Rab1a and Multiple Other Rab Proteins Are Associated with the Transcytotic Pathway in Rat Liver*

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To better understand the function of Rab1a, we have immunoisolated Rab1a-associated transport vesicles from rat liver using affinity-purified anti-Rab1a-coated magnetic beads. A fraction enriched in endoplasmic reticulum (ER) to Golgi transport vesicles (CV2, $\rho = 1.158$) was subjected to immunoisolation, and proteins of the bound and non-bound subfractions were analyzed by Western blotting. To our surprise, we found that immunoisolated vesicles contained not only ER markers (105-kDa form of the polymeric IgA receptor (pIgAR)) but also transcytotic markers (dIgA and the 120-kDa form of pIgAR), suggesting that Rab1a is associated with transcytotic vesicles in rat liver. To investigate this possibility, we used an antibody to the cytoplasmic domain of pIgAR to immunoisolate transcytotic vesicles from a fraction (CV1, $\rho = 1.146$) known to be enriched in these vesicles. Rab1a was detected in the immunoadsorbed subfractions. The composition of the vesicles immunoisolated from the CV1 fraction on anti-Rab1a was similar to that of transcytotic vesicles immunoisolated from the same fraction on pIgAR. Both were enriched in transcytotic markers and depleted in ER and Golgi markers. The main difference between the two was that those isolated on anti-Rab1a appeared to be enriched in post-endosomal transcytotic vesicles, whereas those isolated on pIgAR contained both pre- and postendosomal elements. Analysis of anti-Rab1a isolated vesicles using [$\alpha$-32P]GTP overlay demonstrated the presence of multiple GTP-binding proteins. Some of these were identified by immunoblotting as epithelia-specific Rab17 and ubiquitous Rab1b, -2, and -6. Taken together, these results indicate that: 1) Rab1a is associated with both ER to Golgi and postendosomal transcytotic vesicles, and 2) multiple GTP-binding proteins are associated with each class of isolated vesicle.

Membrane traffic in eukaryotic cells is mediated by vesicular carriers, which bud from a donor compartment and are targeted to and fuse with the appropriate acceptor membrane. The Rab family of small GTP-binding proteins is known to play an important role in the control of these complex events (1–6). It is thought that each Rab protein is associated with a distinct subcellular compartment and associated vesicular carriers to regulate the specificity and directionality of vesicular transport (7).

So far more than 30 Rab proteins have been identified, and some of them have been localized to specific organelles and transport vesicles in mammalian cells (8–12). In addition, in vitro and in vivo studies have demonstrated that each transport step in the exocytic and endocytic pathways involves at least one Rab protein. For example, Rab1a, Rab1b, and Rab2 are located in the ER(1) to Golgi intermediate compartment and cis Golgi cisternae and are required for ER to Golgi and intra-Golgi transport (13–15); Rab4 and Rab5 are present in early endosomes (11, 16) and are involved in regulation of vesicular transport between the plasma membrane (PM) and early endosomes (17–19); and Rab7 and Rab9 are associated with late endosomes and are required for transport to or from late endosomes (20–22).

Although common transport pathways in most cells use ubiquitously expressed Rabs, highly differentiated cells with unique transport pathways may require specific Rab proteins. To date several tissue- or cell type-specific Rabs have been identified (23–26). For example, Rab3a is preferentially expressed in neuroendocrine cells and neurons, where it is specifically associated with synaptic vesicles and appears to mediate neurotransmitter release (23, 24). Rab3d is mainly expressed in adipocytes and pancreatic acinar cells and is thought to control regulated exocytosis of glucose transporter-containing vesicles andzymogen granules (25, 27). Rab17 is specifically expressed in epithelial cells, where it is localized at the basolateral PM and in apical endosomes and is assumed to be involved in regulation of transcytosis across epithelial cells (26).

It is not yet clear whether one Rab or a set of Rabs are required for each transport step. Recent studies have localized more than one Rab in the same vesicular carriers and related intracellular compartments (28, 29), suggesting that multiple Rabs may associate with vesicular carriers operating at the same relay. If multiple Rabs are present on a single class of transport vesicles, are the sets of Rabs relay-specific or is there overlap? To address these questions, isolation and characterization of distinct classes of homogeneous vesicular carriers and identification of their associated Rab proteins are required.

An excellent system for studying this problem is the hepatocyte in which the major intracellular transport pathways (exocytosis, endocytosis, and transcytosis) are well characterized (30, 31). In addition, specific markers (different forms of the polymeric IgA receptor (pIgAR) and dimeric IgA (dIgA)) are available for vesicles operating along these pathways (32–34). In this study we have immunoisolated Rab1a-associated vesicles.

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1 The abbreviations used are: ER, endoplasmic reticulum; B-AF, biotinylated-asialofetuin; dIgA, dimeric IgA; pIgAR, polymeric IgA receptor; PM, plasma membrane; EGF, epidermal growth factor; FCS, fetal calf serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; ARF, ADP-ribosylation factor; IEF, isoelectric focusing; PVDF, polyvinylidene difluoride; ASGPR, asialoglycoprotein receptor; CV1 and CV2, carrier vesicle fractions 1 and 2, respectively.

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ile from rat liver fractions and, to our surprise, found that Rab1a is associated with vesicles involved in transport along both the exocytic and transcytotic pathways, leading us to postulate that transcytosis may be regulated through interactions between specific (Rab17) and ubiquitous Rab8 (Rab1a and others).

