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Absence of Direct Delivery for Single Transmembrane Apical Proteins or Their "Secretory" Forms in Polarized Hepatic Cells

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The absence of a direct route to the apical plasma membrane (PM) for single transmembrane domain (TMD) proteins in polarized hepatic cells has been inferred but never directly demonstrated. The genes encoding three pairs of apical PM proteins, whose extracellular domains are targeted exclusively to the apical milieu in Madin-Darby canine kidney cells, were packaged into recombinant adenovirus and delivered to WIF-B cells in vitro and liver hepatocytes in vivo. By immunofluorescence and pulse-chase metabolic labeling, we found that the soluble constructs were overwhelmingly secreted into the basolateral milieu, which in vivo is the blood and in vitro is the culture medium. The full-length proteins were first delivered to the basolateral surface but then concentrated in the apical PM. Our results imply that hepatic cells lack trans-Golgi network (TGN)-based machinery for directly sorting single transmembrane domain apical proteins and raise interesting questions about current models of PM protein sorting in polarized and nonpolarized cells.

INTRODUCTION

Epithelial cells are a key to the success of multicellular organisms. They line all body organs and serve as selective barriers between two different worlds (the internal and external). They accomplish this essential function by cementing themselves together through intercellular junctions and then restricting distinct activities to one plasma membrane (PM) surface or the other (the basolateral [internal] and apical [external] domains). The functional asymmetry, or polarity, of the two surfaces is matched by compositional polarity, which means that PM molecules are largely restricted to either the apical or basolateral domain. This polarity is achieved and maintained, in part, by polarized membrane traffic. However, it is clear that evolution has used a modular approach to PM protein and lipid trafficking in polarized epithelial cells. Various combinations of membrane traffic produce different patterns of polarity. Furthermore, in a particular epithelial cell, multiple mechanisms may operate to deliver and retain different classes of membrane proteins to a single PM domain. The challenge is to discover the rules of this complex process (reviewed by Yeaman *et al.*, 1999).

The hepatocyte, the major epithelial cell of the liver, performs many crucial functions that stem largely from its strategic position between the blood plasma and the bile. For example, at the basal surface, there is exchange of metabolites with the blood, including transport of small molecules across the membrane and secretion of plasma proteins via fusion of secretory vesicles with the membrane. At the apical surface, there is secretion of bile acids and products of detoxification via apical PM transporters and delivery of immunoglobulin A to bile by fusion of transport vesicles with the apical membrane. These vectorial processes all rely on the asymmetric distribution of PM proteins (reviewed by Ihrke and Hubbard, 1996).

We and others have reported that hepatocytes in vivo direct at least two classes of their newly synthesized apical PM proteins and all of their secretory proteins to the basolateral front; neither single transmembrane domain (TMD)

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Abbreviations used: APN, aminopeptidase N; BC, bile canaliculus; BSA, bovine serum albumin; DPPIV, dipeptidyl peptidase IV; GPI, glycosyl-phosphatidyl-inositol-; HA, hemagglutinin; HSFM, HEPES-buffered serum-free medium; MAL, myelin and lymphocyte; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PI, protease inhibitor; pIgA-R, polymeric immunoglobulin A receptor; PM, plasma membrane; s-, secreted; TGN, trans-Golgi network; TMD, transmembrane domain; vp, viral particles; WH, whole homogenate; wt-, wild type.

nor glycolipid (glycosyl-phosphatidyl-inositol [GPI])-anchored apical PM proteins go directly to the apical front from the trans-Golgi network (TGN; Bartles *et al.*, 1987; Schell *et al.*, 1992). Rather, these PM proteins take a transcytotic route to the apical surface in hepatic cells. This circuitous route is termed "indirect" (Golgi → basolateral surface → apical surface). Studies carried out in different types of polarized epithelial cells have established that transcytosis is a common pathway in most epithelial cells. Until recently, it was considered to be the only route to the apical PM in hepatocytes. However, evidence for the direct in vivo (Kipp and Arias, 2000) and in vitro (Sai *et al.*, 1999) TGN-to-apical PM trafficking of several polytopic PM proteins has been reported.

Given the existence of a direct pathway for at least one class of apical PM proteins in hepatocytes, we asked whether "secretory" forms of single TMD apical proteins might be included as cargo in such transport carriers. Pertinent to our question is published work documenting that in polarized Madin-Darby canine kidney (MDCK) cells the truncated forms of dipeptidyl peptidase IV (DPPIV; Weisz *et al.*, 1992), polymeric immunoglobulin A receptor (pIgA-R; Mostov *et al.*, 1987), and influenza hemagglutinin (HA; Roth *et al.*, 1987), all lacking their TMDs and cytoplasmic tails, are directly targeted from the Golgi to the apical PM domain. In fact, we reported that the apical targeting of soluble DPPIV was more efficient than that of the membrane-anchored form (Weisz *et al.*, 1992). These results suggested to us that the extracellular domains of these proteins contain apical sorting signals that might promote the proteins' inclusion into a membrane carrier going directly to the apical PM in polarized hepatic cells. Thus, we chose to study three full-length apical PM proteins in this topological class, DPPIV, pIgA-R, and HA, and their truncated, extracellular forms. The pIgA-R takes a transcytotic route in all polarized epithelial cells in which it has been studied; molecular dissection has identified structural signals, largely in its 103-amino acid cytoplasmic tail, which direct its circuitous intracellular journey (Mostov, 1994). DPPIV takes the direct route in most epithelial cells other than hepatocytes, but the efficiency varies, with detectable populations taking an indirect route even in MDCK cells (Casanova *et al.*, 1991). The viral membrane protein, HA, has been used as a model for studying direct apical delivery (Misek *et al.*, 1984) but has not been examined in liver.

In this study, we have used recombinant adenovirus to deliver genes to polarized WIF-B cells and hepatocytes in vivo. We report that in WIF-B cells and in vivo soluble recombinant proteins, even the apically secreted soluble pIgA-R, are targeted to the basolateral domain. We conclude that the "direct" TGN-to-apical PM pathway generally does not accept soluble cargo.

MATERIALS AND METHODS

Materials

The Adlox vector, ψ5 DNA, and Cre8 cells were developed by S. Hardy (Hardy *et al.*, 1997). 293 cells were from the Recombinant Adenovirus Core Facility (Johns Hopkins, Baltimore, MD) and Quantum Biotechnologies. Reagents and suppliers were as follows: wild-type (wt)- and secreted (s)-DPPIV cDNAs in PBSSK+ from C. Machamer (Johns Hopkins); HA (Japan strain) cDNA in pCB6, hybridoma cells secreting FC125 monoclonal antibody and rabbit anti-HA serum R584 from M. Roth (University of Texas, Southwestern); wt-pIgA-R cDNA in pCB6 and sIgA-R cDNA in a retrovirus from K. Mostov (University of California, San Francisco). pCDNA3-TA/TOPO with V5 and 6His sequences, α-V5, and α-6His monoclonal antibodies from Invitrogen (Carlsbad, CA); Alexa-labeled antibodies from Molecular Probes (Eugene, OR); and horseradish peroxidase-conjugated antibody from Amersham Pharmacia Biotech (Piscataway, NJ). Molecular biology reagents were from NEB, Invitrogen, or Promega (Madison, WI). Tissue culture reagents were from sources reported in earlier publications. Primer synthesis and DNA sequencing were performed at the Synthesis and Sequencing Facility, Johns Hopkins.

DNA Constructs

All cDNA sequences were subcloned into the Adlox vector, which is engineered with one loxP site, a left inverted terminal repeat followed by a packaging sequence (Ø), the human cytomegalovirus immediate-early promoter, a multiple cloning site, and a right inverted terminal repeat. The Adlox vector serves as the shuttle vector for generating recombinant adenovirus via the Cre-lox system (Hardy, 1997).

