Basolateral Internalization of GPI-anchored Proteins Occurs via a Clathrin-independent Flotillin-dependent Pathway in Polarized Hepatic Cells

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In polarized hepatocytes, the predominant route for apical resident proteins to reach the apical bile canalicular membrane is transcytosis. Apical proteins are first sorted to the basolateral membrane from which they are internalized and transported to the opposite surface. We have noted previously that transmembrane proteins and GPI-anchored proteins reach the apical bile canaliculi at very different rates. Here, we investigated whether these differences may be explained by the use of distinct endocytic mechanisms. We show that endocytosis of both classes of proteins at the basolateral membrane of polarized hepatic cells is dynamin dependent. However, internalization of transmembrane proteins is clathrin mediated, whereas endocytosis of GPI-anchored proteins does not require clathrin. Further analysis of basolateral endocytosis of GPI-anchored proteins showed that caveolin, as well as the small GTPase cdc42 were dispensable. Alternatively, internalized GPI-anchored proteins colocalized with flotillin-2-positive vesicles, and down-expression of flotillin-2 inhibited endocytosis of GPI-anchored proteins. These results show that basolateral endocytosis of GPI-anchored proteins in hepatic cells occurs via a clathrin-independent flotillin-dependent pathway. The use of distinct endocytic pathways may explain, at least in part, the different rates of transcytosis between transmembrane and GPI-anchored proteins.

INTRODUCTION

The plasma membrane of epithelial cells is divided into two domains, apical and basolateral, separated by tight junctions. Each domain has a specific protein and lipid composition, which is correlated with specialized functions. Sorting mechanisms must regulate trafficking of molecules to the appropriate membrane domain. These have been shown to operate along both the biosynthetic and endocytic pathways (reviewed in Rodriguez-Boulan *et al.*, 2005). Newly synthesized proteins are transported to polarized surfaces either by a direct or an indirect route. In the direct pathway, membrane proteins are primarily sorted in the *trans*-Golgi Network (TGN) and targeted directly to the apical or basolateral domain (Rodriguez-Boulan and Powell, 1992). In the indirect pathway, however, TGN-derived carriers are first sent

to the basolateral surface, from where they are selectively internalized and subsequently transcytosed to the opposite apical surface (reviewed in Mostov *et al.*, 2000, Rodriguez-Boulan *et al.*, 2005). In hepatocytes, unlike most simple polarized epithelial cells, the predominant route to the apical bile canalicular (BC) membrane is indirect (reviewed in Aït-Slimane and Hoekstra 2002; Tuma and Hubbard, 2003).

We have shown previously that, in polarized hepatic cells, both GPI-anchored proteins (APs) and single transmembrane (TMD) proteins use the transcytotic pathway. However, transcytosis of GPI-APs requires their integration into detergent-resistant microdomains called rafts, whereas TMD proteins exploit a raft-independent transcytotic pathway (Aït-Slimane *et al.*, 2003). In this study, we noted that transcytotic transport of TMD proteins was considerably more efficient than that of GPI-APs. A similar observation has been made in vivo regarding transcytosis of the GPI-AP 5' nucleotidase (Schell *et al.*, 1992). Whether these variations reflect differences in transport kinetics or use of separate endocytic/transcytotic pathways has not been investigated. It has been reported that newly synthesized apical resident TMD proteins and the transcytosing polymeric immunoglobulin receptor (pIgA-R) follow the same transcytotic pathway (Hemery *et al.*, 1996, Ihrke *et al.*, 1998), but it is not clear whether GPI-APs enter this pathway. Moreover, except for the pIgA-R, which is internalized in clathrin-coated pits, the mechanisms that mediate basolateral internalization of apical resident proteins in hepatocytes are not known. The pIgA-R is endocytosed with tyrosine-based signals, which

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Abbreviations used: AP, anchored protein; APN, aminopeptidase N; BC, bile canalicular; BL, basolateral; CTX, cholera toxin; DPPIV, dipeptidylpeptidase IV; mAb, monoclonal antibody; pAb, polyclonal antibody; Tf, transferrin; TMD, transmembrane domain.

seem to be conventional clathrin-mediated internalization signals (Okamoto *et al.*, 1992). An important difference between the pIgA-R and BC resident proteins is that most of the latter are GPI-APs or TMD proteins with short cytoplasmic tails and no obvious internalization motif. It is generally assumed that in hepatocytes, TMD proteins are internalized via a clathrin-dependent pathway, like the pIgA-R. However, there are no functional studies demonstrating a role for clathrin in basolateral endocytosis of apical resident membrane proteins.

The endocytic pathway of GPI-APs has been extensively investigated in various nonpolarized cell types, but there is still no consensus concerning the mechanisms involved. Several studies performed in Cos-7 and Chinese hamster ovary (CHO) cells have suggested that internalization of GPI-APs is dynamin- and clathrin-independent (reviewed in Chatterjee and Mayor, 2001; Mayor and Riezman, 2004; Perret *et al.*, 2005). Other studies in Cos-7 and MA104 cells found GPI-APs in caveolin (Cav)-positive endocytic structures termed caveolae (Anderson *et al.*, 1992; Nichols, 2002). However, these observations have been challenged in both cell types (Parton *et al.*, 1994; Fujimoto, 1996; Fivaz *et al.*, 2002; Sabharanjak *et al.*, 2002). The group of Mayor proposed that GPI-APs are internalized in specialized compartments called GPI-anchored protein-enriched early-endosomal compartments via cdc42- and ARF1-regulated pathway (Sabharanjak *et al.*, 2002; Kumari and Mayor, 2008). Finally a new pathway involving the scaffolding proteins flotillin-1 and flotillin-2 has been described for the endocytosis of GPI-APs in Cos-7 and CHO cells (Glebov *et al.*, 2006; Frick *et al.*, 2007). Thus far, the mechanisms that mediate the basolateral internalization of apical resident proteins in polarized hepatic cells have not been investigated.