EXPERIMENTAL PROCEDURES

**Materials and Antibodies—**Sprague-Dawley rats (Bantin & Kingman, Fremont, CA), weighing 150–250 g, were used in these experiments. Magnetic beads (M500) were from Dynal Inc. (Lake Success, NY). S107 hybridoma cells were a gift from Dr. D. Bole (University of Michigan). Recombinant Rab1a and Rab1b proteins (15, 35) and antibod- ies to Rab1 in polyclonal probes were generously provided by Dr. W. Balch (Scripps Research Institute); recombinant Rab6 was kindly provided by Dr. B. Goud (Institut Pasteur). Affinity-purified anti-peptide antibodies to Rab1a and Rab6 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Affinity-purified antibody to the cytoplasmic tail of pIgAR was prepared as described (36), affinity-purified anti-peptide antibody against Rab2 was prepared as described (37) by adding [a-32P]GTP overlay was modified from the procedure detailed by Nickel et al. (40). Briefly, PVDF membranes were soaked in 50 mM phosphate buffer, pH 7.6, containing 10 mM MgCl2, 2 mM dithiothreitol, 0.3% Tween 20, 100 mM ATP twice for 15 min each, followed by incubation with [a-32P]GTP (DuPont NEN) in the same buffer at 1 µCi/ml for 2 h. The membranes were washed with the same buffer six times for 2 min each and air-dried. The bound [a-32P]GTP was detected by autoradiography (2 h to overnight exposure) on Kodak X-Omat film.

**Quantitation of Rab1a and Total Protein on Isolated Vesicles—**For quantitation of the amount of Rab1a or Rab6 associated with immunolabeled vesicles, standard curves of recombinant Rab1a or Rab6 with 5- or 10-ng increments were made over a 10–100-ng range. Recombinant proteins and immunolabeling samples were loaded onto the same gels, subjected to SDS-PAGE, and transferred to PVDF membranes. The PVDF membranes were blocked with anti-Rab1 or anti-Rab6 followed by 50 µCi of [125I]protein A (DuPont NEN). The bound [125I]protein A was quantitated on a PhosphorImager, and a standard curve was plotted for each protein. The amount of Rab1a or Rab6 associated with the isolated subfractions was then determined from the standard curve. The numbers shown represent the averages of four gels for Rab1a and two for Rab6.

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**Preparation of Radiolabeled pIgAR and Biotinylated Asialofetuin—**S107 hybridoma cells, which secrete pIgA, were metabolically labeled with 100 µCi/ml Tran35S-label (ICN) for 20 h at which time medium was collected, and pIgA was concentrated with a Centricon 30 concentrator (Amicon) to 0.5 ml (1.5 x 10^6 cpn/ml) for use as a specific transcytotic vesicle marker. Asialofetuin was biotinylated using an immunoprobe biotinylation kit (Sigma) according to the manufacturer’s instructions. After incubation the biotinylated protein was passed through a Sephadex G-25 column (Sigma) according to the manufacturer’s instructions. After separation the gel was removed and equilibrated in 62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, and 10% glycerol (first dimension) in a minigel apparatus (Novex, X-CellII) using 20 mM NAOH as the catholyte and 10 mM H3PO4 as the anolyte for 30 min at 150 V, 2 h at 200 V, and 2 h at 500 V. After separation the gel was removed and equilibrated in 62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, and 10% glycerol for 15–30 min. The gel strips were then cut and applied on the top of 10% SDS-PAGE separated by SDS-PAGE (second dimension) on a Bio-Rad minigel apparatus.

**Immunoblotting—**After electrophoresis on either two-dimensional or one-dimensional gels, the separated proteins were transferred to PVDF membranes (Millipore). The membranes were incubated with primary and then secondary antibodies in 1% FCS/PBS, 0.2% Triton X-100 for 1 h each, washed three times for 15 min each between each incubation, and detected by enhanced chemiluminescence (ECL, Amersham) according to manufacturer’s instructions. Radiolabeled pIgA was detected and quantitated on a PhosphorImager using ImageQuant software.

**Subcellular Fractionation—**Liver homogenization and preparation of cytosol and total microsomal membranes were as described by Bollag and Edelstein (39) with modifications. In brief, liver total microsomes or immunoisolated samples were solubilized in IEF sample buffer (8 M urea, 0.4% amphotol pH 3–10, 2% amphotol pH 5–7, 2% Triton X-100, 1% 2-mercaptoethanol) and loaded onto a slab IEF acrylamide gel consisting of 8 M urea, 0.4% ampholyte pH 3–10, 2% amphotol pH 5–7. The samples were separated by isoelectric focusing (first dimension) in a minigel apparatus (Novex, X-CellII) using 20 mM NaOH as the catholyte and 10 mM H3PO4 as the anolyte for 30 min at 150 V, 2 h at 200 V, and 2 h at 500 V. After separation the gel was removed and equilibrated in 62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, and 10% glycerol for 15–30 min. The gel strips were then cut and applied on the top of 10% SDS-PAGE separated by SDS-PAGE (second dimension) on a Bio-Rad minigel apparatus.