Wt-DPPIV and s-DPPIV were digested with *Hin*dIII and *Xba*I from PBSSK+ and inserted at the same sites in pAdlox. The V5/6His epitope tags were incorporated in-frame at the 3′-end of the open reading frame. Both the wt-pIgA-R and the s-pIgA-R were excised with *Bgl*II and inserted into pAdlox at the *Bam*HI site, generating pAdlox-pIgA-R. V5 and c-myc epitope tags were inserted before the 5′-end of the open reading frame using a fragment that included in tandem the signal sequence of vesicular stomatitis virus glycoprotein, the V5, and c-myc epitopes. The wt-HA was excised with *Hin*dIII and *Bam*HI and subcloned into pAdlox at these sites. The s-HA construct was generated by inserting a stop codon before the TMD of wt-HA. The constructs and positions of epitope tags are shown in Figure 1A; details of the construction are in supplemental material.

Preparation of Recombinant Adenovirus

The recombinant adenoviruses used in this study were generated using the CRE-lox system. Briefly, after subcloning the cDNA of interest in pAdlox, plasmids were purified (Qiagen, Valencia, CA) and the sequences confirmed. Using Lipofectamine-Plus (Invitrogen), Cre8 cells were cotransfected with plasmid DNA (2.1 μg) and DNA (2.1 μg) of the E1-deleted ψ5 virus (adenovirus type 5 engineered with two lox-P sites flanking the packaging site). Following amplification of the recombinant virus in Cre8 cells, a large-scale virus preparation was made from infected 293A cells. The cells were harvested and sedimented (1000 × *g*, 10 min), and the cell pellet was resuspended in 10 ml of frozen/thawed buffer (10 mM Tris-HCl, pH 8, 1 mM $MgCl_2$), freeze-thawed three times, and resedimented. The resulting lysate (~10 ml) was loaded onto a three-step CsCl gradient (1 ml of CsCl in frozen/thawed buffer at 1.25 g/ml, 1 ml at 1.33 g/ml, and 3 ml at 1.45 g/ml), and the gradient was centrifuged (177,000 × *g*, 1 h, 14°C). Viral particles in the 1.33-g/ml region were harvested and either used following G50 column purification or transferred to a reseal tube (Seton Scientific), overlaid with 1.33 g/ml CsCl solution, and centrifuged (246,000 × *g*, 16 h, 14°C). The resulting viral band (1.5–2.5 ml) was dialyzed overnight (Slide-A-Lyzer; Pierce Chemical, Indianapolis, IN) at 4°C against 10 mM Tris-HCl, pH 7.4, 0.9% NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ (3 × 700 ml, with 10% glycerol in the last change) and stored at −80°C. Stock concentrations (viral particles [vp] per milliliter were calculated from OD_{260} × dilution × 1.1 × 10^{12}, with $OD_{260/280}$ of 1.2–1.3.

Cell Culture and Viral Infection

WIF-B cells grown as described by Shanks *et al.* (1994) were seeded at 2 × 10^4 cells/cm^2 on glass coverslips (22 × 22 mm) and infected

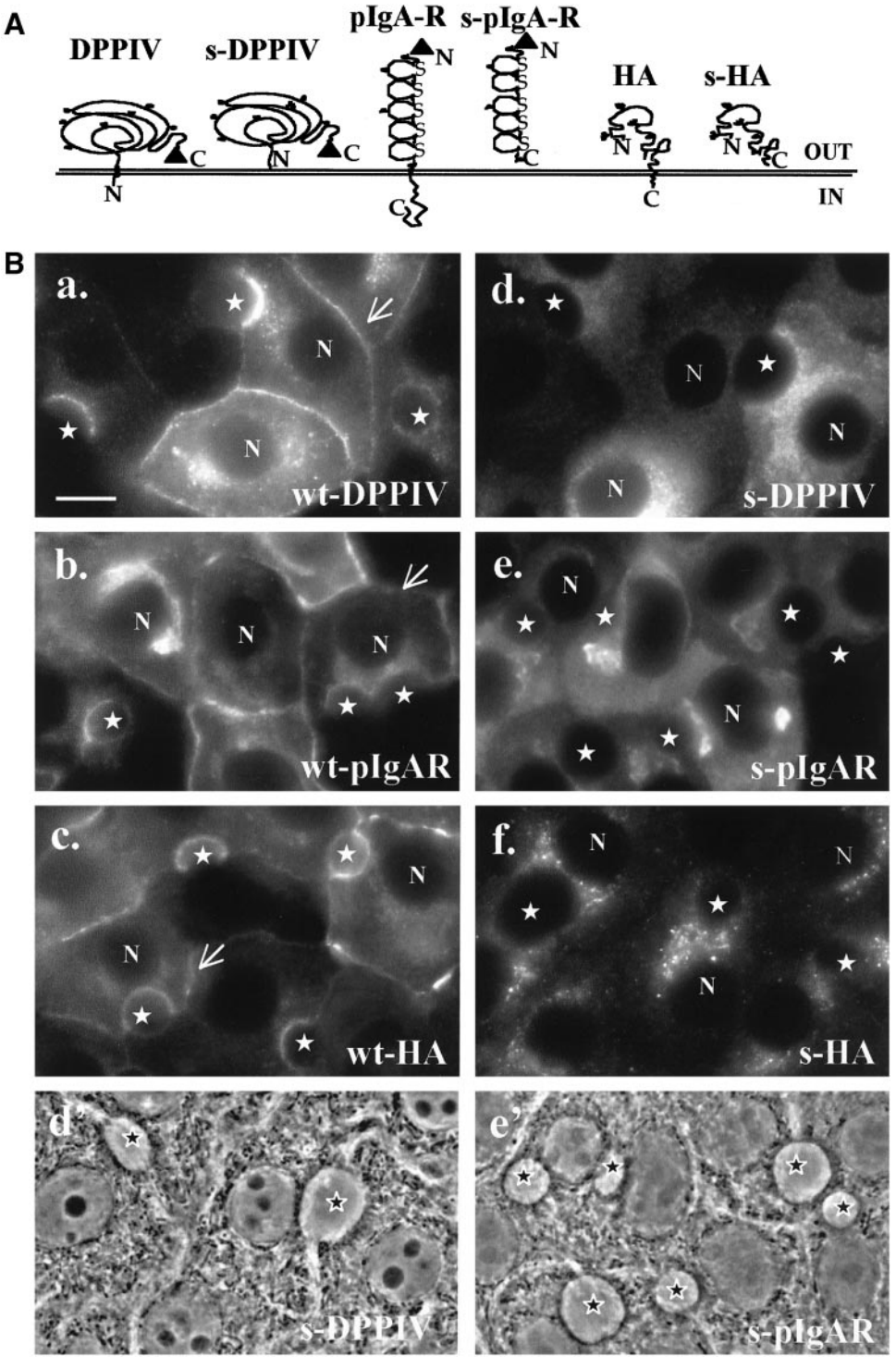

Figure 1. Expression of the wt- and s-forms of DPPIV, pIgA-R, and HA in WIF-B cells. (A) Constructs used in the study. ▲, epitope tags used; s=secretory; N, aminoterminus; C, carboxy terminus. (B) Cells were infected with one of the indicated viruses and fixed 24 h later. The expressed proteins were visualized by indirect immunofluorescence. The wt-proteins (a–c) concentrate at the apical PM (stars) but are also at the basolateral PM (arrows) and in the perinuclear area (N, nucleus). The secretory forms (d–f) are detected only intracellularly and not in the BC space (stars). Phase-contrast images of infected WIF-B cells (e, s-DPPIV; f, s-pIgAR) are shown. Bar, 10 μm.

