyl peptidase (% of 35S-labeled/% immunoblotted) in the second population indicated that these vesicles must transport newly synthesized dipeptidyl peptidase IV. A distinct kind of vesicle was immunoadsorbed from a "carrier-vesicle fraction"; surprisingly, these vesicles contained little 35S-receptor and virtually no dipeptidyl peptidase IV. These results, together with previous kinetic data from in vivo experiments, are consistent with a computer-generated model predicting that newly synthesized dipeptidyl peptidase IV is delivered to basolateral endosomes, which also contain newly synthesized polymeric immunoglobulin A receptor. The two proteins are then transcytosed together to the subapical region.

The plasma membrane (PM)¹ of polarized epithelial cells is separated into distinct domains that have different functions and compositions (2, 3). Newly synthesized integral PM proteins must be delivered to the correct domain to maintain such functional polarity. The route used to deliver newly synthesized apical PM proteins is particularly interesting because it varies in different epithelia (3–5). In hepatocytes, all newly synthesized PM proteins studied so far are transported from the Golgi first to the basolateral PM domain. Apical PM proteins must then be internalized, sorted, and transcytosed to the apical domain (6–8). Caco-2 cells use this indirect route for a few proteins (9, 10), while Madin-Darby canine kidney (MDCK) cells deliver their PM proteins directly from the Golgi to the correct domain (11). However, even MDCK cells can transcytose apical PM proteins if they are missorted to the basolateral surface (12, 13). We want to define this transcytotic pathway: because hepatocytes rely so heavily on transcytosis, we have focused on this cell type.

Fortunately, the transcytosis of one PM protein has been studied extensively in hepatocytes (14, 15); this protein is the polymeric IgA receptor (pIgA-R), which transports IgA from blood to bile. Fig. 1 shows a current view of the intracellular itinerary of newly synthesized pIgA-R. Mature pIgA-R is delivered to the basolateral PM from the Golgi. Both free and ligand-bound receptor are internalized in clathrin-coated vesicles (16–18). Then ligand and receptor are taken to early endosomes; in uptake experiments ligand appears there very quickly (16, 19, 20). Later the ligand (presumably still traveling with the receptor) can be visualized in a subapical tubulovesicular compartment (16, 17, 21, 22). Following a brief delay, pIgA is released into bile, complexed to the ectodomain of the pIgA-R (now called secretory component). Because pIgA-R is cleaved at the apical PM, most of the receptor in the cell is less than 2 h old (23, 24). pIgA-R can be seen in both early endosomes and in the subapical compartment by immunofluorescence (25–27), showing that at steady state there are significant amounts of receptor in both compartments, but not at the apical PM (17, 23). Work in MDCK cells transfected with pIgA-R cDNA has identified multiple signals on the 103-amino acid cytoplasmic tail of pIgA-R that guide the receptor along its complicated journey (27–29).

In contrast to the pIgA-R, many apical PM proteins either have very short cytoplasmic tails with no obvious sorting signals (e.g. dipeptidyl peptidase IV (DPP IV) and aminopeptidase N; Refs. 30–35) or have no cytoplasmic sequences whatsoever (e.g. 5′ nucleotidase, a glycosphatidylinositol (GPI)-anchored protein) (36, 37). Yet all of these proteins are transcytosed in hepatocytes (6, 7). Do they travel with pIgA-R? In an earlier study, we found that bile duct ligation (BDL) slowed the transport of vesicles to the apical PM, and led to accumulation of newly synthesized pIgA-R and apical PM proteins in a common subapical tubulovesicular compartment (1). This result supports the idea that apical PM proteins share at least some of the pIgA-R pathway.

Our goal in this study was to determine if newly synthesized pIgA-R and apical PM proteins are found in the same transcytotic vesicles under normal conditions (38). In this study, we...
Rats were given 20 mCi of Tran-35S-label by saphenous vein injection. No chase was used. After various times, the animals were sacrificed by decapitation and the livers were excised and perfused with ice-cold 0.9% saline in preparation for subcellular fractionation.

125I-Asialo-orosomucoid (ASOR) was prepared by neuraminidase treatment of orosomucoid, iodinated using chloramine T as described (41), and was administered by saphenous vein injection. Both the livers were sacrificed 2 min later, and the livers were excised and perfused (43). Approximately 5 min elapsed between the ASOR injection and the homogenization of the livers.

Preparation of Fractions—Livers were homogenized in 4.3 volumes of 0.25 M sucrose, 3 mM imidazole, pH 7.4 (0.25 M Suc/Im) containing protease inhibitors (1 unit/ml Trasylol, 1 mM phenylmethylsulfonyl fluoride, and 5 μl/ml each of antipain, leupeptin, and benzamidine) by 7 strokes in a Potter homogenizer at 4200 rpm. Microsomal fractions and carrier vesicle fractions (CVF) were prepared as described by Szul et al. (38) with the following modifications. The filtered homogenate was centrifuged at 3500 × g for 10 min (60Ti rotor, Beckman L-7-55) and the supernatant was centrifuged at 180,000 × g for 60 min (60Ti rotor, Beckman L-7-55). The resulting pellet was resuspended in 20 ml of 0.25 M Suc/Im for use in immunoadsorptions and/or analysis. To make CVF, the microsomal pellet was resuspended in 14 ml of 1.22 M Suc/Im and 7 ml was successively overlaid with 8.5 ml of 1.15 M Suc/Im, 0.86 M KCl, 1.5 mM KH2PO4 (PBS). This batch will be called GAM immunoadsorbent. To produce SC166 immunoadsorbent, 1 ml of GAM immunoadsorbent was incubated with 4 mg of SC166 IgG overnight (39) and then washed again in PBS. Both immunoadsorbents were cross-linked with dimethylpimelimidate as described (44) and stored as 50% slurries in PBS/NaCl.