**Immunoprecipitation—**For immunoprecipitation of [35S]pIgAR and [35S]-dIgA from liver fractions, 0.5 ml (for pIgAR) or 0.5 mg of protein (for dIgA) of each fraction was lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate), and antibody for pIgAR (2 µl) or dIgA (1 µg/ml) was added. Samples were subjected to SDS-PAGE and analyzed on a PhosphorImager (Molecular Dynamics) using ImageQuant software. The amount of pIgAR and dIgA was normalized to the total volume and protein concentration, respectively. For immunoprecipitation of Rab1a and Rab6 from immunolabeled vesicles, bound subfractions were lysed in Triton X-100 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100). Antibodies for Rab1a or Rab6 (1 µg/ml) were added, and the lysates were rocked for 2 h at 4°C. After centrifugation, the resulting supernatant (CV1) was collected on protein A-Sepharose. The supernatants depleted of Rab1a or Rab6 were then precipitated with 6% trichloroacetic acid at 4°C for 30 min. Samples were resuspended in equal volumes of SDS sample buffer, and proteins were separated by SDS-PAGE as above.

**Two-dimensional PAGE—**A combination of isoelectric focusing (IEF) and SDS-PAGE was used to resolve proteins in two dimensions as in Bollag and Edelstein (39) with modifications. In brief, liver total microsomes or immunoisolated samples were solubilized in IEF sample buffer (8 M urea, 0.4% amphol pH 3–10, 2% amphol pH 5–7, 2% Triton X-100, 1% 2-mercaptoethanol) and loaded onto a slab IEF acrylamide gel consisting of 8 M urea, 0.4% amphol pH 3–10, 2% amphol pH 5–7. The samples were separated by isoelectric focusing (first dimension) in a minigel apparatus (Novex, X-CellII) using 20 mM NaOH as the catholyte and 10 mM H3PO4 as the anolyte for 30 min at 150 V, 2 h at 200 V, and 2 h at 500 V. After separation the gel was removed and equilibrated in 62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, and 10% glycerol for 15–30 min. The gel strips were then cut and applied on the top of 10% SDS-PAGE separated by SDS-PAGE (second dimension) on a Bio-Rad minigel apparatus.

**Immunolocalization—**After electrophoresis on either two-dimensional or one-dimensional gels, the separated proteins were transferred to PVDF membranes (Millipore). The membranes were incubated with primary and then secondary antibodies in 1% FCS/PBS, 0.2% Triton X-100 for 1 h each, washed three times for 15 min each between each incubation, and detected by enhanced chemiluminescence (ECL, Amersham) according to manufacturer’s instructions. Radiolabeled pIgA was detected and quantitated on a PhosphorImager using ImageQuant software.
phenomenon to enrich ER to Golgi transport vesicles, CV1 contains very few of these vesicles and is enriched in transcytotic vesicles.

Continuation of CV1 and CV2 with other organelles has also been examined using protein disulfide isomerase (ER), α-mannosidase II (Golgi), and B-AF (endosomes) as markers. CV1 contains a small amount of the total protein disulfide isomerase and α-mannosidase II (1.5 and 11%, respectively) in the total microsomes and ~30% of the total B-AF. By contrast, the percentages of the total of these three markers in CV2 are 40, 30, and 10%, respectively (data not shown). Therefore, CV1 and CV2 represent fractions highly enriched in transcytotic and ER to Golgi vesicular carriers, respectively, and because of this are appropriate as starting material for immunoisolation of the respective carriers.

**Rab1a Is Tightly Membrane-associated in Rat Liver**—Rab1a has been localized by immunogold labeling to the membranes of clusters of vesicles located between the ER and the Golgi region of NRK cells (14, 42) and has been shown to be required for ER to Golgi and intra-Golgi transport (14, 15, 35). Since Rab1a is known to be associated with ER to Golgi transport vesicles, we decided to use it to isolate this population of vesicles.

Rab1a is anchored to the membrane via geranylgeranyl moieties covalently attached at cysteine residues located at the carboxyl terminus (43–45). According to current models of Rab function (1, 5), Rab proteins could be found either in the cytosol or associated with membranes. The distribution of Rab1a in liver fractions was determined by immunoblotting. As reported for cultured cells (43, 46), in rat liver most of the Rab1a is associated with membranes at steady state (Fig. 1, bottom panel, compare Cyt and TM), and it has a broad distribution across the gradient. The association of Rab1a with membranes is resistant to treatment with either high pH (100 mM Na2CO3, pH 11) or high salt (1 M KCl) (data not shown). Therefore, it behaves as an integral membrane protein. The tight association of Rab1a with membranes permitted us to use it to immunoisolate Rab1a-associated vesicles using an affinity-purified, anti-Rab1a antibody.

**Characterization of Rab1a-specific IgG**—The antibody used for immunosialation was affinity-purified IgG raised against a 19-amino acid peptide near the carboxyl terminus of Rab1a (amino acids 181–199; AAGGAEKSNVKIQSTPVKQS). Sequence analysis of the 32 known Rab shows that there is no homology of the peptide to any other Rab. Rab1a and Rab1b are the closest isoforms, which share 92% identity (47) and differ in only 14 amino acids, 9 of which are restricted to amino acids 180–199. Therefore this antibody was expected to be specific for Rab1a. Western blotting on purified recombinant Rab proteins confirmed that it detected Rab1a but not Rab1b (Fig. 2A), indicating this antibody can discriminate between the two proteins. In total microsomes the antibody reacted with a single protein, which co-migrated with recombinant Rab1a and could be detected by p68, a well characterized polyclonal antibody that recognizes both Rab1a and Rab1b (13).