We have investigated the mechanisms involved in the endocytic/transcytotic transport of apical resident TMD proteins and GPI-APs in polarized hepatic cells. We have compared the basolateral internalization of two GPI-APs, CD59 endogenously expressed, and GPI-green fluorescent protein (GFP) stably transfected in HepG2 cells, with that of two endogenous TMD proteins, dipeptidyl-peptidase IV (DPPIV) and aminopeptidase N (APN). Our data show that in polarized hepatic cells, GPI-APs and TMD proteins exploit distinct mechanisms for their endocytic/transcytotic transport. We demonstrate that internalization of TMD proteins is dynamin and clathrin dependent, whereas internalization of GPI-APs is dynamin dependent but clathrin independent. Furthermore, we found that flotillin-2 RNA interference (RNAi) inhibited basolateral endocytosis of GPI-APs without affecting internalization of TMD proteins. We propose that flotillin-2 is one determinant of the clathrin-independent endocytic pathway of GPI-APs in polarized hepatic cells.

MATERIALS AND METHODS

Reagents and Antibodies

Culture media, Alexa Fluor 488 (AF488)-labeled secondary antibodies, and Alexa Fluor 594-labeled transferrin (AF594-Tf), cholera toxin B subunit (AF594-CTX), and Topro-3 were purchased from Invitrogen (Cergy-Pontoise, France). Fluorescent secondary antibodies were from Jackson ImmunoResearch Laboratories (Montluçon, France). Peroxidase-conjugated secondary antibodies were from Rockland Immunochemicals (Gilbertville, PA). The enhanced chemiluminescence (ECL) detection kit was from GE Healthcare France (Orsay, France). The small-interfering RNA (siRNA)-clathrin heavy chain duplex was purchased from Eurogentec (Liège, Belgium). The siRNA-flotillin-2 duplex was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and control siRNA was obtained from Dharmacon RNA Technologies (Lafayette, CO). All other reagents were obtained from Sigma (St. Quentin-Fallavier, France) unless otherwise noted. The polyclonal antibodies (pAbs)

anti-actin and anti-clathrin heavy chain were from Santa Cruz Biotechnology. The pAbs anti-flotillin-2 and anti-caveolin-1 were from BD Biosciences (Le-Pont-de-Claix, France). The monoclonal antibody (mAb) anti-CD59 was from Abcam (Cambridge, United Kingdom). The mAb anti-GFP was from Roche Diagnostics (Meylan, France). The pAb against GFP was raised in the laboratory by Jean-Louis Delaunay (UMRS 938, CDR Saint-Antoine, Paris, France). The pAb against APN was provided by Prof. Ann Hubbard (John Hopkins University, Baltimore, MD). The mAb directed against human DPP-IV (HBB3/775) was provided by Prof. Hans-Peter Hauri (Biozentrum der Universität Basel, Basel, Switzerland).

Cell Culture

HepG2 cells were grown at 37°C in DMEM supplemented with 10% heat-inactivated (56°C; 30 min) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, under a 5% CO₂ air atmosphere. For microscopy and biochemistry experiments, HepG2 cells were grown onto glass coverslips and 60-mm dishes, respectively.

DNA Constructs, Transfection, and Clonal Selection

The GPI-GFP construct produced by fusion of the folate receptor GPI-anchoring sequence with GFP was a kind gift from Dr. S. Mayor (National Centre for Biological Sciences, Bangalore, India). Flot2-GFP was a kind gift from Dr. R. Tikkanen (University Clinic, Frankfurt-am-Main, Germany). The Cav-1-GFP was from Dr. A. Le Bivic (Institut de Biologie du Développement de Marseille, Faculté des Sciences de Luminy, Marseille, France). GFP constructs of cdc42 N17, rac N17, and rhoA N19 were obtained from Dr. S. Etienne-Manneville (Institut Pasteur, Paris, France). GFP construct of Eps15 DIIIΔ2 (control) and Eps15 EΔ95/295 (dominant-negative Eps15) were from Dr. A. Benmerah (Institut Cochin, Paris, France). GFP construct of dynamin 2 (control) and dynamin 2K44 (dominant-negative Dyn) were from Dr. M. McNiven (Mayo Clinic, Rochester, MN). HepG2 cells were transfected using nucleofector II (Amaxa, Cologne, Germany) using program 22 (solution V) according to the manufacturer's instructions. After 48 h, selection was started by addition of 1 mg/ml Geneticin (G418, Invitrogen, Cergy-Pontoise, France). Stable clones were isolated after 3 wk by using cloning cylinders. Positive cells were screened by GFP fluorescence.