Immunoadsorption of 35S-Labeled Fractions—After an overnight fast, rats were given 20 mCi of Tran-35S-label and sacrificed at 25 or 110 min. Then either microsomes or CVF were prepared. Similar fractions were obtained from rats whose bile ducts had been ligated 24 h before injection of 35S-label; these rats were sacrificed at 110 min. The secreted Golgi-derived vesicles from binding to the SC166 immunoadsorbent, two rats were given 1.0 mg of CHX/100 g of body weight 15 min after injection of radioactivity and fractions were prepared 95 min later. The combined labeling and CHX chase time was 110 min.

Immunoadsorptions were performed at 4°C as shown in Fig. 2. For most experiments, 5.6 mg of microsomes or 1.4 mg of CVF diluted into 14 ml of 0.25 M sucrose, 150 mM NaCl, PBS, pH 7.4, 1% bovine serum albumin (Suc/PBS/BSA), were immunoadsorbed in 15-ml conical tubes. Generally, immunoadsorptions from microsomes used 4 times more protein than immunoadsorptions from CVF. Variations are noted in the figure legends. The fractions were first preadherent by addition of fixed Staphylococcus aureus (from ATCC) (43), that had been washed three times in Suc/PBS/BSA (700 μl of 22.5% SA/14 ml of microsomes or CVF). This mixture was incubated on a platform shaker at 200 oscillations/min for 1.25 h. The samples were spun at 3000 × g for 15 min (3750 rpm, Beckman GS-6R centrifuge, GH-3.8 rotor). The pellet was rinsed three times by resuspension-centrifugation, first in 7 ml of Suc/PBS and then in 7 ml of Suc/PBS (twice). All the rinses were discarded. The final pellet was designated “precip.” The preadherent supernatant (from the initial centrifugation) was transferred to a new tube and incubated as described above, but with affinity-purified anti-ASGP-R antibody (84 μg/ml). Fixed S. aureus was added, and the incubation was continued for a second hour. The sample was centrifuged as above. The washed S. aureus pellet was designated the “first ASGP-R immunoadsorption” (A1). The A1 supernatant was transferred to a fresh tube containing affinity-purified anti-ASGP-R antibody, and the process was repeated. The resulting S. aureus pellet was designated the “second ASGP-R immunoadsorption” (A2). The A2 supernatant was split into two 7-ml aliquots, and 490 μl of either SC166 or GAM immunoadsorbent (50% slurry) was added to an aliquot. The mixtures were incubated overnight (12–16 h) with end over end rotation, centrifuged at 300 × g for 3 min and were rinsed as described above. The SC166 and GAM samples were centrifuged at the lowest
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Starting Vesicles (Microsomes or CVF)

Incubate with anti-ASGP-R antibodies/Fixed Staph A

Spin

Washed Pellet = Preclear

Incubate with anti-ASGP-R antibodies/Fixed Staph A

Spin

Washed Pellet = A1

Incubate with anti-ASGP-R antibodies/Fixed Staph A

Spin

Washed Pellet = A2

Incubate with GAM immunoadsorbent

Spin

Washed Pellet

Incubate with SC166 immunoadsorbent

Spin

Washed Pellet = SC166

Fig. 2. The protocol for immunoadsorption of vesicles. Vesicular fractions were sequentially incubated once with fixed S. aureus, twice with anti-ASGP-R antibodies and fixed S. aureus, and then with SC166 or GAM immunoadsorbent. After each incubation, the samples were centrifuged and the pellets were washed as described under “Materials and Methods.” The preclear contains vesicles that adhere to S. aureus nonspecifically, A1 and A2 contain vesicles with DPP IV-R, and SC166 contains any vesicles with pIg-R but without ASGP-R. GAM serves as a control for vesicles that adhere to the linker antibody or Sepharose beads.

RESULTS

Characterization of the Immunoadsorbed Samples—We used in vivo metabolic labeling combined with vesicle immunoadsorption to test the hypothesis that apical PM proteins are transcytosed with pIg-R. Our starting material, the microsomal fraction, contained Golgi-derived vesicles (indicated by the presence of sialyl transferase [ST]), basolateral endosomes (indicated by the presence of ASGP-R, transferrin receptor [TF-R], and 125I-ASOR internalized for 5 min) and transcytotic vesicles (indicated by the presence of 125I-pIgR internalized for 60 min). Because pIg-R, our target for immunoadsorption, is most likely present in all of these compartments (50, 51), we designed a protocol that specifically isolated transcytotic vesicles (Fig. 2). The preclear step removed vesicles that stuck to S. aureus nonspecifically. The next two immunoadsorption steps removed membranes that contained ASGP-R. ASGP-R is abundant in early endosomes, but it is also at the basolateral PM (52) and in late endosomes (41). Thus, vesicles derived from these compartments would be found in the A1 and A2 samples. Any remaining vesicles that contained pIg-R but lacked ASGP-R would then be bound by the SC166 immunoadsorbent. The GAM immunoadsorbent served as a control for nonspecific binding. Comparison of the amounts of a particular protein in each of the immunoadsorbed samples gave us a quantitative picture of its distribution in the biosynthetic pathway. S. aureus was used for the first immunoadsorptions because we knew from previous work that we could remove all the endosomes in this manner. However, we did not use S. aureus for selecting pIg-R vesicles, because it shows higher nonspecific binding than does Sepharose.