To rule out the possibility that anti-Rab1a antibody may cross-react with other Rab proteins that have the same mobility as Rab1a, total microsomes were subjected to two-dimensional electrophoresis, followed by immunoblotting with anti-Rab5 (44% of the expected pI (pH 6.0) and mobility of Rab1a (48) was detected (Fig. 2B). The same spot can be detected by p68 (data not shown). Since the likelihood that two different proteins could have the same pl and mobility is very low, we concluded that the anti-Rab1a is specific for Rab1a.

**Imunoisolation of Rab1a-associated Vesicles**—The CV2 fraction (d = 1.158) was chosen as the starting material for immunosialation because it is enriched in ER to Golgi transport vesicles. Anti-Rab1a-coated magnetic beads were incubated

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**RESULTS**

**Characterization of Rat Liver Fractions**—Subcellular fractions obtained from rat liver total microsomes by flotation in a discontinuous sucrose gradient have been characterized previously (36). To obtain more enriched ER to Golgi and transcytotic vesicle fractions, we modified the fractionation procedure and separated the crude vesicular fraction into two carrier vesicle fractions, CV1 and CV2.

Characterization of these fractions was carried out using pIgAR and dIgA as markers. dIgA is present only in transcytotic vesicles. pIgAR has three forms with different mobilities and locations (32–34) (see Fig. 1): a 105-kDa ER form (endo H-sensitive), a 116-kDa fully glycosylated Golgi form (endo H-resistant), and a 120-kDa phosphorylated form, which is a marker for the transcytotic pathway (34). To determine the forms of pIgAR present in CV1 and CV2, Tran35S-label was injected into the portal vein, and 15 or 30 min later the liver was removed, homogenized, and fractionated. Interestingly, the transcytotic vesicles of hepatic cells are much more enriched in Rab1a than are CV1 and CV2.

For quantification of the total protein bound to immunoadsorbents, starting material and non-bound subfractions were spun at 100,000 x g to remove the serum proteins added (as blocking agents) during immunosialation. The membrane pellets were resuspended in PBS, and the protein concentration was determined by BCA assay. The total protein bound to the beads was determined by subtracting non-bound from the starting material. The numbers were normalized to μg of protein/μg of beads (mean ± S.D., n = 3).

**Electron Microscopy**—Vesicles immunolabeled on magnetic beads were fixed, stained and sectioned as described previously (36, 41). In brief, beads were fixed for 45 min in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M cacodylate-HCl buffer, pH 7.2, followed by 1% OsO4 in the same buffer for 1 h. Samples were stained in block with 2% uranyl acetate, dehydrated, and embedded in Epon. Thin sections were cut, stained with 2% uranyl acetate and lead citrate, and examined in a Philips CM-10 electron microscope.

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**FIG. 1.** CV2 and CV1 are enriched in ER to Golgi transport vesicles and transcytotic vesicles, respectively. Tran35S-label or 35S-dIgA were injected into the portal vein, and 15 or 30 min later the liver was removed, homogenized, and fractionated as described under “Experimental Procedures.” pIgAR or dIgA were immunoprecipitated from this fraction and separated by SDS-PAGE, and analyzed on a PhosphorImager. ~80% of the 105-kDa form of the pIgAR is found in CV2 and residual microsomes, indicating that CV2 is enriched in ER to Golgi transport vesicles. pIgAR has three forms with different mobilities and locations (32–34) (see Fig. 1): a 105-kDa ER form (endo H-sensitive), a 116-kDa fully glycosylated Golgi form (endo H-resistant), and a 120-kDa phosphorylated form, which is a marker for the transcytotic pathway (34). To determine the forms of pIgAR present in CV1 and CV2, Tran35S-label was injected into the portal vein, and 15 or 30 min later the liver was removed and processed for fractionation. After 15 min, ~35% of the newly synthesized 105-kDa form of the pIgAR was found in the CV2 fraction and another 45% was associated with the residual microsomes, indicating that CV2 is enriched in ER to Golgi transport vesicles (Fig. 1, top panel). CV1 contains little (~8%) of the 105- or 116-kDa forms, suggesting that this fraction contains relatively few ER to Golgi and intra-Golgi transport vesicles. The 120-kDa mature form is not seen at 15 min after injection of the label (37). However, 30 min after injection ~36% of the 120-kDa form of the receptor was found in CV1 (data not shown). Similarly, ~35S-dIgA was injected into the portal vein to label transcytotic vesicles of hepatic cells, most (~54%) of the dIgA was recovered in the CV1 fraction at 30 min after injection and only 11% in CV2 (Fig. 1, middle panel). These results indicate that whereas CV2 is enriched in ER to Golgi transport vesicles, CV1 contains very few of these vesicles and is enriched in transcytotic vesicles.

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**FIG. 1.** CV2 and CV1 are enriched in ER to Golgi transport vesicles and transcytotic vesicles, respectively. Tran35S-label or 35S-dIgA were injected into the portal vein, and 15 or 30 min later the liver was removed, homogenized, and fractionated as described under “Experimental Procedures.” pIgAR or dIgA were immunoprecipitated from this fraction and separated by SDS-PAGE, and analyzed on a PhosphorImager. ~80% of the 105-kDa form of the pIgAR is found in CV2 and residual microsomes (RM), and >90% of the 116-kDa form is in Golgi light (GL) and Golgi heavy (GH) (top panel). CV1 contains little (~8% and 4%, respectively) of either form. Most of the dIgA is found in CV1 (54%), and less in Golgi heavy (18%) and CV2 (11%) (middle panel). Rab1a determined by immunoblotting has a broad distribution across the gradient (bottom panel).
microsomes from rat liver (both Rab1a and Rab1b. Both antibodies detect a single band in total but not Rab1b, whereas the polyclonal antibody p68 (13) recognizes Rab1a antibody and detection by ECL. Anti-Rab1a recognizes Rab1a were separated by SDS-PAGE, followed by immunoblotting with anti-Rab1adetected with ECL. Anti-Rab1a recognizes a single spot with the expected pI and mobility of Rab1a in two-dimensional gels (55).