Indirect Immunofluorescence and Confocal Microscopy

Cells were washed with phosphate-buffered saline with 0.5 mM CaCl₂ and 1 mM MgCl₂ (PBS⁺), fixed with 4% paraformaldehyde for 1 min at 4°C, and permeabilized in methanol for 10 min at 4°C. After blocking in 1% PBS/bovine serum albumin (BSA), cells were incubated for 1 h at room temperature with primary antibodies. After three washes in PBS, cells were incubated for 1 h with fluorescently labeled secondary antibodies. After three washes in PBS, cells were incubated for 10 min with RNase A and then for 1 min with Topro-3 to stain nuclei. Confocal imaging was acquired with a TCS SP2 laser-scanning spectral system (Leica, Wetzlar, Germany) attached to a Leica DMR inverted microscope. Optical sections were recorded with a 63/1.4 immersion objective. Laser scanning confocal images were collected, and analyzed using the online Scan Ware software. Figure compilation was accomplished using Adobe Photoshop 5.5 and Adobe Illustrator 10 (Adobe Systems, Mountain View, CA).

Internalization Assays

Internalization assays were performed in two different ways according to a previously published protocol (Ait-Slimane *et al.*, 2003). In the first method, HepG2 cells were washed three times with HEPES-buffered (20 mM, pH 7.0) serum-free medium (HSFM). Cell surface antigens were labeled at 4°C for 30 min with specific primary antibodies diluted in HSFM/0.2% BSA. After surface labeling, cells were extensively washed with HSFM/0.2% BSA, placed in prewarmed complete medium, and incubated at 37°C for the indicated times. In the second method, HepG2 cells were continuously labeled with anti-CD59, DPPIV, or APN antibodies at 37°C for the indicated times. Non-internalized antibody-antigen complexes were removed by acid washing (200 mM glycine and 150 mM NaCl, pH 2.5) before fixation, permeabilization, and staining with fluorescently labeled secondary antibodies. For CTX and Tf labeling, HepG2 cells were incubated at 37°C for the indicated times with 10 µg/ml AF594-CTX or 30 µg/ml AF594-Tf. Excess CTX or Tf at the cell surface was removed by acid stripping. Fluorescence was examined by confocal scanning microscopy.

Analysis of Colocalization and Quantification of Total Fluorescence

To quantify the level of colocalization, 20–30 cells per experimental condition were randomly selected on the same coverslip among those that showed a well resolved pattern for the anti-CD59- or anti-DPPIV-labeled structures. The laser power and the levels for detector amplification were optimized for each channel before starting acquisition of the images. The ImageJ (National Institutes of Health, Bethesda, MD) 1.41 measure colocalization function was used to calculate the percentage of colocalization between the different

Figure 1. GPI-APs and TMD proteins are internalized and transcytosed with different kinetics in HepG2 cells. (A) Endogenous DPPIV, APN, CD59, and stably expressed GPI-GFP present at the BL membrane were continuously labeled with specific antibodies for 20 min at 37°C. The cells were fixed, permeabilized, and the trafficked antibody–antigen complexes visualized with fluorescently labeled secondary antibodies. (B) Antibodies to DPPIV and CD59 were bound simultaneously to the BL membrane of HepG2 cells at 0°C. After unbound antibody was removed, the cells were incubated at 37°C for 20 min and then fixed and permeabilized. Primary antibodies were visualized by AF488-conjugated (DPPIV) or Cy3-conjugated (CD59) secondary antibody. Right, merged image of DPPIV (left; green) and CD59 (middle; red). BCs are marked by asterisks. Bars, 10 μ m.

probes. To quantify the amount of internalized cargo, 20–30 control and siRNA or dominant-negative mutant expressing cells per coverslip were randomly selected and imaged using the confocal microscope. All the images were taken with identical acquisition parameters. The amount of internalized cargo in treated cells was measured using ImageJ 1.41 and expressed as a percentage of that internalized in control cells.

Western Blot

Transfected cells were washed with PBS⁺ and lysed on ice for 30 min in TNE buffer (20 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, pH 7.4) containing 1% (wt/vol) Triton X-100 in the presence of a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Protein content was determined using the detergent-compatible bicinchoninic acid reagent (Interchim, Montluçon, France), with BSA as the standard, according to the manufacturer's instructions. Equal amounts of protein were directly processed for SDS-polyacrylamide gel electrophoresis on 7.5% polyacrylamide gels. Immunoblotting was performed using the appropriate primary antibodies followed by horseradish peroxidase-conjugated mouse-specific secondary antibody. Development of peroxidase activity was performed with the ECL detection kit (GE Healthcare France).

RESULTS

GPI-anchored Proteins and Single Transmembrane Domain Proteins Are Transcytosed with Different Efficiencies

We compared the kinetic of transcytosis of two GPI-APs, CD59 and GPI-GFP, to that of two TMD proteins, DPPIV and APN. CD59, DPPIV, and APN are endogenously expressed in HepG2 cells. The fusion protein GPI-GFP was stably expressed after transfection. Transcytosis was monitored by continuously labeling the basolateral pool of selected apical proteins for 20 min at 37°C. The cells were fixed, permeabilized, and the trafficked antibody–antigen complexes visualized with secondary antibodies. Figure 1A shows that, after 20 min, the GPI-APs were concentrated in small punctate structures particularly visible in the area

directly underneath the basolateral membrane, whereas no apical staining was detected. By contrast, the TMD proteins were present mainly at the apical plasma membrane, and in the subapical area. To directly compare the kinetics of internalization of GPI-APs and TMD proteins, we followed the trafficking of CD59 and DPPIV simultaneously. HepG2 cells were incubated for 15 min at 0°C with antibodies to CD59 and DPPIV to allow their binding to the basolateral surface. Trafficking was initiated by raising the temperature to 37°C. After 20 min, the cells were processed as in Figure 1A. As shown in Figure 1B, both CD59 and DPPIV became internalized as reflected by the increase of the intracellular punctate staining. However, staining for CD59 remained largely at the periphery of the cells, whereas staining for DPPIV was observed deeper in the cells and at the level of the membrane (see the merged picture). These results show that GPI-APs and TMD proteins have different kinetics of internalization at the basolateral membrane of polarized hepatic cells.