Recovery of pIg-R—Fig. 3A shows an immunoblot of the pIg-R that was found in samples which were analyzed after the initial sedimentation (i.e., no washing). pIg-R was present in each immunoadsorbed sample, but the amount varied dramatically (Fig. 3B). The preclear and A1 samples contained >65% of the pIg-R. Of the total microsomal pIg-R, the SC166 sample consistently contained >10%, which was 6-fold more than in the GAM control. ~7% of the pIg-R remained unbound. Overall, we could account for 80–100% of the pIg-R through the four steps of the immunoadsorption protocol. However, only 30% of the microsomal pIg-R was recovered in comparable samples that had been washed prior to analysis (A1 + A2 + SC166 ~ 23% unbound ~ 7%). The difference between washed and unwashed samples was predominantly in the A1 and A2 samples, suggesting to us that immunoadsorbed vesicles may have broken during the S. au-
membranes during the washes. The pIgA-R, indicating the adsorbed vesicles probably break and lose after each immunoadsorption resulted in the loss of as much as 50% of starting pIgA-R in unwashed samples; however, washing the vesicles of this waste is the immature ER form. We could account for 80–100% of the starting pIgA-R in unwashed samples, because there was no obvious difference between the starting and unbound material (data not shown). We also determined the distributions of DPP IV and Tf-R throughout the immunoadsorption. The SC166 sample contained 99% of the homogenate pIgA-R, which was 2.5 times more DPP IV than was found in the GAM sample (range 2–3.3-fold). In contrast, only 0.03% of the homogenate Tf-R was found in either the SC166 or GAM sample. We found that Tf-R was predominantly in the A1 sample (data not shown), suggesting that this recycling receptor was present in the vesicles that contained the ASGP-R. Neither cathepsin D nor \( \beta \)-glucuronidase activity, markers of late endosomes and lysosomes (41), was present in the immunoadsorbed SC166 sample. However, both activities were found in the immunoadsorbed A1 and A2 samples. These results indicate that the SC166 samples did not include either early or late endosomes, in contrast to the A1 and A2 samples, which contained both.

**Electron Microscopy of the Immunoadsorbed Samples**—Examination of the immunoadsorbed samples by electron microscopy revealed the presence of small vesicles bound to SC166 (Fig. 4A); very few vesicles were seen in the GAM control (Fig. 4B). The SC166 vesicles ranged from 60 to 150 nm in diameter and were smaller and more uniform than the tubules and vesicles present in the A1 and A2 samples (43; data not shown). These samples also contained a few vesicles with attached ribosomes. However, both the endosomal (A1 and A2) and transcytotic (SC166) vesicles were minor components of total microsomes, because there was no obvious difference between the starting and unbound material (data not shown).

**Newly Synthesized DPP IV Is in Vesicles That Contain Newly Synthesized pIgA-R**—Next, we analyzed the distributions of

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**Fig. 3. Characterization of samples immunoadsorbed from microsomes.** A, immunoblot of pIgA-R in the immunoadsorbed samples. 400 \( \mu g \) of microsomes were used in the immunoadsorption protocol as described under "Materials and Methods" and in Fig. 2, except that gel samples were made directly from unwashed pellets at each step. 50% of the preclar and A1 and 25% of the unbound sedimentable material are shown on this immunoblot. B, recovery of pIgA-R in the various samples. Bands from immunoblots like the one shown in Fig. 2A were quantified by video densitometry. This graph shows the average of three experiments. 25% of the pIgA-R from microsomes was in the preclar, 36% was in the A1 sample, 12% in the SC166 sample, but only 2% was in the GAM sample. 6% of the pIgA-R remained unbound; most of this was the immature ER form. We could account for 80–100% of the starting pIgA-R in unwashed samples; however, washing the vesicles after each immunoadsorption resulted in the loss of as much as 50% of the pIgA-R, indicating the adsorbed vesicles probably break and lose membranes during the washes. C, \( 125^I \)-ASOR was removed by immunoadsorption with S. aureus and anti ASGP-R antibodies. Animals were given \( 125^I \)-ASOR for 2 min before sacrifice; the labeled ASOR should be in early endosomes at this time. Microsomes were prepared and used in the immunoadsorption protocol described in A. The graph shows the distribution of the sedimentable ASOR in unwashed immunoadsorbed samples (100% = the sedimentable \( 125^I \)-ASOR found in microsomes, 90% of the total ASOR). Essentially no ASOR is left after A2, showing that early endosomes have been efficiently removed. The pattern seen here, preclar + A1 > A2 binding, is characteristic of the binding of membranes containing ASGP-R. As seen in B, 12% of the microsomal pIgA-R was found in immunoadsorbed SC166 samples, demonstrating the presence of vesicles containing pIgA-R but not ASGP-R.