As expected, vesicles immunoisolated from CV1 with beads coated with anti-pIgAR contained the transcytotic content marker dIgA and the mature (120-kDa) form of pIgAR (Fig. 4, lane 3). However, to our surprise, a considerable amount of the mature, 120-kDa form was also present in the same subfraction. Since the mature form of pIgAR is associated only with transcytotic carriers (33), this finding suggested that the population of vesicles immunoisolated from the CV2 fraction with anti-Rab1a includes not only vesicles involved in ER to Golgi transport, but also those involved in transcytosis.

Rab1a Is Associated with Transcytotic Vesicular Carriers—To further investigate this possibility, we immunoisolated transcytotic vesicles from CV1 (d = 1.146), the fraction enriched in transcytotic vesicular carriers, using an antibody against the cytoplasmic domain of Rab1a (37) and assayed for the presence of Rab1a. The 120-kDa form of pIgAR and 35S-dIgA were used as markers for the transcytotic pathway (37).

Neither Rab1a nor pIgAR are associated with the transcytotic markers. The CV1 fraction, which is enriched in transcytotic vesicles, was incubated with magnetic beads coated with either anti-Rab1a (lanes 1–3) or anti-pIgAR (lanes 4–6) as described under “Experimental Procedures.” The starting material (SM), non-bound (NB), and bound (B) fractions were processed for immunoblotting with anti-pIgAR and anti-Rab1a as in Fig. 3 or for autoradiography for another transcytotic marker, 35S-dIgA. Rab1a is found in the bound subfractions after immunolocalization on either anti-Rab1a (lane 3) or anti-pIgAR (lane 6). The pIgAR found in the bound subfractions is primarily the 120-kDa form, with a small amount of the 116-kDa form. The 105-kDa form is barely detectable in the bound subfraction. dIgA is also found in the bound subfractions (lanes 3 and 6).

We next immunoisolated vesicles from the CV1 fraction on anti-Rab1a and compared their composition with those isolated from the same fraction on anti-pIgAR. The results were similar; dIgA was found only in the bound fraction (Fig. 4, lane 6). To verify that this band is indeed Rab1a, high resolution two-dimensional PAGE followed by immunoblotting was performed. A single spot at the expected pI and mobility of Rab1a was detected in the bound fraction (data not shown). These results provide further evidence that Rab1a is associated with transcytotic vesicles.
<1% of the marker, whereas the corresponding bound fraction from CV2 accounts for ~36%.

For quantitation of the Rab1a associated with the isolated vesicles, recombinant Rab1a was titrated to make a standard curve, and the amount of Rab1a present on the vesicles was determined using 125I-protein A. The amount of Rab1a associated with vesicles immunoisolated via the two antibodies from CV1 was comparable; under conditions in which >95% of Rab1a was recovered in the bound fraction (e.g., Fig. 4), 1.249 ± 0.158 and 1.253 ± 0.350 ng of Rab1a/μg of starting material were associated with vesicles isolated on anti-Rab1a and anti-pIgAR, respectively.

To validate that Rab1a is truly a component of transcytotic vesicles, we compared the density of Rab1a present on these vesicles isolated from CV1, which is enriched in transcytotic vesicles, with those isolated from CV2, which is enriched in ER-to-Golgi transport vesicles. The amount of total protein and Rab1a bound to the immunoadsorbents after incubation with CV1 or CV2 was determined using 125I-protein A. The amount of Rab1a associated with vesicles immunoisolated via the two antibodies from CV1 was comparable; under conditions in which >95% of Rab1a was recovered in the bound fraction (e.g., Fig. 4), 1.249 ± 0.158 and 1.253 ± 0.350 ng of Rab1a/μg of starting material were associated with vesicles isolated on anti-Rab1a and anti-pIgAR, respectively.

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with a diameter of 60–100 nm. There is no major difference in the size and morphology of vesicles immunoprecipitated on Rab1a from the two fractions. Control beads coated with normal IgG lacked any associated vesicular elements (Fig. 6).

Multiple Small GTP-binding Proteins Are Found in Vesicles Immunoprecipitated via Anti-Rab1a or Anti-pIgAR—The experiments described above demonstrated that a reasonably homogeneous subpopulation of transcytotic vesicles can be immunoprecipitated from the CV1 fraction using anti-Rab1a. We next set out to determine how many small GTP-binding proteins are associated with this class of vesicles. After immunoprecipitation, samples were analyzed by $[^{32}P]GTP$ overlay. We found at least three major bands that were selectively associated with the bound subfraction, whereas most of the more slowly migrating bands and small amounts of the other bands remained in the non-bound subfraction (data not shown). We also found multiple small GTP-binding proteins associated with the bound subfraction immunoprecipitated on anti-pIgAR, with the pattern being quite similar to that obtained after immunoprecipitation on anti-Rab1a. These data indicate that multiple specific small GTP-binding proteins are associated with vesicles immunoprecipitated via either anti-Rab1a or anti-pIgAR.