7–9 d later, when optimal polarity was established. Briefly, medium was aspirated, the cells were rinsed (phosphate-buffered saline [PBS] with 1 mM $MgCl_2$), and viral dilutions were made in 200 μl of OptiMEM (Invitrogen) and applied to each coverslip. Control, noninfected cells were treated with OptiMEM. The optimal viral concentration for >90% expression was 5×10^9 vp/200 μl. After incubation at 37°C for 30 min, the virus was aspirated, fresh medium was added, and the cells were returned to 37°C for 24–48 h before analysis.

Immunofluorescence Microscopy

Cells were fixed with cold 4% paraformaldehyde (PFA) in PBS, permeabilized in methanol (Mevel-Ninio and Weiss, 1981), and processed for single- or double-indirect immunofluorescence (Ihrke *et al.*, 1993) with the following primary antibodies: rabbit α-rat DPPIV (II-2 at 1:5000, so only exogenous protein is detected), α-HA (FC125 culture supernatant at 1:250), and α-V5 IgG (at 1:300). Secondary antibodies (Alexa α-rabbit or α-mouse) were used at 5–8 μg/ml. The cells were examined by epifluorescence (Axioplan universal microscope, Carl Zeiss, Thornwood, NY). In some experiments, infected cells were treated with 50 μM leupeptin added each hour for 4 h before fixation. The labeled cells were viewed by confocal laser scanning microscopy (Noran Oz) using narrow band emission filters (525 and 605 nm) to eliminate channel cross-talk. Images were imported into Photoshop (Adobe Systems, Mountain View, CA) for processing.

Antibody-Trafficking Assay

Twenty-four hours after viral infection, cells were rinsed with HEPES-buffered serum-free medium (HSFM), chilled to 4°C for 5 min, and then incubated for 15 min at 4°C with either α-HA (R584 at 1:100) or α-V5 (1:100) in HSFM plus 2 mg/ml bovine serum albumin (BSA). The cells were washed extensively with the HSFM plus BSA and returned to 37°C in complete medium. At various times, the cells were rinsed briefly with PBS, fixed as described above, washed, blocked with 1% BSA/PBS, and incubated with the appropriate secondary Alexa antibody for 30 min. In some experiments, fixed cells were labeled first with antibody to an apical PM protein followed by a mixture of secondary antibodies.

Immunochemical Methods

Medium and cell extracts were harvested and processed for immunoprecipitation. The medium was cleared of cell debris (134,000 $\times$ *g*, 45 min, 4°C) and the supernatant (5 ml) was mixed with 1.25 ml of a 5$\times$ stock of solubilization buffer with a final composition of 0.025 M $NaPO_4$, pH 7.4, 0.3 M NaCl, 0.5% Triton X-100 supplemented with 20 mM octylglucoside and protease inhibitors (PIs; final concentrations of 100 KIU/ml aprotinin, 1 μg/ml pepstatin, 5 μg/ml each of antipain and leupeptin, and 1 mM each of benzamidine and phenylmethylsulfonyl fluoride). Cells were incubated in 1 ml of cold solubilization buffer for 30 min, centrifuged (15,000 $\times$ *g*, 4°C, 10 min), and the supernatant was immunoprecipitated. Cell and medium extracts were incubated for ~16 h at 4°C with the following antibodies: α-V5 (1 μg/ml) to immunoprecipitate DPPIV and pIgA-R; and FC125 ascites (1:100) to immunoprecipitate HA. Protein A-Sepharose beads (100 μl of 20% slurry) were added for 4 h. Further processing followed previously described methods (Bartles *et al.*, 1987). For immunoblot analysis, nitrocellulose membranes were blocked with 5% skim milk in PBS/0.1% Tween-20 and incubated with primary antibodies (monoclonal α-V5, 1:5000; polyclonal α-HA, 1:1000; polyclonal α-c-myc, 1:1000) and then horseradish peroxidase-conjugated secondary antibodies, and the immunoreactivity was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Metabolic Labeling of WIF-B Cells

WIF-B cells on coverslips were infected with recombinant adenovirus and 24 h later incubated in cysteine- and methionine-free medium (1 h at 37°C) followed by a 5-min "pulse" with the same medium (200 μl) containing 100 μCi ^{35}S-cysteine (for the pIgA-R labeling) or Tran-^{35}S label (for DPPIV and HA). The pulse was terminated by rinsing with Hanks' balanced salt solution (HBSS) and the "chase" was begun by addition of complete medium (37°C, 3 ml/well) containing unlabeled cysteine (64 μg/ml) and methionine (20 μg/ml). At various times, medium was collected onto ice, and the cells were rinsed briefly with HBSS and transferred to fresh wells containing 2.5 mM EGTA in HBSS (1 ml/well) supplemented with 5 mM NaN_3 and 50 mM 2-deoxyglucose. They were incubated twice at 37°C for 15 min (1 ml each), rinsed (1 ml), transferred to fresh wells containing ice-cold PBS, and extracted with 2 $\times$ 0.5 ml of solubilization buffer plus PIs. One-third of each fraction was processed for immunoprecipitation. After separation by SDS-PAGE and transfer to nitrocellulose membranes, the radioactivity in the ^{35}S-polypeptides was measured (phosphor imager, Fuji, Tokyo, Japan). Images were imported into Adobe Photoshop, and band intensities (after background subtraction) were imported into Excel. The means of duplicate values were calculated and expressed as percentages of the total at each time point.

Adenoviral Infection In Vivo

Young adult male Sprague Dawley rats (125–150 g) or mice (20–30 g, Charles River Breeding Labs, Wilmington, MA) under ether anesthesia were given injections of 5×10^{10} or 5×10^9 vp, respectively, into the saphenous vein or retro-orbital sinus. Three days later, rats were anesthetized (6.3 mg ketamine/0.6 mg xylazine/100 g body weight), their bile ducts were cannulated (PE10 tubing, Intramedic; BD Biosciences San Jose, CA), and bile was collected into ice-cold tubes containing PIs. After injection of ^{35}S-amino acids (1.0 mCi/100 g body weight), blood was collected every 30 min and diluted 1:10 with saline containing 10$\times$ PIs, and the cells were removed by centrifugation (10 min, 15,000 $\times$ *g*). Bile was collected continuously over 30-min intervals. At the end of the experiment, the liver was excised, rapidly perfused with ice-cold saline, and homogenized in 4 volumes of 0.25 M sucrose and 10 mM Tris-HCl, pH 7.2. An aliquot of the homogenate was diluted 1:4 into solubilization buffer plus PIs, and a detergent extract was prepared. The resulting supernatant (termed whole homogenate, WH) was used for immunoprecipitation. Aliquots of bile (100 μl), serum (10 μl), and liver WH (200 and 400 μl) brought to 500 μl in solubilization buffer were incubated overnight with α-V5 (1 μl) or FC125 ascites (10 μl) and further processed. The density of ^{35}S-bands in the autoradiograms was measured and normalized to the total volumes of the samples.

Immunofluorescence In Vivo

The livers of infected rats and mice were perfused via the portal vein with PBS and then with ice-cold 2% PFA/PBS. Tissue blocks (~5 $\times$ 20 mm) were postfixed in 2% PFA/PBS for 2 h, immersed in 0.5 M sucrose/PBS overnight, embedded in OCT medium (Miles, (Elkhart, IN), and quickly frozen in isopentane cooled in liquid N_2. Sections (7 μm thick) were cut at −20°C on a cryostat (Leica, (Wetzlar, Germany), transferred to slides, and stored at −20°C. For immunofluorescence, the sections were fixed in ice-cold methanol (5 min), washed in PBS (three times for 5 min each at room temperature), and blocked with 1.5% BSA/PBS/0.3% Triton X-100 (30–45 min), which was the buffer used thereafter. Primary antibodies were added for 3–6 h (mouse α-V5-FITC 1:100, rabbit α-aminopeptidase N [APN] 1:100), followed by three rinses for 5 min each, incubation with Alexa-α–rabbit secondary antibody, three rinses for 5 min each, and a final rinse in PBS. Specimens were mounted and viewed by laser confocal microscopy.