**Fig. 4. Electron micrographs of the immunoadsorbed samples.** Immunoadsorbed samples were fixed and processed for EM without washing. A, small vesicles (60–150 nm in diameter) were bound to the SC166 immunoadsorbent. The inset shows a random selection of bound vesicles. Usually only single vesicles were bound to the beads, although occasionally aggregates were present. B, a few vesicles were seen on the GAM immunoadsorbent. Bar = 0.5 \( \mu m \).
DPP IV and pIgA-R in samples immunoadsorbed from liver microsomes of rats labeled with Tran\(^{35}\)S-label for 110 min in vivo. After 110 min of labeling, microsomes were prepared and 2600 \(\mu\)g were used in the immunoadsorption protocol as described under "Materials and Methods" and in Fig. 2. The vesicles bound to the immunoadsorbents were solubilized, divided into portions, either DPP IV or pIgA-R was immunoprecipitated and gel samples were made from the immunoprecipitates. The preclear, A1, and A2 samples were run on 1 gel, while the SC166 and GAM samples were run on another. Each gel contained a standard curve of immunoprecipitated homogenate and 2 dilutions of microsomes. A, fluorogram showing \(^{35}\)S-labeled DPP IV. Various amounts of each immunoadsorbed sample (indicated by Ad) were loaded to obtain bands of similar densities; the caption shows how much sample was loaded on the gel (equivalent \(\mu\)g of starting microsomes). B, fluorogram showing \(^{35}\)S-labeled DPP IV. Various amounts of each immunoadsorbed sample (indicated by Ad) were loaded to obtain bands of similar densities; the caption shows how much sample was loaded on the gel (equivalent \(\mu\)g of starting microsomes). C, fluorogram showing \(^{35}\)S-labeled pIgA-R. D, immunoblot showing the distribution of pIgA-R in the various immunoadsorbed samples. D. Immunoblot showing the distribution of pIgA-R in the various immunoadsorbed samples. A substantial amount of pIgA-R is found in the SC166 sample, while very little is seen on GAM immunoadsorbent. Presumably the pIgA-R found in the A1 and A2 samples is in early basolateral endosomes, a compartment known to be involved in the transcytosis of pIgA-R.

DPP IV and pIgA-R are transcytosed together in rat hepatocytes.
We quantified the amounts of 35S-labeled and immunoreactive DPP IV—

...were treated with CHX for 95 min before the preparation of microsomes. 400 µg of these microsomes were used in the immunoadsorption protocol, gel samples were made without immunoprecipitation, and the resulting blots were probed with polyclonal anti-sialyl transferase antibodies. A, ST was present in immunoadsorbed SC166 samples from control microsomes, indicating that Golgi-derived vesicles were present. B, ST was substantially depleted from SC166 samples immunoadsorbed from microsomes of CHX-treated animals. The amount of ST in microsomes was slightly increased by CHX treatment. (40 ± 9% of the homogenate ST was in control microsomes; 60 ± 20% of homogenate ST was in microsomes from CHX-treated animals.)

**FIG. 6.** Vesicles containing sialyl transferase do not bind to SC166 immunoadsorbent after in vivo CHX treatment. Animals were treated with CHX for 95 min before the preparation of microsomes. 400 µg of these microsomes were used in the immunoadsorption protocol, gel samples were made without immunoprecipitation, and the resulting blots were probed with polyclonal anti-sialyl transferase antibodies. A, ST was present in immunoadsorbed SC166 samples from control microsomes, indicating that Golgi-derived vesicles were present. B, ST was substantially depleted from SC166 samples immunoadsorbed from microsomes of CHX-treated animals. The amount of ST in microsomes was slightly increased by CHX treatment. (40 ± 9% of the homogenate ST was in control microsomes; 60 ± 20% of homogenate ST was in microsomes from CHX-treated animals.)

**FIG. 7.** The immunoadsorption of DPP IV and HA321 from liver microsomes of CHX-treated animals given Tran (label for 110 min in vivo). Microsomes from CHX-treated animals were used in the immunoadsorption protocol and analyzed like those in Fig. 5. A, fluorogram showing 35S-labeled DPP IV. After CHX treatment, the immunoadsorption of Golgi-derived vesicles was greatly reduced, but the amount of labeled DPP IV in the immunoadsorbed SC166 sample did not decrease. The distribution of 35S-labeled DPP IV in the immunoadsorbed samples was not affected by CHX treatment. B, immunoblot showing the distribution of DPP IV. The distribution of total DPP IV in the immunoadsorption was unchanged by CHX treatment. Because the amount of labeled DPP IV in vesicles bound to SC166 was not decreased by CHX treatment, the newly synthesized DPP IV in this sample was not from Golgi-derived vesicles. C, fluorogram showing newly synthesized HA321. All of these samples were run on a single gel. Newly synthesized HA321 was found in the A1 sample, not in the SC166 sample. D, immunoblot showing the distribution of HA321, these samples were also run on a single gel. There was no 35S-labeled and little immunoreactive HA321 in the SC166 sample (in fact, an examination of the blot shows mainly background staining). This suggests that the vesicles containing DPP IV and plgA-R that bind to SC166 immunoadsorbent arise from a compartment involved in transcytosis of apical proteins, after separation from basolateral proteins.

HA321 was never detected in the SC166 sample. (A small amount of HA321 (0.05% of homogenate) was detected in the immunoblot shown in Fig. 7, but in another sample there was no immunoreactive protein.)

We did not use CHX routinely because it altered the subcellular distribution of plgA-R and changed the morphology of many hepatocytes (data not shown).