Identification of the Small GTP-binding Proteins Associated with Transcytotic Vesicles—To determine which Rabs associate with immunoprecipitated transcytotic vesicles, we first examined two epithelia-specific Rabs: Rab17 and Rab18 (26, 29). Most of the Rab17 was present in the bound fractions of vesicles immunoprecipitated on anti-Rab1a (Fig. 7, lane 3) or anti-pIgAR (Fig. 7, lane 6). In contrast, Rab18 was detected only in the non-bound subfractions (lanes 2 and 5). These data suggest that epithelia-specific Rab17 is associated with transcytotic vesicles, whereas Rab18 is associated with some other type(s) of vesicular carrier, which remains to be identified.

In addition to the epithelia-specific Rabs, we investigated the presence of ubiquitously expressed Rabs (Rabs1b, -2, and -6) and ARF in vesicles immunoprecipitated on anti-Rab1a. Although Rab1b has the same distribution as Rab1a in NRK cells (15, 42), we found that Rab1b has a broader distribution than Rab1a in rat liver. Rab1b was found in comparable amounts in both the non-bound (lanes 2 and 5) and bound (lanes 3 and 6) subfractions by immunoblotting, whereas Rab1a was detected only in the bound subfractions (lanes 3 and 6). Rab2 and Rab6 were also distributed between the nonbound and bound fractions (lanes 3 and 6), suggesting that these Rabs are also associated with elements of the transcytotic pathway. In contrast, ARF was found only in the non-bound subfractions (lanes 2 and 5). The smaller amounts of Rab2 and Rab6 remaining in the non-bound subfractions (lanes 2 and 5) are presumably associated with other populations of vesicular carriers.

To rule out the possibility that the presence of multiple Rabs in the bound subfractions was due to nonspecific binding of trace quantities of Rabs to the matrix of the immunoadsorbents, we carried out additional immunoprecipitation experiments with Rab6 and quantitated the amount of Rab6 associated with the bound vesicles. Rab6 was used as a representative Rab because antibody suitable for immunoprecipitation was available. After immunoprecipitation on anti-Rab1a or anti-pIgAR, the isolated vesicles were lysed in Triton X-100 lysis buffer, and immunoprecipitation with anti-Rab6 was performed on the solubilized proteins. A single ~24-kDa band was precipitated (Fig. 8, lanes 5 and 10). The same band was depleted from the supernatants (Fig. 8, lanes 4 and 9). When the amount of Rab6 associated with isolated transcytotic vesicles

FIG. 7. Vesicles immunoprecipitated on anti-Rab1a or anti-pIgAR share the same Rab profile. Immunoprecipitation was carried out on either anti-Rab1a (lanes 1–3) or anti-pIgAR (lanes 4–6) as described in Fig. 4, and the presence of ARF and various Rab proteins (indicated on the right) was determined by immunoblotting. Rab17 and most of the Rab2 and Rab6 are associated with vesicles immunoprecipitated on anti-Rab1a (lane 3) or anti-pIgAR (lane 6). Rab18 is evenly distributed between the bound (B, lanes 3 and 6) and non-bound (NB, lanes 2 and 5) subfractions, whereas Rab18 and ARF are found primarily in the non-bound subfractions. SM, starting material.

FIG. 6. EM morphology of vesicles immunoprecipitated on anti-Rab1a and anti-pIgAR. Vesicles immunoprecipitated on magnetic beads were fixed, stained in block with uranyl acetate, and processed for routine electron microscopy as described under “Experimental Procedures.” A, low magnification view showing that the beads coated with anti-Rab1a isolated from CV2 are uniformly covered with small (60–100 nm) vesicles, which are relatively homogeneous in size and morphology. B, control sample in which nonspecific IgG was coupled to the beads shows minimal binding of vesicular structures to the beads. Vesicles isolated from either the CV1 fraction (D) or the CV2 fraction (E) on anti-Rab1a or from the CV1 fraction on anti-pIgAR (E) are similar in size and morphology. Note that tubular structures (arrows) are occasionally seen (E). Bar, 0.5 μm (A, E) or 0.1 μm (B–D).
Rab1a in the Transcytotic Pathway

Fig. 8. Immunoprecipitation of Rab6 from vesicles isolated on anti-Rab1a or anti-plgAR. Immunoisolation was carried out on anti-Rab1a (lanes 1-5) or anti-plgAR (lanes 6-10) as described in Fig. 4. After immunosolation, the bound membranes were solubilized in Triton X-100 lysis buffer, divided into equal portions, followed by immunoprecipitation (IP) with either anti-Rab6 (lanes 4, 5, 9, and 10) or anti-Rab1a (lanes 2, 3, 7, and 8) as control. An aliquot of the bound membranes (B, lanes 1 and 6), immunoprecipitates (P, lanes 3, 5, 8, and 10) and supernatants (S, lanes 2, 4, 7, and 9) were separated by SDS-PAGE and detected by [α-32P]GTP overlay. IA, immunoadsorbent; SF, subtraction.

was quantitated by immunoblotting using 125I-protein A for detection, ~1.292 ± 0.44 and 1.111 ± 0.06 ng of Rab6/μg of starting material was detected in vesicles isolated on anti-Rab1a and anti-plgAR, respectively. These results indicate that Rab6 is associated with immunosolated transcytotic vesicles at a level comparable to that of Rab1a (see "Rab1a Is Associated with Transcytotic Vesicular Carriers").