GPI-anchored Proteins and Single Transmembrane Domain Proteins Are Internalized in Distinct Vesicles

In previous studies, we and others have shown that, in polarized hepatic cells, the transcytotic transport of GPI-APs occurs through a raft-mediated pathway, whereas TMD proteins do not exploit such transport mechanism (Aït-Slimane *et al.*, 2003; Nyasae *et al.*, 2003). This observation suggests that GPI-APs and TMD proteins may use different internalization mechanisms at the basolateral membrane. To test this hypothesis, we performed cotrafficking experiments using antibodies to transcytotic proteins, and we searched for colocalization with markers of known endocytic pathways. As shown in Figure 2A, nearly perfect colocalization was

Figure 2. GPI-APs and TMD proteins are internalized in distinct vesicles. HepG2 cells were allowed to internalize AF594-labeled Tf (A and B) or AF594-labeled CTX (C and D) with antibodies to DPPIV or CD59 for 5 min at 37°C. At the end of the 37°C incubation, CTX, Tf, and antibody–antigen complexes remaining at the basolateral surface were removed by acid washing as described in *Materials and Methods*. Cells were fixed, permeabilized, and internalized antibodies were labeled with AF488-conjugated secondary antibodies. (A and C) Merged confocal pictures of the indicated internalized proteins and fluorescent probes. (B and D) Quantification of Tf or CTX colocalization with the indicated endocytosed apical resident proteins shown in A and C. The percentage of internalized cargo was calculated as described in *Materials and Methods* by using multiple confocal sections of at least 20 cells in three independent experiments. Bars, 10 μ m.

observed between DPPIV and Tf, a well established marker for the clathrin pathway, whereas only few vesicles contained with CD59 and Tf. Quantification of the results showed that >80% of DPPIV but only ~20% of CD59 colocalized with Tf. Similar to DPPIV, APN colocalized with Tf, whereas GPI-GFP behave like CD59 (Figure 2B). We also compared internalization of GPI-APs and TMD proteins with CTX which, depending on cell type, can be internalized via clathrin-dependent and/or -independent mechanisms (Torgersen *et al.*, 2001; Sandvig and van Deurs, 2002; Nichols, 2003). In HepG 2 cells, basolateral (BL) internalized CTX did not colocalize with Tf or with the early endosome marker EEA1 (data not shown), suggesting that BL uptake of CTX occurs via a clathrin-independent pathway. It is interesting that the GPI-AP CD59 was internalized in structures positive for CTX, whereas no colocalization was detected between DPPIV and CTX (Figure 2C). GPI-GFP also colocalized with CTX but APN did not, as shown in the quantitative analysis (Figure 2D). These results show that TMD proteins are likely to be internalized via clathrin-coated vesicles, whereas GPI-APs are excluded from Tf-positive structures and internalized in CTX-positive vesicles.

Basolateral Internalization of GPI-APs Is Eps15 Independent, Dynamin Dependent

The observation that GPI-APs were excluded from Tf-containing vesicles suggested that their basolateral endocytic/transcytotic transport occurs through a clathrin-independent pathway. To ascertain that clathrin is not involved in the basolateral internalization of GPI-APs, we transfected HepG2 cells with a dominant-negative isoform of Eps15 (EΔ95/295), which interferes with clathrin-coated pit assembly (Benmerah *et al.*, 1999). Figure 3A shows that the basolateral internalization of CD59 and CTX was not obviously impaired by expression of the Eps15 mutant. On the contrary, Tf internalization, a positive control of clathrin function was dramatically reduced, as well as DPPIV internalization. These morphological observations were quantified by measuring the relative fluorescence intensity present in control cells and cells expressing the Eps15 mutant (Figure 3C). Internalization of Tf and DPPIV was reduced from ~90 to 10%, whereas internalization of CD59 and CTX were minimally affected, shifting from ~90 to 75%.

Dynamin has been shown to regulate many vesiculation events at the plasma membrane, both clathrin dependent

and clathrin independent (McNiven *et al.*, 2000). To investigate the role of dynamin in the endocytic process of GPI-APs, we transiently transfected HepG2 cells either with the wild-type dynamin (Dyn^{WT}) or with the mutant defective in GTP binding and hydrolysis (Dyn^{K44A}). Figure 3B shows that Tf, DPPIV, CTX, and CD59 were efficiently internalized in Dyn^{WT} cells. In contrast, the internalization of all markers was considerably reduced in cells overexpressing the GTP-deficient mutant Dyn^{K44A}. Quantitative analysis of internalized fluorescence (Figure 3D) confirmed that endocytosis of Tf and DPPIV was almost abolished in Dyn^{K44A} cells and that internalization of CTX and CD59 was also strongly reduced, dropping to <20%. Together, these results show that internalization of GPI-APs is mediated by a clathrin-independent, dynamin-dependent mechanism.