RESULTS

WIF-B Cells Are Easily Transduced by Recombinant Adenovirus and They Retain Their Polarity

Our first challenge was to establish a reproducible transfection method for expressing exogenous proteins in fully differentiated hepatic cells. We found that a recombinant adenovirus system was most effective in delivering genes to polarized WIF-B cells and hepatocytes in vivo. A transduced WIF-B cell population (50–80%) routinely expressed detectable levels of a recombinant protein. Although expression levels varied within a population, retention of cell polarity was excellent, as shown in the phase images of Figure 1B.

wt- and s-Recombinant Proteins Show Different Patterns of Expression in WIF-B Cells

Polarized WIF-B cells were infected with recombinant adenoviruses encoding the six proteins and examined by indirect immunofluorescence 24 h later. Because both DPPIV and pIgA-R are endogenous hepatic proteins, we added epitope tags to the ends of their extracellular domains (C terminus for DPPIV and N terminus for pIgA-R). The viral protein HA was used without tags. In the infected cells, the recombinant wt-proteins were localized predominantly to the apical PM domain, as shown by the bright fluorescence staining of the PM lining dilated spaces between cells. We have shown that these cysts are functionally equivalent to bile canaliculi in vivo, hence the term "BC" (Figure 1B, a–c, stars). There was also wt-protein expression at the basolateral PM domain (arrows), which likely represents the transcytosing population. In contrast, the secreted forms were concentrated near the nucleus in a Golgi-like pattern, with no PM-associated fluorescence (Figure 1B, d–f). Such results were consistent with secretion of these forms into the medium. Given our prediction that the three secreted proteins might be released into the apical (BC) space (stars), we were struck by their absence from this extracellular compartment at any time following infection (24–72 h).

To ensure that soluble recombinant proteins could be detected in the BC spaces, we treated WIF-B cells with the microtubule-depolymerizing drug, nocodazole, 2 h before fixation. Others have shown that newly synthesized plasma proteins, which are normally secreted into the circulation in vivo with high fidelity, are redirected into bile after microtubule disruption (Godfrey *et al.*, 1982). The Golgi also disperses under these conditions. As shown in Figure 2A, polarized WIF-B cells behaved similarly after nocodazole treatment, with endogenous albumin and recombinant s-DPPIV detected near the BC space as well as throughout the WIF-B cytoplasm. These results indicated that, if the soluble forms of the apical PM proteins were directly secreted from the apical PM, we would have detected them.

Recombinant wt-Proteins at the Basolateral Surface Are Transcytosed to the Apical PM Domain

The presence of membrane-anchored apical proteins at the basolateral PM in WIF-B cells suggested either that they were randomly targeted to both PM domains or that the basolateral PM pool was in transit to the apical PM. To test this latter hypothesis, we performed an antibody-trafficking assay. In previous work, we had established conditions in which only the basolateral PM population of apical PM proteins was labeled by antibodies added to the medium of live WIF-B cells (Ihrke *et al.*, 1998). The presence of tight junctions prohibited access and binding of antibodies to their cognate antigens at the apical PM. Using this assay we reported that endogenous pIgA-R and DPPIV were transcytosed across polarized WIF-B cells (Ihrke *et al.*, 1998). We obtained similar results for recombinant wt-pIgA-R and DPPIV in this study. It was particularly important to examine the dynamics of the basolateral pool of wt-HA, because this protein serves as the model for a "directly targeted" apical PM protein (Misek *et al.*, 1984). wt-HA-expressing cells were labeled at 4°C, warmed to 37°C to allow trafficking of the antibody-antigen complexes, then fixed, permeabilized, and stained with secondary antibody to detect the trafficked antibody. Figure 2B shows the time-dependent movement of basolaterally labeled HA to the apical PM. At 0 min, wt-HA was present only along the basolateral PM, but by 30 min it was detected near the apical PM in intracellular punctae, which most likely represent the subapical compartment that transiently accumulates transcytosing apical PM proteins (Barr and Hubbard, 1993; Ihrke *et al.*, 1998). By 90 min, wt-HA had reached the apical surface and had correspondingly decreased at the basolateral PM. The kinetics were similar to those of other apical PM proteins we have studied (Ihrke *et al.*, 1998). This result strongly indicated that wt-HA present at the apical surface was derived, at least in part, from the basolateral domain via transcytosis.

Recombinant Proteins Are Expressed in WIF-B Cells and Secretory Forms Accumulate in the Culture Medium

We next determined the biochemical levels of recombinant proteins remaining cell associated versus secreted. Cells and medium were collected 24 h after adenovirus infection and analyzed by immunoprecipitation and immunoblotting. As seen in Figure 2C, the wt-proteins and soluble proteins were easily detected in transduced cells; recombinant DPPIV and pIgA-R were expressed at ~50- to 100-fold over their endogenous counterparts. As described earlier (Weisz *et al.*, 1992), overexpressed wt-DPPIV exhibited two electrophoretic forms, representing the precursor and mature proteins. Cells transduced with wt-HA displayed predominantly the mature form, although prolonged exposures revealed the precursor (Figure 2C, inset). The presence of multiple wt-pIgA-R bands in cells prevented direct identification of the immature and mature forms, as reported previously for the rabbit protein (Kuhn and Kraehenbuhl, 1981). As expected, wt-DPPIV and wt-HA were predominantly cell associated, as was the full-length form of pIgA-R. A truncated form of pIgA-R was also present in the medium. In contrast, the secreted forms were detected both in the media and in cells. However, in each case, the immature forms were only associated with cells, whereas the mature forms were overwhelmingly detected in the media. Controls consisting of immunoprecipitates from uninfected cells or their medium were uniformly negative.