Based on these experiments, we conclude that 1) multiple Rab proteins, including specialized and ubiquitous Rabs, apparently associate with transcytotic carrier vesicles; and 2) Rab1a and Rab6 are present in transcytotic vesicles with comparable stoichiometry.

DISCUSSION

In the present work we have used subcellular fractionation of rat liver to obtain fractions enriched in either ER to Golgi transport vesicles (CV2) or transcytotic vesicles (CV1) and, in the case of CV1, de-enriched in other cell components. A specific subset of transport vesicles was further purified from these fractions by immunoisolation on anti-Rab1a antibody. In characterizing the immunosolated vesicles, we found that: 1) Rab1a is not only associated with ER to Golgi transport vesicles, but also is present on transcytotic vesicles; 2) multiple Rabs, including epithelia-specific Rab17 and ubiquitous Rab1b, -2, and -6, are found on the membranes of transcytotic vesicles; and 3) ARF is not found in association with transcytotic vesicles.

Rab1a Is Associated with Transcytotic Vesicles as Well as ER to Golgi Transport Vesicles—The initial goal of this study was to isolate ER to Golgi transport vesicles using Rab1a as the target. For this purpose we utilized an antibody that is Rab1a-specific and does not cross-react with any other Rabs, including the closely-related protein, Rab1b. The specificity of anti-Rab1a for Rab1a was established experimentally by immunoblotting on recombinant Rab1a and -1b proteins as well as on total microsomes after separating the proteins by high resolution two-dimensional PAGE.

To obtain a highly purified preparation of ER-to-Golgi transport vesicles, we prepared a fraction, CV2, from rat liver enriched in ER to Golgi carrier vesicles and subjected this fraction to immunoisolation on anti-Rab1a-coated magnetic beads. Using plgAR as a marker, to our surprise, we detected in the bound fraction not only the 105-kDa/ER form of plgAR, but also the 120-kDa form of plgAR, which is found primarily in transcytotic vesicles. This suggested that, in addition to being associated with ER to Golgi transport vesicles, Rab1a is also present on vesicular elements associated with the transcytotic pathway in rat liver.

To further investigate this possibility, we carried out immunoisolation on a fraction, CV1, known to be enriched in transcytotic vesicles. Once again we found that vesicles immunosolated on anti-Rab1a contained the two best transcytotic markers: dIgA and its receptor, the 120-kDa form of plgAR. Moreover, Rab1a was detected by immunoblotting in transcytotic vesicles immunosolated on anti-plgAR-coated magnetic beads, and its presence was confirmed by high resolution two-dimensional PAGE. This method has been used to analyze the complexity of the large superfamily of small GTP-binding proteins. At least 28 small GTP-binding proteins (20–25 kDa), including Rab1a, have been mapped by their relative pI and mobility (55). Our experiments suggest that the density of Rab1a in transcytotic vesicles is comparable to that on ER to Golgi vesicles, indicating that Rab1a is truly associated with transcytotic vesicles.

The association of Rab1a with transcytotic vesicles was unexpected, because Rab1a has been well characterized in cultured NRK cells, where it was localized to pre-Golgi and Golgi elements by immunogold labeling and shown to be required for ER to Golgi and intraGolgi transport (14, 35, 42). The finding that Rab1a is associated with transcytotic vesicles in hepatocytes, which are highly polarized epithelial cells, suggests that the distribution of Rab proteins may vary in different cell types.

Vesicles Isolated on Anti-Rab1a Are Post-endosomal Transcytotic Vesicles—Sztul et al. (37) have previously immunosolated transcytotic vesicles from a carrier vesicle-enriched fraction using an antibody against the cytoplasmic domain of plgAR. We have modified the fractionation procedure to obtain a more enriched fraction of transcytotic vesicles, CV1, which contains ~54% of the transcytotic marker (dIgA) but ~8% of the ER and Golgi markers (105-kDa and 116-kDa plgAR, respectively). Immunoisolation carried out with either anti-Rab1a- or anti-plgAR-coated magnetic beads yielded a reasonably homogeneous population of transcytotic vesicles based on the presence of transcytotic markers (dIgA and the 120-kDa plgAR) and the absence of ER (calreticulin, 105-kDa plgAR) and Golgi (sialyltransferase) markers. Further analysis of the two populations of immunosolated vesicles suggests that those isolated on anti-plgAR are involved in both pre-endosomal and post-endosomal steps in transcytosis, whereas those isolated on Rab1a consist mainly of post-endosomal vesicles involved in transport from endosomes to the apical (bile canalicular) PM.