Effect of Clathrin Heavy Chain Depletion on the Basolateral Internalization of GPI-APs

To obtain further support for a clathrin-independent mechanism of GPI-APs internalization in polarized hepatic cells, we inhibited expression of the clathrin heavy chain in HepG2 cells. We used a siRNA duplex that targets segment 3311-3333 of the clathrin heavy chain (Hinrichsen *et al.*, 2003). Control cells were transfected with a scrambled siRNA. To quantitate the reduction of clathrin heavy chain expression, transiently transfected HepG2 cells were lysed in SDS-sample buffer and analyzed by immunoblotting with mAb directed against the clathrin heavy chain. Three days after transfection, the signal from the clathrin heavy chain was strongly reduced (Figure 4A). We chose this time point to study the effect of clathrin depletion on the endocytic/transcytotic transport of GPI-APs and TMD proteins using the antibody trafficking assay. Transiently transfected HepG2 cells were incubated for 15 min at 37°C with antibodies to transcytosed proteins. The cells were fixed and permeabilized, and the trafficked antibody–antigen complexes were visualized with secondary antibodies. Cells were subsequently stained with a mAb directed against the clathrin heavy chain. Immunofluorescence showed that typical dot-like staining of clathrin was strongly reduced in many but not all cells (Figure 4B), thus making it possible to observe both clathrin-expressing and clathrin-depleted cells within the same field. Cells that did not express clathrin did not internalize Tf or DPPIV (Figure 4B). By contrast clathrin-depleted cells still internalized CTX as well as CD59. The

Figure 3. Effect of Eps15 and dynamin dominant-negative mutants on the BL endocytosis of GPI-APs and TMD proteins. HepG2 cells were transfected with Eps15 DIIIΔ2 (Eps15 CT), Eps15 EΔ95/295 (Eps15 MT), Dyn2WT, or Dyn2K44, all tagged with GFP. Three days after transfection, cells were incubated for 5 min at 37°C with AF594-labeled Tf or AF594-labeled CTX or for 30 min at 37°C with antibodies to DPPIV or CD59. After an acid washing to remove CTX, Tf, and antibody-antigen complexes remaining at the basolateral surface, cells were fixed, permeabilized, and internalized antibodies were visualized by Cy3-conjugated secondary antibodies and examined by confocal microscopy. (A and B) Confocal pictures. Cells expressing the Eps15 or dynamin construct are delineated. (C and D) The amount of internalized Tf, CTX, DPPIV, and CD59 was measured in cells transfected with Eps15 (C) or dynamin (D) and expressed as a percentage of that internalized in control cells. Black bars represent cells transfected with Eps15 CT (C) or Dyn2 WT (D), and open bars represent cells transfected with Eps15 (C) and dynamin (D) dominant-negative mutant. Quantification of three independent experiments is shown. BCs are marked by asterisks. Bars, 10 μ m.

results of intracellular fluorescence quantification (Figure 4C) gave values very similar to those obtained with the Eps15 mutant (Figure 3C). These results provide additional support for a clathrin-independent way of internalization of GPI-APs at the BL surface of polarized hepatic cells.

The Small GTPase *cdc42* Is Not Involved in the Basolateral Endocytosis of GPI-APs in Polarized Hepatic Cells

The Rho family of small GTPases has been involved in several endocytic pathways, including pinocytosis and

transcytosis (reviewed in Ellis and Mellor, 2000). Among them, *cdc42* has been involved in the internalization of GPI-APs (Sabharanjak *et al.*, 2002; Chadda *et al.*, 2007; Kumari and Mayor, 2008). To examine whether *cdc42* was involved in the regulation of clathrin-independent endocytosis of GPI-APs, we analyzed the internalization of both CD59 and CTX in HepG2 cells expressing a dominant-negative isoform of *cdc42* (*cdc42N17*). We analyzed in parallel the internalization of Tf and DPPIV. In agreement with the previously published data showing that *cdc42* was involved

Figure 4. Basolateral internalization of TMD proteins but not GPI-APs is clathrin-mediated. (A) Western blot analysis of clathrin heavy chain (HC) depletion in HepG2 cells. Lysates were prepared 72 h after transfection with clathrin siRNA or control siRNA. Actin is used as an internal control. (B) 72 h after transfection, HepG2 cells treated with clathrin siRNA were incubated for 5 min at 37°C with AF488-labeled Tf or AF488-labeled CTX or for 15 min at 37°C with antibodies to DPPIV and CD59. At the end of the 37°C incubation, CTX, Tf, and antibody-antigen complexes remaining at the basolateral surface were removed by acid washing. Cells were fixed and permeabilized, and internalized antibodies were visualized by AF488-conjugated secondary antibody. Subsequently, the cells were stained for clathrin HC and examined in confocal microscopy. Nuclei are stained with Topro-3. Arrows point to cells in which the expression of clathrin HC is down-expressed. (C) The amount of internalized Tf, CTX, DPPIV, and CD59 was measured as in Figure 3. Black bars represent CT cells and open bars represent cells transfected with clathrin siRNA. Quantification of three independent experiments is shown. BCs are marked by asterisks. Bars, 10 μ m.