The SC166 Samples Are Enriched in Newly Synthesized DPP IV—We quantified the amounts of 35S-labeled and immunoreactive DPP IV in the samples from the sequential immunoadsorptions (Fig. 8). Since we would predict that transcytotic vesicles would contain predominantly newly synthesized molecules, the ratio of newly synthesized to total protein should be higher in these vesicles than in homogenate. This ratio will be higher for a protein with a long half-life and low synthetic rate, like DPP IV; most of the DPP IV protein is at the apical PM and very few newly synthesized molecules are in transit to that surface (24). In contrast, older plgA-R is removed once it reaches the apical PM (22, 54, 55). Thus, most of the plgA-R within the hepatocyte is newly synthesized; this means that the specific activity of plgA-R within transport vesicles is never much greater than the specific activity of homogenate. The ratio ([35S]-labeled protein, % of homogenate/immunoblotted protein, % of homogenate), which we called the specific activity, indicates the relative enrichment of newly synthesized molecules in a sample in comparison to homogenate.

Microsomes contained about half the 35S-labeled DPP IV from homogenate, but showed little increase in specific activity (Fig. 8A). Although the early endosomal samples (A1 + A2) contained 10% of 35S-labeled DPP IV, the specific activity did not increase (Fig. 8B). In contrast, the SC166 samples had less 35S-labeled DPP IV (3%), but this DPP IV had a specific activity 25 times higher than homogenate (Fig. 8C, note the change of scale on the specific activity axis). Such a high specific activity indicated that we had immunoadsorbed vesicles involved in the transport of newly synthesized DPP IV 110 min after synthesis. It is formally possible that these vesicles contained older unlabeled plgA-R, while other vesicles in the SC166 samples contained the newly synthesized plgA-R. However, the short half-life of plgA-R in hepatocytes predicts that no membranes would contain primarily unlabeled plgA-R.

The Vesicles Containing Newly Synthesized DPP IV Are Not Present in a “Carrier” Vesicle Fraction—We also examined immunoadsorptions from the CVF, because previous studies indicated that it was enriched in transcytotic vesicles (38, 56). By immunoblot analysis, CVF also contains early endosomes, Golgi derived vesicles and a small amount of PM (data not shown). Approximately 50% of the plgA-R from the CVF was found in A1 + A2 samples and another 20% bound to SC166 immunoadsorbent, accounting for the majority of the plgA-R in the starting fraction (data not shown).

Fig. 9 shows the distribution of DPP IV and plgA-R immunoadsorbed from CVF. The immunoadsorbed A1 + A2 samples were similar to those adsorbed from microsomes. However, we found very little newly synthesized DPP IV in the SC166 sample.

We also performed immunoadsorptions using CVF from BDL rats. There was no increase in the amount of newly synthesized DPP IV immunoadsorbed by SC166, showing that the subapical compartment was not in this fraction. In addition, the specific activity of the DPP IV was low in all of the samples that were immunoadsorbed from CVF. (The quantification of DPP IV in the SC166 samples from CVF is given in Fig. 10.) We also examined immunoadsorptions from animals labeled for 90 min, to look for transcytotic vesicles that might have already passed through a compartment found in the 110-min CVF. However, the results matched those of the 110-min labeling experiments (data not shown).

The SC166 samples from CVF consistently contained 1–2% of both the 35S-labeled and total plgA-R. Moreover, there was less 35S-labeled plgA-R in immunoadsorptions of CVF from rats labeled for 25 min than in similar immunoadsorptions from rats labeled for 90 or 110 min. Finally, SC166 samples from CVF did not contain HA321 (data not shown). These characteristics indicated that the SC166 samples from CVF contained vesicles involved in the transport of plgA-R after it had passed through the basolateral PM; however, these vesicles lacked newly synthesized DPP IV.
The characteristics of the immunoadsorbed samples depend on the fractions used, not on the treatment of the animal. The amounts of pIgA-R and DPP IV found in SC166 samples immunoadsorbed from the same fraction appears to be similar in all our treatment groups. Because the variation between animals given different treatments was as great as the variation between groups, we combined the data from all the groups.

In immunoadsorptions of microsomes from control, BDL-, or CHX-treated animals labeled for 110 min, 3% of the 35S-labeled DPP IV was found in the SC166 samples with a specific activity of 25-times homogenate (Table I) (average of data from 6 animals). These vesicles also contained an average of 13% of the 35S-labeled pIgA-R. In contrast, in immunoadsorptions of CVF from control or BDL animals labeled for 110 or 90 min, only 0.07% of the 35S-labeled DPP IV was found in the SC166 samples with a specific activity of 1.4-times homogenate (Table II) (average of data from 5 animals). These vesicles contained DPP IV and pIgA-R are transcytosed together in rat hepatocytes.

## FIG. 8.
Quantification of DPP IV in samples immunoadsorbed from microsomes. Bands from fluorograms and immunoblots like those shown in Figs. 5 and 7 were quantified by video densitometry (data from BDL animals are also shown). The darkness of each band was compared to a standard curve of homogenate to obtain the percentage of the starting homogenate found in the immunoadsorbed samples. 35S values were calculated from fluorograms, and total amounts were calculated from immunoblots. The specific activity was then calculated by taking the ratio of 35S-labeled protein to total protein for each sample. Each bar shows data from a different animal. A, DPP IV in microsomes. About 40% of the 35S-labeled DPP IV from homogenate was in the microsomal fraction; slightly less was found in microsomes from CHX-treated animals. The specific activity of the DPP IV in microsomes was about the same as homogenate (1.2 times homogenate overall), so the microsomal fraction was not enriched in newly synthesized DPP IV. B, DPP IV in the A1 and A2 samples. Membranes that contained ASGP-R also contained about 10% of the newly synthesized DPP IV from homogenate was in the microsomal fraction; slightly less was found in microsomes from CHX-treated animals. The specific activity of the DPP IV in microsomes was about the same as homogenate (1.2 times homogenate overall), so the microsomal fraction was not enriched in newly synthesized DPP IV. C, DPP IV in the SC166 samples. 3% of the newly synthesized DPP IV from homogenate was found in the immunoadsorbed SC166 samples. But the specific activity of this DPP IV was 25 times that of homogenate (note the change of scale on the right y axis), indicating these samples do not contain a compartment(s) transporting newly synthesized DPP IV. Moreover, neither the amount nor the specific activity was decreased by CHX treatment, indicating that this DPP IV was in a post-Golgi compartment.