Transcytosis starts at the basolateral (sinusoidal) PM, where the plgAR and its ligand are internalized into cells via coated vesicles and transported to early endosomes where they are sorted from other membrane and content proteins. Transcytotic vesicles then bud off from endosomes and transport plgAR with bound plgA to the apical (bile canalicular) domain of the PM (50, 52, 53). Although the early stages of transcytosis from internalization at the basolateral PM to sorting at the early endosome have been well characterized in hepatocytes, the late steps of the pathway in the apical region are less clear. In Madin-Darby canine kidney cells, it has been shown that dIgA is transcytosed and delivered to an apical endosome before reaching the apical PM (56, 57). In hepatocytes it has been shown that plgAR is transported to a subapical compartment, where it colocalizes with several apical membrane proteins (58). By immunodepletion of endosomes (using ASGPR as the target) followed by immunadsorption for transcytotic vesicles (using plgAR), Barr et al. (54) have recently isolated a population of vesicles containing transcytosed dipeptidyl peptidase IV and plgAR. Kinetic data and computer-generated models suggest that the isolated fraction constitutes the subapical compartment. We have also detected dipeptidyl peptidase IV in...
vesicles isolated on both anti-Rab1a and anti-pGAR. We do not know whether our immunosolated vesicles are postendosomal transport vesicles or represent vesicles derived from the subapical compartment or a mixture of the two.

Contamination or Multiplicity of Rabs per Relay?—In principle the presence of a Rab protein (Rab1a) in different vesicular carriers could reflect intercontamination. This can be ruled out in the case of Rab1a, since its ratio to exocytic and transcytotic markers is clearly different. To test the possibility that there are more than one Rab per relay of transport carriers, we examined the association of small GTP-binding proteins with the isolated vesicles by [32P]GTP overlay and by immunoblotting with anti-Rab1a and antibodies to other small GTP-binding proteins. We found that multiple small GTP-binding proteins were present on transcytotic vesicles, including Rab1b, -2, -6, and -17 as well as Rab1a. Several pieces of evidence indicate that the presence of multiple Rabs on the transcytotic vesicles was not due to nonspecific binding; (i) none of these Rabs were detected on magnetic beads coated with nonspecific IgG (see Fig. 3), (ii) Rab6, a representative of these Rab proteins, can be immunoprecipitated from isolated vesicles (see Fig. 8), and (iii) the amount of Rab6 associated with the transcytotic vesicles is comparable with that of Rab1a.

Little is known about the molecular mechanisms by which Rabs regulate membrane traffic. Over 30 Rabs have been identified in mammalian cells. As there are less than 30 relays of transport carriers, multiple Rab proteins may be associated with the same carrier vesicles and function together in vesicular transport.

Supporting this idea, multiple Rabs have also been found in other transport pathways. For example, at least seven small GTP-binding proteins were found to be associated with synogen granule membranes (59–61), and Rab3b, -18, -20, and -22, as well as Rab4 and Rab5 are associated with early endosomes (11, 16, 29, 62). The function of most of these Rabs is unknown.

Rab Proteins Associated with More Than One Population of Vesicular Carriers—We have identified at least four Rabs (Rab1b, -2, -6, and -17) on vesicles isolated from CV1 on anti-Rab1a. Rab17 is expressed only in epithelial cells in the kidney, liver, and intestine and has been localized to the basolateral PM and apical endosome region by electron microscopy (26). Our finding that Rab17 associates with transcytotic vesicles suggests that it may be involved in transcytosis. In contrast, Rab18, another Rab protein highly expressed in epithelia, was found only in the non-bound subfractions.

Additional Rabs found on the isolated transcytotic vesicles, i.e. Rab1b, -2, and -6, are ubiquitously expressed in all cell types and have been localized to pre-Golgi and Golgi membranes, intermediate compartment, and medial and trans Golgi and trans Golgi network, respectively (14, 24). Our data suggest that similar to Rab1a, Rab1b, -2, and -6 are associated with more than one population of transport vesicles operating in both the exocytic and transcytotic pathways.

It is not clear why these ubiquitous Rab proteins are associated with the transcytotic pathway. It is possible that each transport step requires one “major” Rab (specific Rab) and a set of “helper” Rabs (common Rabs). Each of these ubiquitous Rabs may serve as a “major” Rab in a particular step in the exocytic pathway and a “helper” Rab in the transcytotic pathway, and interactions among multiple Rabs may control the specificity and directionality of vesicular transport. Another possible explanation is that a given Rab controls (as a switch or kinetic proof reader) the entry of a factor into the fusion/targeting complex. Since these complexes have common components in addition to specific components, the same Rab (e.g. Rab1a) could be found at multiple relays.

In support of this hypothesis, some Rab proteins have been found in two transport pathways. For example, in addition to its function in the endocytic pathway, Rab4 is associated with glucose transporter (Glut4)-containing vesicles in adipocytes, and its distribution is modified by insulin. Upon stimulation with insulin, Rab4 is released from the membrane of Glut4-containing vesicles into the cytosol and Glut4 is transported to the cell surface, suggesting that Rab4 may be involved in insulin stimulated translocation of Glut4-containing vesicles (63).

Rab5 has been found to be associated with both apical and basolateral endosomes in polarized Madin-Darby canine kidney cells (64), to be involved in endocytosis at both the apical and basolateral PM, and to be required for homotypic fusion of apical or basolateral endosomes in vitro (64, 65). Yet fusion between the two sets of endosomes or mixing of the respective contents does not occur (66–68). This indicates that Rab5 alone would not be sufficient to target vesicles to either apical or basolateral compartments. Targeting may be controlled through interaction between Rab5 and other regulatory proteins, which could be other Rabs located in the same vesicular carriers.

Our data have implicated a network of Rab proteins in the transcytotic pathway in rat liver. Important questions to be addressed in future studies are what role they play in the transcytotic pathway and how they function together. It may be possible to address these questions experimentally by using mutant Rab proteins and determining their effect on transcytotic transport in polarized cultured epithelial cells.

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