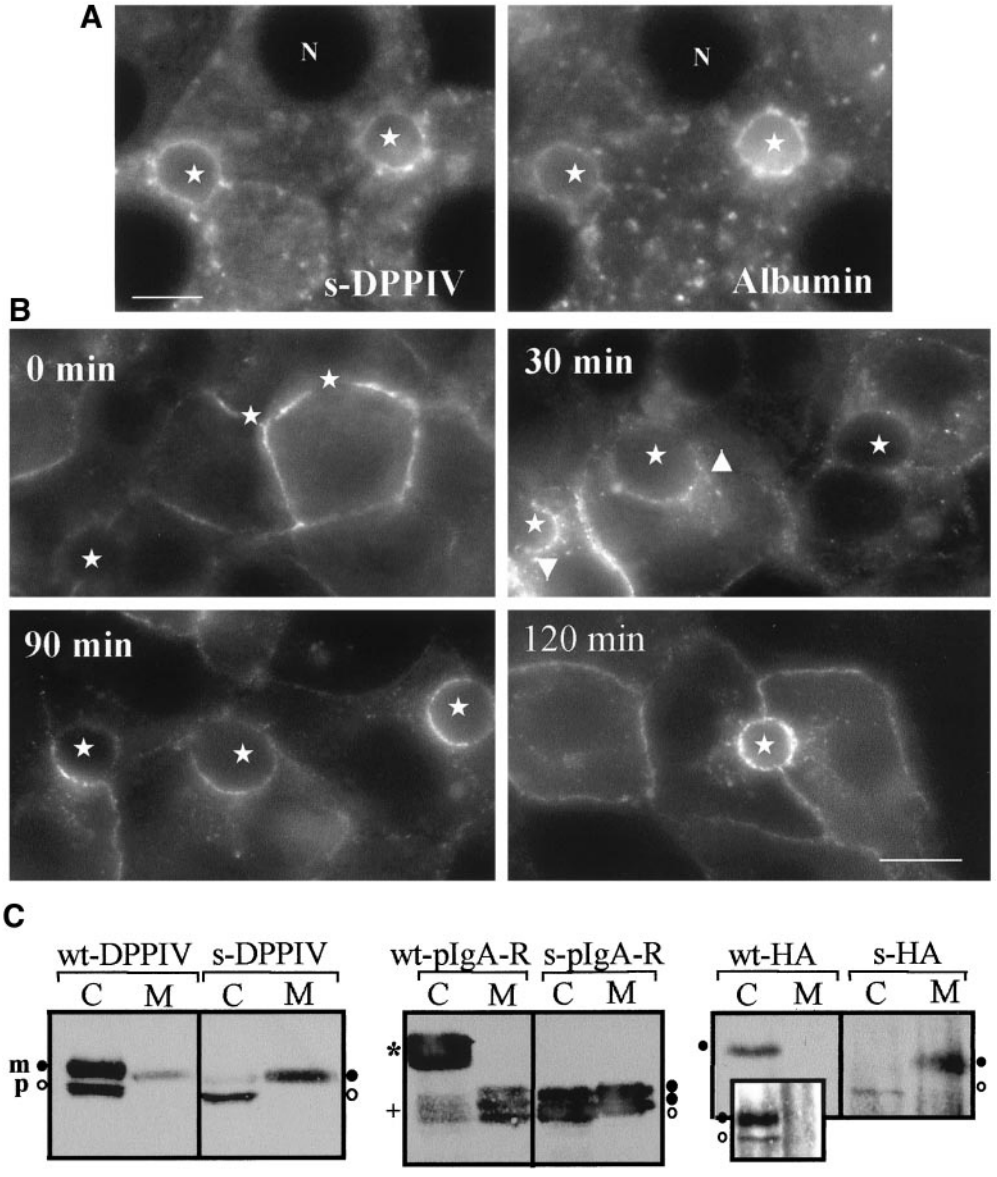

Figure 2. Behavior of wt- and s-recombinant proteins in WIF-B cells. (A) Dispersal. Infected WIF-B cells treated with nocodazole (33 μM) for 2 h before fixation were double-labeled with α-V5 to detect s-DPPIV (left) and α-rat serum albumin (right). The Golgi is dispersed and the s-proteins ring the BC (stars). N, nucleus. Bar, 10 μm. (B) Transcytosis. HA expressed at the basolateral surface was labeled with α-HA (R584, 4°C for 15 min) and chased at 37°C for the indicated times before fixation and visualization by secondary antibody. Note the absence of labeling at the apical PM after 0 min of chase (stars) and the progressive increase with longer times at 37°C. White arrowheads point to puncta near the apical PM. Bar, 10 μm. (C) Maturation and secretion. Cells were infected with the indicated virus and 24 h later, cell extracts (100%) and conditioned media (20%) were immunoprecipitated and processed for immunoblot analysis. C, cell; M, medium; ●, mature (m); ○, precursor (p); *, full-length pIgA-R; +, truncated p-IgA-R.

Recombinant Secretory Proteins Are Released Directly into the Culture Medium of Polarized WIF-B Cells, whereas Transcytosed wt-pIgA-R Is Cleaved and Released at the Apical PM

Our immunoblotting results (Figure 2C) showed that the secreted forms of DPPIV, HA, and pIgA-R were present in the medium of WIF-B cells. However, the surface at which the secreted forms were released could not be established by this long-term (24 h) experiment, because of possible leakage into or out of the BC space. Furthermore, the cleaved product of wt-pIgA-R was also detected in the medium, even though in vivo its cleavage and release occurs exclusively at the apical PM (Musil and Baenziger, 1988) One interpreta-

tion of these results was that proteins secreted into the bile space might have leaked through WIF-B tight junctions during the long incubation. Therefore, we performed short-term metabolic labeling experiments to determine the sites of release of the three secretory proteins and wt-pIgA-R in WIF-B cells. We labeled cells for 5 min with ^{35}S-amino acids and then chased from 0–180 min. At each time point, the medium was collected and the cells were incubated with EGTA to disrupt the tight junctions, thereby releasing apically secreted contents. Metabolic inhibitors were included to stop further membrane traffic (including secretion) during the EGTA treatment (Casciola-Rosen and Hubbard, 1991). After this incubation, the cells were lysed, extracted, and immunoprecipitated along with the two media fractions, and the radioactivity content of each was analyzed.

We focused first on the wt-proteins (Figure 3). At the end of the 5-min pulse, only the immature ^{35}S-labeled forms were evident; at longer times, the mature ^{35}S-labeled forms appeared and increased in intensity as the immature forms disappeared. The appearance of a truncated form of ^{35}S-wt-pIgA-R extracellularly (Figure 3B) showed that cleavage indeed took place, albeit to a lesser extent than that in vivo. This cleaved form first appeared in the EGTA fraction at 30 min of chase and continued to increase there to a maximum of 20% of the total ^{35}S-protein at 60 min. Small amounts were also detected in the culture medium at 45 min of chase but did not change over the rest of the experiment. These results demonstrated that the cleaved form first appeared in the apical medium with minimal leakage and/or appearance in the basolateral medium. Interestingly, a significant fraction of the mature wt-DPPIV was also released, but more was found in the culture medium than in the EGTA fraction (Figure 3A). Mature ^{35}S-wt-HA was never detected extracellularly (Figure 3C).

The secretory forms were examined in a similar way (Figure 4). The relative maturation of the cellular forms varied somewhat. Mature ^{35}S-s-pIgA-R (Figure 4C) was easily seen by 15 min, whereas mature ^{35}S-s-DPPIV and s-HA were detectable in cells only after 30 min of chase (Figure 4, A and B). Likewise, ^{35}S-s-pIgA-R appeared earlier in the media (30 min) than did the other two ^{35}S-s-proteins. Although ^{35}S-s-proteins were detected in the EGTA samples (Figure 4, e), the amounts of ^{35}S-s-DPPIV and ^{35}S-s-HA remained very low (~1–4%) in this fraction throughout the chase period, and they progressively increased in the culture medium. This was not the case for ^{35}S-s-pIgA-R, which was detected in the EGTA fraction as early as 15 min but only in the medium beginning at 30 min. Thereafter it increased in the culture medium.

The presence of ^{35}S-proteins in the EGTA fraction prompted us to assess the efficacy and limitations of this method for collecting apical material. We determined the secretion kinetics of newly synthesized albumin, an endogenous hepatic protein that is constitutively secreted from the basolateral PM domain of hepatocytes. Surprisingly, we found ^{35}S-albumin's behavior to be most similar to that of ^{35}S-s-pIgA-R (compare Figure 4D to 5C), i.e., low levels were first detected in the EGTA (apical) fraction at 15 min and increased to ~14% at 45 min. ^{35}S-albumin was detectable in the culture (basolateral) medium only at 30 min, after which the levels increased dramatically. These results suggested several possibilities. Either EGTA treatment was damaging a small fraction of the cells early in the chase period and releasing contents, apical secretion was actually occurring, or the metabolic inhibitors were not effective. We eliminated the last possibility by determining that ~2–4% of ^{35}S-albumin was released in the presence of the inhibitors when EGTA was omitted. Thus, either cellular damage or limited apical secretion of rapidly processed proteins (s-pIgA-R and albumin) occurred.