## FIG. 9.
Immunoadsorption of DPP IV and pIgA-R from liver CVF of animals given Tras5-S-label for 110 min in vivo. After 110 min of labeling, a CVF was prepared, used in the immunoadsorption protocol and analyzed as described under “Materials and Methods” and Fig. 5. A, fluorogram showing 35S-labeled DPP IV. These samples were all run on a single gel. Newly synthesized DPP IV was found in the immunoadsorbed A1 sample, but very little was in the immunoadsorbed SC166 sample. B, immunoblot showing the distribution of DPP IV. These samples were also run on a single gel. The A1 sample contained a significant amount of immunoreactive DPP IV. C, fluorogram showing 35S-labeled pIgA-R. There were vesicles containing labeled pIgA-R bound to the SC166 immunoadsorbent. D, immunoblot showing the distribution of pIgA-R. A substantial amount of pIgA-R from CVF, both 35S-labeled and immunoreactive, was found in the immunoadsorbed A1 and SC166 samples. There was very little newly synthesized DPP IV in the immunoadsorbed SC166 sample.

## FIG. 10.
Quantification of DPP IV in SC166 samples immunoadsorbed from CVF. Bands from fluorograms and immunoblots like those shown in Fig. 9 were quantified by video densitometry (data from BDL animals are also shown) and analyzed as described in Fig. 8. Each bar shows data from a different animal. Very little newly synthesized DPP IV was found in the SC166 samples; only 0.1% of the homogenate DPP IV. Furthermore the specific activity of this DPP IV was low (1.4 times homogenate). Thus, we did not find newly synthesized DPP IV being transported with pIgA-R in vesicles found in the CVF.

The characteristics of the immunoadsorbed samples depend on the fractions used. Not on the treatment of the animal—The amounts of pIgA-R and DPP IV found in SC166 samples immunoadsorbed from the same fraction appears to be similar in all our treatment groups. Because the variation between animals given different treatments was as great as the variation between groups, we combined the data from all the groups. In immunoadsorptions of microsomes from control, BDL-, or CHX-treated animals labeled for 110 min, 3% of the 35S-labeled DPP IV was found in the SC166 samples with a specific activity of 25-times homogenate (Table I) (average of data from 6 animals). These vesicles also contained an average of 13% of the 35S-labeled pIgA-R. In contrast, in immunoadsorptions of CVF from control or BDL animals labeled for 110 or 90 min, only 0.07% of the 35S-labeled DPP IV was found in the SC166 samples with a specific activity of 1.4-times homogenate (Table II) (average of data from 5 animals). These vesicles contained...
DPP IV and plgA-R Are Transcytosed Together in Rat Hepatocytes

DISCUSSION

Three Kinds of Vesicles Transport plgA-R from Basolateral to Apical PM in Hepatocytes; Only Two of These Carry DPP IV—We were able to immunoadsorb three biochemically distinct vesicles that all appeared to be involved in the transport of newly synthesized plgA-R between the basolateral and apical PM. First, we found vesicles that contained ASGP-R, a large amount of newly synthesized plgA-R (18% of homogenate), and a substantial amount of newly synthesized DPP IV (10% of homogenate). Next we found vesicles that lacked ASGP-R but contained a substantial amount of newly synthesized plgA-R (13% of homogenate) and some newly synthesized DPP IV (3% of homogenate). Finally, there were vesicles in the CVF that contained a small amount of newly synthesized plgA-R (2% of homogenate), but lacked both ASGP-R and DPP IV. Only the middle group, the vesicles with plgA-R and DPP IV, contained DPP IV with a high specific activity, a feature we expected in vesicles transporting newly synthesized apical PM proteins.

What subcellular compartments involved in the transcytosis of plgA-R give rise to these three kinds of vesicles?

A Computer Model of the Transport of Newly Synthesized DPP IV—We used the Stella modeling program to explore the movement of DPP IV through the biosynthetic pathway (Fig. 11). The model is based on simple assumptions. We assumed that exit from a compartment is random and proportional to the amount in the compartment, so we have used first order rate constants to model movement between compartments. However, synthesis in the ER required a discrete residence time, because most proteins cannot leave the ER until they have attained the proper conformation (57, 58). The kinetics of the maturation and movement of DPP IV determined in earlier in vivo studies from this laboratory were used to define the rate constants used in the model (6, 7).

We determined the steady state distribution of DPP IV and the distribution of a pulse 110 min after synthesis in a model that included the known biosynthetic compartments and only one transcytotic compartment between the basolateral and apical PM (Fig. 11A). This initial choice was based on our identification of the SC166-immunoadsorbed vesicles as the only obvious compartment that was involved in the transcytosis of newly synthesized DPP IV. The movement of DPP IV through the biosynthetic pathway predicted by this model is slightly different from that found in the experimental studies (6, 7) (Fig. 11B). In particular, DPP IV arrives at the apical PM more quickly than was found experimentally, and the peak at the basolateral PM is smaller than the data indicates, although it occurs at the correct time. It is important to remember that the experimental data are from in vivo studies that involved single point determinations from a series of animals; this means that the exact distribution of 35S-labeled DPP IV is not well defined experimentally. In addition, the amounts in the various compartments are often estimates based on the recoveries in the fractions. Given the uncertainty in the experimental data, the model does a good job of predicting the rate of DPP IV maturation and arrival at the PM.