Figure 5. The Rho family GTPase *cdc42* is not involved in the basolateral internalization of GPI-APs. (A) HepG2 cells were transfected with *cdc42N17* tagged with GFP. After transfection, cells were incubated for 5 min at 37°C with AF594-labeled Tf or AF594-labeled CTX or for 15 min at 37°C with antibodies to DPPIV or CD59. At the end of the 37°C incubation, CTX, Tf, and antibody–antigen complexes remaining at the basolateral surface were removed by acid washing. Cells were fixed, permeabilized, and internalized antibodies were visualized by Cy3-conjugated secondary antibodies and examined in confocal microscopy. (B) The amount of internalized Tf, CTX, DPPIV, and CD59 was measured as in Figure 3. Black bars represent CT cells, and open bars represent cells transfected with *cdc42N17*. Quantification of three independent experiments is shown. BCs are marked by asterisks. Bars, 10 μ m.

in the clathrin-mediated endocytosis of IgA in MDCK cells (Rojas *et al.*, 2001), we found that clathrin-mediated endocytosis of Tf and DPPIV was inhibited in cells expressing the dominant-negative isoform of *cdc42* (Figure 5A). In contrast, the *cdc42* mutant did not prevent endocytosis of CTX and CD59 (Figure 5A). These results were confirmed by the quantitative analysis of internalized fluorescence (Figure 5B), suggesting that in polarized hepatic cells *cdc42* is not involved in the BL internalization of GPI-APs.

Caveolin-1 Is Not Involved in the Basolateral Endocytosis of GPI-APs in Polarized Hepatic Cells

Because HepG2 cells do not express caveolin-1 endogenously (Fujimoto *et al.*, 2000; Fu *et al.*, 2004; Figure 6A), the possibility was raised that this protein may be required for efficient endocytosis of GPI-APs at the BL surface of HepG2 cells. To test this hypothesis, we stably transfected HepG2 cells with a cDNA encoding the caveolin-1-GFP fusion protein (Cav-1-GFP). Expression of Cav-1-GFP was first analyzed by Western blot using specific anti-caveolin-1 and anti-GFP antibodies. A single band of 48 kDa was detected in HepG2/Cav-1-GFP cells (Figure 6A). No expression of caveolin-1 protein was detected in nontransfected HepG2 cells (Figure 6A). The pattern of distribution of Cav-1-GFP in HepG2 cells was analyzed by confocal microscopy. As shown in Figure 6B, transfected cells displayed Cav-1-GFP as a punctate pattern at both the BC surface and the BL membrane, in addition to significant intracellular staining. To determine whether caveolin-1 is involved in the BL internalization of GPI-APs, we performed the antibody-traffic assay. Cav-1-GFP-expressing cells were incubated continuously with antibodies to CD59. After 15 min at 37°C, the cells were fixed, permeabilized, and incubated with fluorescently labeled secondary antibodies. As shown in Figure 6C, there was no significant colocalization between Cav-GFP and CD59 as evidenced by the presence of distinct green and red vesicles throughout the cytoplasm. As expected, similar experiments comparing the internalization of Tf or DPPIV with caveolin did not show any colocalization (Figure 6C). These results suggest

that basolateral internalization of GPI-APs does not occur via caveolae, even in caveolin-expressing HepG2 cells.

Figure 6. Basolateral internalization of GPI-APs and TMD proteins occurs independently of caveolin-1. (A) Western blot of nontransfected (lane 1) and caveolin-1-GFP-transfected HepG2 cells by using anti-caveolin-1 antibody (lane 2) and anti-GFP antibody (lane 3). The expected size of the fusion protein is 48 kDa. (B) caveolin-1-GFP-expressing cells were fixed and the subcellular distribution of caveolin-1-GFP was analyzed by confocal microscopy. (C) caveolin-1-GFP-expressing cells were incubated for 5 min at 37°C with AF594-labeled CTX or AF594-labeled Tf or for 15 min at 37°C with antibodies to CD59 or DPPIV. At the end of the 37°C incubation, cells were fixed and permeabilized, and internalized antibodies were visualized by Cy3-conjugated secondary antibodies and examined by confocal microscopy. BCs are marked by asterisks. Bars, 10 μ m.

Figure 7. Basolateral internalization of GPI-APs involves flotillin-2. (A) Antibodies to CD59 and DPPIV were bound simultaneously to the basolateral surface of flotillin-2-GFP-expressing cells at 0°C. After unbound antibody was removed, the cells were incubated at 37°C for 30 min and then fixed and permeabilized. Primary antibodies were visualized by Cy3-conjugated (CD59) or Cy5-conjugated (DPPIV) secondary antibodies. Right, merged image of the confocal field is shown. (B) Western blot showing reduced flotillin-2 expression in cells transfected with flotillin-2 siRNA. Clathrin is used as an internal control. (C) Three days after transfection with flotillin-2 siRNA or control siRNA, HepG2 were fixed, permeabilized, and stained for flotillin-2 with anti-flotillin-2 polyclonal antibody. (D) After transfection with flotillin-2 siRNA or control siRNA, HepG2 cells were incubated for 5 min at 37°C with AF594-labeled Tf or AF594-labeled CTX or for 15 min at 37°C with antibodies to DPPIV or CD59. At the end of the 37°C incubation, CTX, Tf, and antibody-antigen complexes remaining at the basolateral surface were removed by acid washing. Cells were fixed and permeabilized, and internalized antibodies were visualized by Cy3-conjugated secondary antibody and examined in confocal microscopy. The amount of internalized Tf, CTX, DPPIV, and CD59 was measured as in Figure 3. Black bars represent cells transfected with control siRNA and open bars represent cells transfected with flotillin-2 siRNA. Quantification of three independent experiments is shown. (E) HepG2 cells transfected with flotillin-2 siRNA (a–d) or control siRNA (a'–d') were surface labeled with anti-CD59 (b and b') and anti-DPPIV (d and d') antibodies (0°C for 30 min), and chased at 37°C for 60 min before fixation. Internalized antibodies were visualized with Cy3-conjugated antibodies. Flotillin-2 was stained with anti-flotillin-2 and AF488-conjugated secondary antibody. BCs are marked by asterisks. Bars, 10 μ m.