Wt-Recombinant Apical PM Proteins Are Expressed Predominantly in Liver Hepatocytes In Vivo

Our combined observations in polarized WIF-B cells of the steady-state morphological localizations of the three apical wt-proteins and the low-to-absent apical release of the secreted forms suggested that these six proteins were first delivered to the basolateral PM domain. However, we could not exclude the possibility that low amounts were indeed delivered directly to the apical PM domain but were either below the detection limits of the assay or (less likely) quickly leaked out of the BCs. Therefore, we carried out experiments in vivo, where there is less potential for mixing of fluids secreted into the apical (bile) and basolateral (blood) environments. To determine which organs expressed the recombinant proteins, we administered recombinant adenovirus encoding wt-DPPIV, because this membrane protein would remain associated with cells in whole tissue homogenates. Three days after infection, we analyzed five organs and found that the liver contained >95% of the total expressed protein (in supplemental material).

To properly interpret our data, we next used immunofluorescence to determine which cell types in the liver expressed the recombinant proteins. Unfortunately, we were unable to reliably detect HA, but we could detect wt-DPPIV and wt-pIgA-R, which were separately expressed in mice. Because each construct carried a V5 epitope, we stained liver sections directly with α-V5-FITC, thus avoiding nonspecific staining of sinusoidal lining cells with secondary α-mouse antibodies. We found that wt-pIgA-R was expressed predominantly in hepatocytes and not Kupffer or endothelial cells lining the sinusoids (Figure 5). As shown in Figure 5, A and C, protein expression varied among the hepatocytes but was easily detectable in intracellular locations and at the sinusoidal surface. To confirm that lack of apical PM staining was due to proteolytic cleavage of wt-pIgA-R at that location, we administered the PI leupeptin at 30-min intervals for 4 h before sacrifice. We previously reported that leupeptin inhibits cleavage and subsequent release of the ectodomain of endogenous pIgA-R upon its arrival at the apical PM (Barr and Hubbard, 1993). Figure 5, B and D, show that in livers so treated recombinant wt-pIgA-R colocalized with an endogenous apical PM protein, APN, at the apical PM.

Soluble Recombinant Proteins Are Quantitatively Secreted into the Blood In Vivo, whereas the Cleaved Ectodomain of wt-pIgA-R Is Released into the Bile

Having established that the recombinant protein expression in liver was predominantly in hepatocytes, we performed pulse-chase metabolic labeling experiments to determine the distributions of the ^{35}S-labeled proteins in the blood, bile, and livers of animals infected 3 d previously. The results are

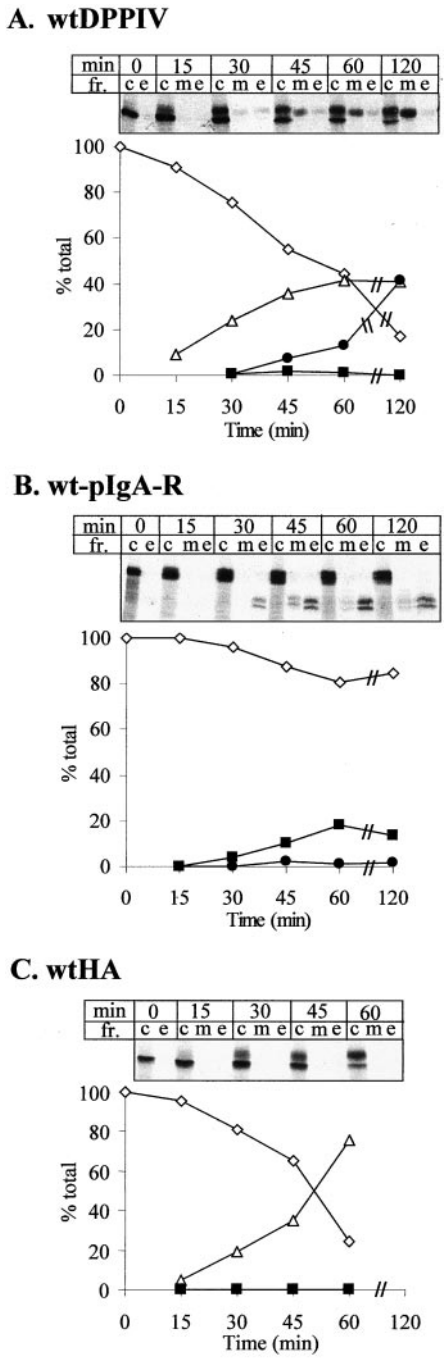

Figure 3. Quantitative analysis of recombinant wt-protein expression in WIF-B cells. WIF-B cells infected with the indicated adenovirus were pulse-labeled with ^{35}S-amino acids for 5 min and chased for up to 120 min. Samples were collected at 15-min intervals for the first hour and each hour thereafter. The ^{35}S-proteins were immunoprecipitated from equal portions of the collected samples and processed for autoradiography. fr., fraction; c, cell extracts; m, medium (basolateral secretion); e, EGTA extract (apical secretion). Each value in the graphs is expressed as a percentage of the total (c + m + e = 100%) at each time point. ◇, precursor; △, mature; ●, medium; ■, EGTA extract. Values are averages of duplicates from the same experiment as shown in the gels. Two experiments gave similar results.

presented in Table 1. The amounts of the ^{35}S-s-proteins detected in the plasma were dramatically greater than the cumulative amounts in bile: ~55% of the total ^{35}S-s-DPPIV was found in the blood circulation at the end of the 2-h experiment and ~45% was still associated with the liver, leaving only ~0.25% in the bile. ^{35}S-s-pIgA-R gave comparable results throughout a 3-h chase. Although the amounts of recombinant ^{35}S-s-proteins in bile were higher than that of endogenous ^{35}S-albumin (Table 1, experiment 1), the finding that <0.5% of the total secreted ^{35}S-s-proteins was detected in bile indicated that little direct secretion occurred at the apical PM in vivo. In contrast to ^{35}S-s-DPPIV, the recombinant ^{35}S-wt-DPPIV remained overwhelmingly associated with the liver (93%) at the end of a 2-h chase. Interestingly, more ^{35}S-wt-DPPIV than ^{35}S-s-DPPIV was released into bile (0.8%), consistent with reports that this resident protein is cleaved at the apical PM (Scott and Hubbard, 1992). Six percent of ^{35}S-wt-DPPIV was also detected in the circulation, perhaps representing cleavage and release at the hepatocyte basolateral PM or at the PM of transduced liver sinusoidal lining cells. Finally, we compared the behaviors of recombinant and endogenous ^{35}S-wt-pIgA-R in the same livers (Table 1, experiments 8 and 9). Both proteins were processed similarly, with the majority of released ^{35}S-labeled proteins being found in the bile (Figure 5E) and of a size appropriate to that of the pIgA-R cleavage product. Detectable amounts of cleaved, recombinant, but not endogenous ^{35}S-wt-pIgA-R were also present in blood, perhaps reflecting release from extrahepatic sources.

DISCUSSION

Using a recombinant protein approach, we have documented for the first time that polarized hepatic cells use the basolateral secretory route exclusively for the first leg of the post-Golgi transport of newly synthesized single TMD apical PM proteins. We expressed three pairs of apical PM proteins whose extracellular domains are targeted directly to the apical PM domain in MDCK cells and found in hepatic cells that the soluble constructs were overwhelmingly secreted into the basolateral milieu, which in vivo is the blood and in vitro is the culture medium. The full-length proteins were concentrated in the apical PM but also showed basolateral PM expression. The latter population could be followed across the cell to the apical PM using an antibody-trafficking assay. These combined results are consistent with the exclusive use of transcytosis by single TMD apical PM proteins in polarized hepatic cells.

Our study has several important implications. First, the fact that recombinant apical PM proteins and their soluble cognates behave similarly in vitro and in vivo reinforces our assertion that the WIF-B cells are a good surrogate for hepatocytes in vivo. Second, the exclusion of recombinant soluble proteins from post-TGN vesicles carrying newly synthesized polytopic apical PM proteins directly to the apical PM implies that sorting into this newly reported pathway is more highly regulated than the TGN-based apical sorting mechanisms in MDCK cells. Finally, our results suggest that a sorting mechanism(s) for at least one class of PM proteins is not present in the TGN of hepatic cells. This last point is relevant to current models of PM protein sorting in polarized and unpolarized cells.