The amount of newly synthesized DPP IV in the transcytotic compartment and the specific activity calculated from the model are shown in Fig. 11C. The model predicts that 5.6% of the DPP IV made in a 15-min pulse should be in this compartment 110 min after synthesis. We found an average of 3% of the labeled DPP IV from homogenate in the SC166 sample from microsomes. Since we may have lost bound membrane during the washes, the amount we found is in good agreement with the model. Although we did not find much 35S-labeled DPP IV, it is not an unreasonably small amount. The specific activity in the transcytotic compartment in the model is 100 times that of homogenate, while the specific activity in the SC166 samples was 25 times homogenate, indicating that the experimental sample has more unlabeled DPP IV than would be predicted, based on our simple model. However, if we had immunoadsorbed 2% of the 35S-labeled plgA-R, much less than was found in the samples from microsomes.

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### Table I

| Sample         | DPP IV (% of homogenate) | Total SA | 35S | | SA |
|----------------|--------------------------|----------|-----|-----|-----|
| Microsomes     | 43 ± 11                  | 37 ± 12  | 1.2 | 89 ± 23 | 88 ± 23 |
| Im A1          | 8 ± 2                    | 5.4 ± 1.5| 1.5 | 13 ± 4 | 9 ± 6 |
| Im A2          | 2 ± 0.9                  | 1.1 ± 0.8| 2.0 | 5 ± 2 | 2.2 ± 1.5 |
| Im SC166       | 3 ± 2                    | 0.12 ± 0.06| 25 | 13 ± 6 | 10 ± 9 |
| Im GAM         | 0.2 ± 0.2                | 0.04 ± 0.03| 5  | 1 ± 0.8 | 1 ± 0.8 |

### Table II

| Sample         | DPP IV (% of homogenate) | Total SA | 35S | | SA |
|----------------|--------------------------|----------|-----|-----|-----|
| CVF            | 8 ± 4                    | 6 ± 2    | 1.3 | 15 ± 5 | 15 ± 6 |
| Im A1          | 2.2 ± 0.6                | 2.1 ± 0.7| 1.0 | 3.7 ± 1 | 4 ± 2 |
| Im A2          | 0.25 ± 0.07              | 0.2 ± 0.2| 1.2 | 0.5 ± 0.6 | 0.8 ± 0.1 |
| Im SC166       | 0.07 ± 0.04              | 0.05 ± 0.02| 1.4 | 1.7 ± 0.4 | 1.7 ± 0.7 |
| Im GAM         | 0.01 ± 0.01              | 0.01 ± 0.01| 1.0 | 0.1 ± 0.1 | 0.1 ± 0.1 |

### Bands from immunoblots and fluorograms like those shown in Figs. 5 and 7 were quantified by video densitometry. The darkness of each band was compared to a standard curve of homogenate to obtain the percentage of the total homogenate found in the immunoadsorbed samples. 35S values were calculated from fluorograms and total amounts were calculated from immunoblots. The specific activity (SA) is the ratio of 35S to total amounts. If the variation between animals in a treatment group was larger than the variation between groups, the data from the different groups was combined. Values for DPP IV and pIgA-R were the average of measurements from control, BDL-, and CHX-treated animals. Values for DPP IV are the average of measurements from control and BDL animals (n = 4); samples from CHX-treated animals contained less plgA-R and were not used (range for Im SC166 = 0.01–0.07). Values for pIgA-R are the average of measurements from the control and BDL animals (n = 4); samples from CHX-treated animals contained less plgA-R and were not used (range for Im SC166 = 0.01–0.07).
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FIG. 11. Analysis of the transcytosis of DPP IV using a Stella model. A, a schematic drawing of the model. The boxes represent intracellular compartments, and the arrows represent the movement of DPP IV from one compartment to the next. The cloud at the beginning signifies the input of newly synthesized protein, while the cloud at the end signifies degradation. The ER conveyor is a mathematical construct that retains newly synthesized DPP IV for a set period (4 min) before allowing movement to the Golgi. All other transport steps are modeled as first order processes. There is only one long-lived transcytotic compartment. B, comparison of the model to the in vivo kinetics of DPP IV biosynthesis. These graphs show data from earlier studies on the maturation and movement of newly synthesized DPP IV from the ER to the apical PM in comparison to the kinetics calculated from the model. The left graph shows movement from the ER through the Golgi, while the right side shows movement through the basolateral PM to the apical PM. The model matches the kinetics of DPP IV biosynthesis fairly well, although the model brings DPP IV to the apical membrane a little quickly. The model also predicts a smaller amount of newly synthesized DPP IV at the basolateral membrane at 70 min. However, the data show only that the peak of basolateral DPP IV occurs around 70 min; we obtained the peak amount by subtracting the amounts in the ER and Golgi at this time from 100%. If a substantial amount of newly synthesized DPP IV is basolateral endosomes, as is indicated by the immunoadsorption data, then the amount at the basolateral PM decreases. Adding basolateral endosomes to the kinetic model also delays the arrival of newly synthesized DPP IV at the apical PM, so the revised model fits the data better. C, characteristics of DPP IV at 110 min after synthesis. The model predicts that 6% of the newly synthesized DPP IV should be in the subapical compartment at this time. We found about 3% of the 35S-labeled DPP IV in the immunoadsorbed SC166 samples, so the model indicates that this small amount is not unreasonable. The model predicts that DPP IV in all the biosynthetic compartments should have a high specific activity and that in the transcytotic compartment the specific activity should be even higher than the 25-fold increase we found in the SC166 samples. However, even a very small amount of apical PM in our immunoadsorbed samples would decrease the specific activity dramatically because of the large amount of unlabeled DPP IV at the apical surface.