Involvement of Flotillin in the Basolateral Internalization of GPI-APs in Polarized Hepatic Cells

Recent reports have described a new clathrin- and caveolin-independent endocytic pathway in mammalian cells. The two closely related proteins flotillin-1 and flotillin-2 serve as markers for this pathway (Glebov *et al.*, 2006; Frick *et al.*, 2007; Payne *et al.*, 2007). Hepatic cells express flotillin-2 but not flotillin-1 (our unpublished data; Volonté *et al.*, 1999). To test the possible involvement of flotillin-2 in the clathrin- and caveolin-independent internalization of GPI-APs, we first checked whether internalized CD59 colocalized with flotillin-2, in flotillin-2-GFP-expressing cells. Cells were incubated with antibodies to CD59 or DPPIV for 30 min at 0°C. Trafficking was initiated by raising the temperature to 37°C. After 30 min, the cells were fixed, permeabilized, and incubated with fluorescently labeled secondary antibodies. As shown in Figure 7A, after 30 min at 37°C, abundant puncta containing both flotillin-2-GFP and CD59 were observed at the periphery of the cells. In contrast, DPPIV, which is excluded from these structures, was observed deeper in the cells and at the BC membrane, where it colocalized with the steady-state localized flotillin-2-GFP. Moreover, after a short period of internalization (2 min), CTX but not Tf nearly 100% colocalized with flotillin-2-GFP within the same vesicles (data not shown). To test whether flotillin-2 is required for the BL internalization of GPI-APs in HepG2 cells, we used siRNA to knock down the expression level of flotillin-2. The

knockdown efficiency was tested by immunoblotting as well as by immunofluorescence. As shown in Figure 7B, 72 h after transfection, the flotillin-2 signal was strongly reduced, whereas the clathrin signal, used as an internal control had not changed. Compared with control cells in which flotillin-2 fluorescence was present at the BC membrane, at the BL surface and to a lesser extent in intracellular structures, cells transfected with flotillin-2 siRNA did not show detectable signal for flotillin-2 (Figure 7C). We analyzed the effect of flotillin-2 knockdown on the BL internalization of DPPIV and CD59. The efficiency of entry was determined using the assay described above for the effect of clathrin knockdown. Tf and CTX were used as markers for the clathrin-dependent and clathrin-independent pathways, respectively. Compared with control cells, cells transfected with siRNA against flotillin-2 showed ~70% decrease of CTX and CD59 internalization, whereas DPPIV and Tf entry were not inhibited by flotillin-2 knockdown (Figure 7D). To further determine whether flotillin-2 is required for the endocytic-transcytotic transport in polarized hepatic cells, we monitored the antibody-trafficking assay in control and siRNA treated. Cells were processed as in Figure 7A, except that the cells were incubated at 37°C for 60 min and were subsequently stained with a mAb directed against flotillin-2. As shown in Figure 7E, in control cells CD59 was largely detected in punctate structures visible in the cytoplasm, in the subapical area and at the BC membrane, whereas in

siRNA-treated cells, CD59 remained largely at the BL surface and no BC staining was detected (Figure 7E). By contrast, a prominent and homogeneous distribution of the TMD protein DPPIV at the BC surface was apparent in both control and siRNA-treated cells (Figure 7E). Together, these experiments suggest that flotillin-2 plays a role in the basolateral internalization of GPI-APs in polarized hepatic cells.

DISCUSSION

In previous studies, we have made the observation that, in polarized hepatic cells, the transcytotic efflux of apical resident GPI-APs and TMD proteins occurs with different kinetics, suggesting that distinct classes of transcytosing proteins may use separate internalization mechanisms. In this study, we took endogenous CD59 and transfected GPI-GFP as representatives of apical GPI-APs in HepG2 cells, and the endogenous DPPIV and APN as apical TMD proteins. We show that TMD proteins are internalized through a clathrin-dependant mechanism, whereas internalization of GPI-APs is clathrin-independent but requires both dynamin and flotillin.