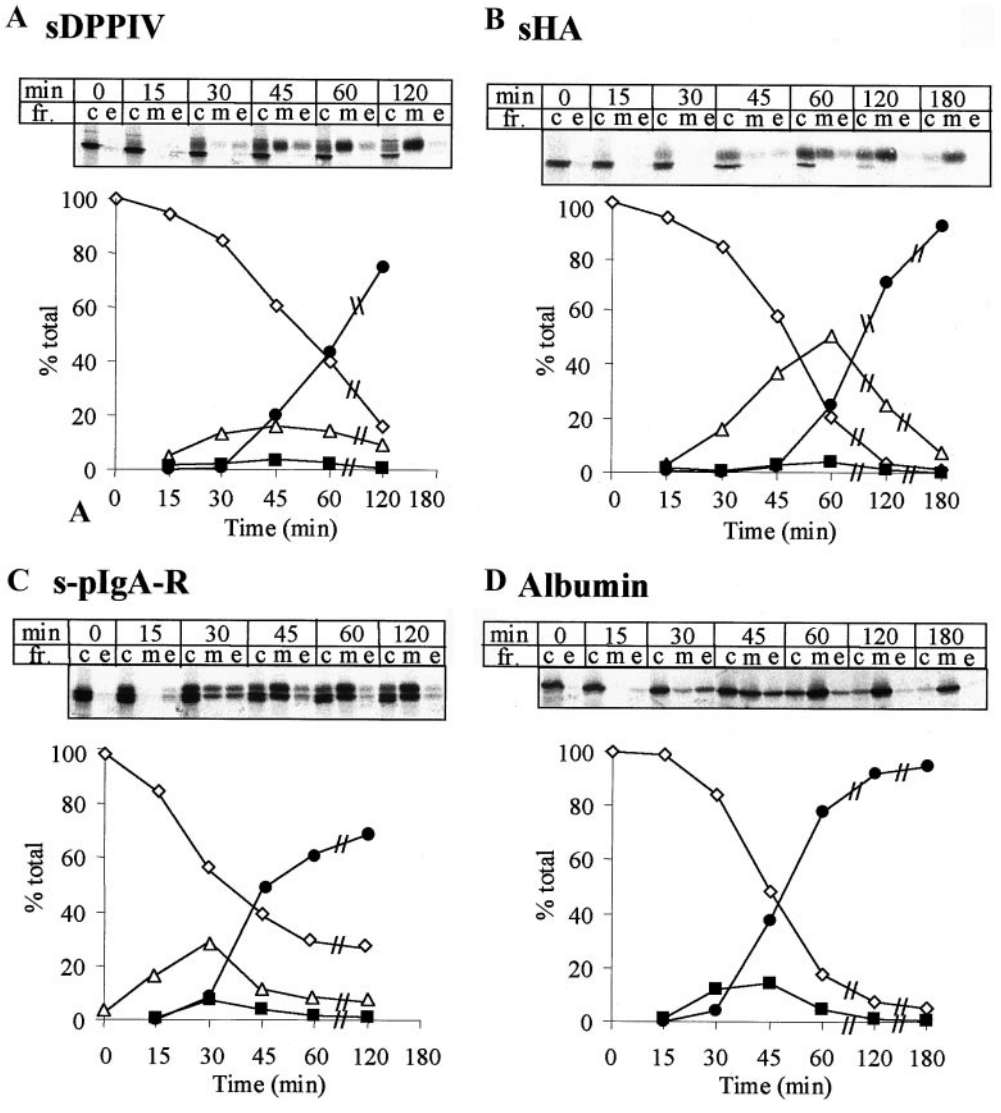

Figure 4. Quantitative analysis of recombinant s-protein expression in WIF-B cells. WIF-B cells infected with the indicated adenovirus were pulse-labeled with ^{35}S-amino acids for 5 min and chased up to 180 min. ^{35}S-labeled samples were processed as described in Figure 7 legend. D: Endogenous ^{35}S-albumin was also immunoprecipitated from the same samples in some of these experiments. Symbols are described in Figure 7 legend. Two experiments gave similar results.

WIF-B Cells Are a Good In Vitro Model of Polarized Hepatocytes

The advantages of studying PM protein traffic in cell lines are numerous, the principal one being ease of manipulation. However, achieving high and reproducibly uniform polarity in cultured hepatic cells has been problematic. We initially studied hepatic PM protein trafficking in vivo, to establish a standard against which in vitro systems could be measured. The subsequent generation of WIF-B cells and characterization of their polarized phenotype, followed by a comparison of their PM protein-trafficking routes to those in vivo, assured us of their usefulness for more detailed mechanistic studies. Another hepatic line, the HepG2 cells, is proving useful for studies of membrane lipid transport (Hoekstra *et al.*, 1999).

Despite the advent of polarized hepatic lines, quantitative in vitro studies of the mechanisms of PM protein sorting and trafficking in these cells have been hindered by several limitations. First, the structural organization of polarized hepatocytes prohibits direct access to the apical PM, which is confined between adjacent cells and bounded by tight junctions. Hence, determination of the rates and extents of membrane protein transport to the two PM domains using accepted methods (e.g., surface biotinylation in MDCK cells) is not feasible. However, in this study we successfully quantitated polarized secretion of soluble proteins by WIF-B cells in vitro. Our results show quite clearly that exogenous and