DPP IV and plgA-R are transcytosed together in rat hepatocytes. The transcytosis of plgA-R occurs via the subapical compartment. The specific activity of DPP IV in the subapical compartment is significantly higher than in other compartments, indicating that the two molecules are transcytosed together. This supports the hypothesis that DPP IV and plgA-R are co-trafficked through the same pathway.
and A2 samples (10% of the \(^{35}\)S-labeled DPP IV and 5% of the total DPP IV, with a relative specific activity of 2 times homogenate) cannot be fit into our current kinetic model. The model predicts that the relative specific activity of all the biosynthetic compartments, including the ER, should be about 30 times that of homogenate. Moreover, putting a significant amount of the total DPP IV (more than 2%) into any biosynthetic compartment at steady state prevents the pulse of newly synthesized DPP IV from reaching the apical PM for 6 h. Since we know that ASGP-R is present in many intracellular compartments, it is likely that the A1 and A2 samples contain a mixture of vesicles and that the DPP IV in these samples is present in two kinds of vesicles, some that contain mainly labeled DPP IV and some that contain older, unlabeled DPP IV.

The A1 and A2 samples contain markers of several compartments, including Golgi (about 5% of the homogenate ST), basolateral PM (1–2% of the homogenate HA321), early endosomes (80% of the sedimentable ASOR in microsomes), and late endosomes (2.5% of cathepsin D, 1.5% of \(\beta\)-glucuronidase). Only early endosomes are present in sufficient quantity in the A1 + A2 samples to account for 10% of the newly synthesized DPP IV. We speculate that the unlabeled DPP IV in A1 + A2 is from late endosomes. Internalized material from the basolateral and apical surfaces meet at the level of late endosomes in MDCK cells (59), so this compartment could contain proteins from both PM domains in hepatocytes. Thus, the unlabeled DPP IV found in A1 and A2 could be older DPP IV internalized from the apical PM and on its way to degradation (23).

If the model is adjusted to show transit of newly synthesized DPP IV through basolateral endosomes, newly synthesized DPP IV leaves the basolateral PM earlier and arrives at the apical PM later. Thus, the revised model fits the experimental kinetic data better. It also predicts the movement of pIgA-R from the Golgi to the apical PM fairly accurately (data not shown).

There Is a Transport Step Where \(\text{pIgA-R} \text{ and } \text{DPP IV} \text{ Are Separate} \text{— In this study we also found vesicles that contained labeled pIgA-R, little labeled DPP IV, and yet had many characteristics of vesicles involved in the late transport of pIgA-R. This is consistent with earlier studies showing that vesicles immunoadsorbed from CVF were enriched in mature forms of pIgA-R, contained dimeric IgA and therefore were transcytotic vesicles (38). Antibodies generated against components of samples immunoadsorbed from CVF recognize a 108-kDa protein that has been implicated in vesicle fusion throughout the biosynthetic pathway (38, 56, 60); thus, these vesicles could be carriers of newly synthesized pIgA-R. However, the low recovery of labeled pIgA-R from a similar starting fraction (CVF) in our study (2% of homogenate), together with our failure to find labeled DPP IV in immunoadsorptions of CVF from BDL ani-

The HA321 data indicated that only a small amount of the basolateral PM in microsomes was immunoadsorbed. This appears to contradict our results with Tf-R and ASGP-R, since these proteins were removed from the starting microsomes and they are certainly present in basolateral PM as well as early endosomes. However, the explanation lies in the relative distributions of the various proteins in the two compartments. If 20% of the Tf-R is at the PM (73, 74) and 25% of the PM was recovered in microsomes, then about 5% of the total receptor present in microsomes was from basolateral PM (30–40% of the total Tf-R is in microsomes) presumably the rest was from early endosomes. If we assume that all of the HA321 in microsomes represents basolateral PM and only 5% was bound to our immunoadsorbent, then 90–95% of Tf-R from basolateral PM or about 5% of the total receptor should remain unbound. We found only 1% of the Tf-R left unbound or about 1/5 of the amount we expected. One possible explanation is that the places in the basolateral PM where recycling receptors like ASGP-R and Tf-R are clustered were more efficiently immunoadsorbed, resulting in an almost complete removal of these proteins, while the rest of the basolateral PM remained unbound.

\(^1\text{DPP IV and pIgA-R Are Transcytosed Together in Rat Hepatocytes}\)}
proteins proceeds with the same kinetics. This suggests that DPP IV is both internalized and sorted into the transcytotic pathway efficiently. In contrast, 5′ nucleotidase is transcytosed very slowly, as if internalization or sorting is difficult for GPI-anchored proteins. Thus, while the same transcytotic pathway may be used by all proteins, the interactions with the machinery may be quite different. Finally, if DPP IV is internalized very slowly, as if internalization or sorting is difficult for GPI-A-R and ASGP-R. Clearly, more may be quite different.

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