Internalization of TMD Proteins but Not GPI-APs Is Clathrin Mediated

Clathrin-dependent endocytosis is by far the better understood mechanism for internalization. Clathrin assembly can be driven by a variety of sorting motifs present in membrane receptors that can thus mediate their own endocytosis (Conner and Schmid, 2003). The mechanism of internalization of GPI-APs or TMD proteins that do not bear internalization motifs remains elusive. Apical resident proteins like DPPIV and APN have very short cytoplasmic tails with only six and eight amino acids, respectively. It is therefore unlikely that their cytoplasmic tails contain a signal for incorporation into coated pits. Nevertheless, we found that BL endocytosis of APN and DPPIV was strongly inhibited by clathrin depletion or by expression of Eps15 dominant-negative mutants. In contrast, BL endocytosis of GPI-APs was little affected by clathrin depletion. These observations indicate that APN and DPPIV, but not GPI-APs, are able to enter coated pits. These differences may be linked to their affinity for distinct membrane microdomains. Indeed, GPI-APs preferentially partition into cholesterol-enriched membrane microdomains, called rafts (Ait-Slimane *et al.*, 2003; Nyasae *et al.*, 2003). Experiments using fluorescence resonance energy transfer have shown that cholesterol-dependent clusters of CTX bound to GM1 and GPI-APs are excluded from clathrin-coated pits (Nichols, 2003). TMD proteins, which do not cluster in rafts may enter coated pits by diffusion and make opportunity of the efficient clathrin-mediated endocytic pathway to reach endosomal sorting compartments and achieve transcytosis, whereas GPI-APs would not be able to enter coated pits readily.

The Non-Clathrin Endocytic Pathway of GPI-APs

That GPI-APs are preferentially localized in rafts makes caveolae a good candidate for their endocytosis. Like rafts, these small invaginations are enriched in cholesterol and sphingolipids and can be isolated as detergent-resistant membranes (reviewed in Parton and Simons, 2007). They have been implicated in endocytosis of CTX and GPI-APs (Kurzchalia and Parton, 1999). However, formation of caveolae depends on the presence of caveolin, and not all cells do express caveolin. In HepG2 cells, we did not observe significant colocalization of GPI-APs and caveolae after induction of caveolae by caveolin overexpression. This makes

it unlikely that caveolae could represent an endocytic pathway for GPI-APs in hepatic cells.

Several other raft-dependent entry mechanisms have been described that may be used by GPI-APs and/or raft-associated proteins (Mayor and Riezman, 2004; Mayor and Pagano, 2007). The raft-associated IL-2 receptor is endocytosed via a dynamin and RhoA-dependent mechanism in lymphocytes (Lamaze *et al.*, 2001). In contrast, Sabharanjak *et al.* (2002) found that internalization of GPI-APs was independent of dynamin and RhoA and was regulated by another small GTPase, cdc42, in CHO and Cos-7 cells. In our study, the entry of GPI-APs was dynamin dependent but did not seem to involve cdc42. Furthermore, we did not observe any effect of dominant-negative mutants of the Rho and Rac GTPases on the BL internalization of GPI-APs (our unpublished data). These different results suggest that endocytosis of GPI-APs and raft-associated proteins may be differentially regulated in different cell types or that more than one mechanism is involved in endocytosis of raft-associated proteins.

Involvement of Flotillin in Basolateral Endocytosis of GPI-APs

Flotillin-1 and flotillin-2, also known as reggie-2 and reggie-1, have been identified as plasma membrane-associated proteins that cocluster with GPI-APs in noncaveolar raft membrane microdomains (Lang *et al.*, 1998; Stuermer *et al.*, 2001). Flotillin-1 was recently implicated in the clathrin- and caveolin-independent endocytosis of GPI-APs and CTX in Cos-7 cells (Glebov *et al.*, 2006); and of proteoglycan-binding ligands in HeLa cells (Payne *et al.*, 2007). Moreover, a recent report suggested that flotillin-1 and flotillin-2 coassemble to generate membrane microdomains, and this coassembly was necessary to induce membrane invagination and vesicle budding (Frick *et al.*, 2007). In HepG2 cells, we observed a large colocalization between internalized CD59 and flotillin-2-GFP but not between internalized DPPIV and flotillin-2-GFP. Moreover, immediately after entry, CTX but not Tf, nearly 100% colocalized with flotillin-2-GFP within the same vesicles (data not shown). BL internalization of CD59 and CTX were strongly inhibited after down-expression of flotillin-2, proving that in polarized hepatic cells, the BL uptake of GPI-APs occurs via a flotillin-dependent pathway. It has been reported that the flotillin-dependent internalization of CTX and GPI-APs is dynamin independent (Glebov *et al.*, 2006), whereas another study showed that the entry of proteoglycan-binding ligands is dynamin and flotillin dependent (Payne *et al.*, 2007). Here, we showed that BL endocytosis of GPI-APs and CTX requires both flotillin and dynamin. These differences suggest that flotillin may play a role in several endocytic pathways, both dynamin dependent and independent.

It is generally considered that clathrin-dependent endocytosis is a very fast process ($t_{1/2} < 10$ s), and that clathrin-independent endocytosis proceeds more slowly ($t_{1/2} > 1$ min), with caveolae being the slowest ($t_{1/2} > 20$ min) (Conner and Schmid, 2003). Our results are in agreement with flotillin-dependent endocytosis being slower than the clathrin-dependent mechanism. In these conditions, BL internalization of GPI-APs would be less efficient than BL internalization of TMD proteins. These differences may explain, to a large part, the different kinetics of transcytosis of these two classes of membrane proteins in HepG2 cells. One major question for the future is whether the clathrin-dependent pathway of TMD proteins and the flotillin-dependent pathway of GPI-APs converge in endosomes and whether they use common transcytotic compartments.

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