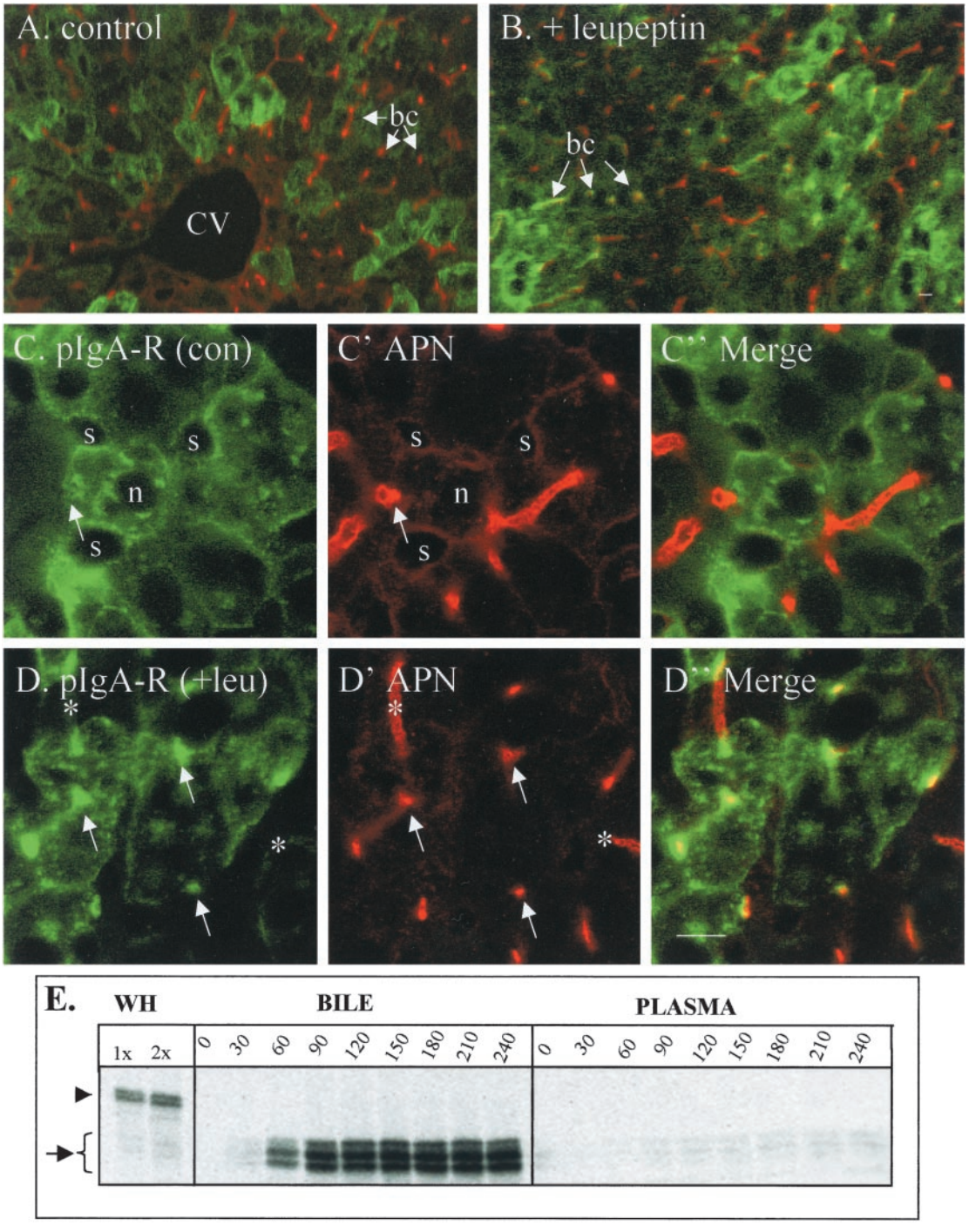

Figure 5. Recombinant wt-pIgA-R expressed in vivo reaches the apical surface of hepatocytes and is cleaved. Mice injected with V5-wt-pIgA-R adenovirus were sacrificed 3 d later, without (A and B, control) or with (C and D, +leupeptin) ip injection of 1 mg/100 gm body weight leupeptin administered every 30 min for 4 h. 7 um fixed sections were labeled with mouse α-V5-FITC (green) and rabbit α-APN then Alexa- α-rabbit antibody (red). Cells labeled with the α-V5 are those infected. In panel A, there is no overlap between the red and green signals, indicating the absence of pIgA-R at the apical PM of infected cells. However, in panel B, from an animal treated with leupeptin, recombinant wt-pIgA-R is seen at the apical PM and overlaps with endogenous APN. (bc, bile canaliculi). Rows C and D are enlarged views of untreated (C) and leupeptin-treated (D) livers. Symbols: n, nuclei; s, sinusoidal space; arrows, bile canaliculi; asterisks in D and D', bile canalicular PM in uninfected cells. Bars, 20 um (A and B) and 10 um (C and D). E. Rats infected with adenovirus were pulse-chased with ^{35}S-cysteine. ^{35}S-pIgA-R was immunoprecipitated with α-V5 from WHs, bile and plasma collected at the indicated times. 15% of each bile sample and 0.05% of each plasma sample are represented in the individual lanes in the autoradiogram. Symbols: arrowhead, mature forms of wt-pIgA-R; arrow, cleaved forms.

Table 1. Distribution of ^{35}S-proteins in vivo

Protein	Percent of total		
	Bile	Plasma	Liver
Secretory proteins			
s-DPPIV			
Expt. 1	0.1	52	48 (2 h)
Expt. 2	0.3	53	46 (2 h)
Expt. 3	0.3	57	43 (2 h)
s-pIgA-R			
Expt. 5	0.3	73	27 (4 h)
Expt. 6	0.2	63	37 (4 h)
Expt. 7	0.2	56	44 (3 h)
Endogenous albumin			
Expt. 1	0.1	98	2 (2 h)
Full-length Proteins			
wt-DPPIV			
Expt. 4	0.8	6	93 (2 h)
wt-pIgA-R			
Expt. 8	14*	5*	81 (4 h)
Expt. 9	33*	2*	65 (4 h)
Endogenous pIgA-R			
Expt. 8	36*	0	64 (4 h)
Expt. 9	16*	0	84 (4 h)

Rats were infected with recombinant adenovirus; 3 days later, ^{35}S-tran-label or ^{35}S-cysteine (pIgA-R) (1 mCi/100 g body weight) was administered iv. Blood was collected periodically and bile continuously. At the end of the experiment, the liver was excised. Newly-synthesized recombinant proteins were immunoprecipitated with anti-V5 antibody (DPPIV and pIgA-R); endogenous proteins were immunoprecipitated with anti-rat serum albumin or anti-pIgA-R. The immunoprecipitates were processed as described in MATERIALS AND METHODS and were visualized by autoradiography. The amount of ^{35}S-protein in each sample was calculated by densitometry and normalized for the total sample size. Numbers in parentheses indicate the duration of the individual experiments.

* Cleaved form of wt-pIgA-R (see Figure 5E).

endogenous molecules are secreted into the basolateral milieu with preferences from 35:1 to >100:1 (endpoint values in Figure 5 graphs). Although the fidelity is lower than in vivo (Table 1, plasma to bile ratios of 200:1–1000:1), the WIF-B polarized secretion index compares favorably to those of other model epithelial cells (e.g., MDCK and Caco-2 [Kondor-Koch *et al.*, 1985; Traber *et al.*, 1987]).

A second limitation in the use of hepatic cell lines has been expression of recombinant exogenous proteins. Traditional transient transfection methods are particularly detrimental to the polarized phenotype, because they are often toxic to cells. Stable transfections are feasible but the efficiency is extremely low. We successfully used recombinant adenoviruses to express both wt- and s-proteins in WIF-B cells and in rat liver in vivo. Adenoviruses can infect fully differentiated, nondividing cells, and they preferentially target to liver, presumably because of the discontinuous nature of the endothelium lining the blood sinusoids in this organ, which allows access of virus particles to receptors present on the hepatocyte (Jaffe *et al.*, 1992; Wilson, 2001). Although expression of the gene products is transient, it is high and reasonably uniform; most importantly, retention of polarity is excellent.

Soluble Cargo Is Excluded from the Newly Described TGN-Apical PM Route in Polarized Hepatic Cells

Until recently, there was no evidence for a direct TGN-to-apical route in hepatocytes. However, several multispanning apical PM transporters appear to take such a route in polarized WIF-B cells and in vivo (Sai *et al.*, 1999; Kipp and Arias, 2000). In light of our present results, the existence of a direct pathway for at least one class of new apical PM proteins raises several questions. First, why are single TMD apical PM proteins excluded from it? We know that the hepatic apical PM proteins contain sorting signals, because they are directly delivered to the apical PM when expressed in simple epithelial cells, such as MDCK. Therefore, the relevant sorting machinery must be either absent from hepatocytes or present outside of the TGN. If present, is it in basolateral endosomes? If absent, how do the proteins achieve their steady-state concentration at the apical PM?

A second and equally puzzling question is why the extracellular portions of the three single TMD proteins were excluded from apically targeted vesicles. Three possibilities are: 1) the apical sorting mechanism for these domains is absent from hepatocytes; 2) the amount of traffic going directly to the apical PM during the time course of our experiments is very low; and 3) the shape of the carriers (e.g., high surface to volume ratio) prohibits soluble material from being passively included in the lumen. The last two possibilities can be addressed with careful quantitative biochemical and morphological studies.

Finally, it is puzzling that the EGTA fractions (bile equivalent) from metabolically labeled WIF-B cells contained small but detectable amounts of only two of the four newly synthesized soluble proteins we analyzed, and only at early times of chase. This differential behavior is probably not a function of higher expression levels (and therefore, increased detection) of albumin and s-pIgA-R, because the levels of s-HA and s-DPPIV were not significantly lower. Perhaps the experimental manipulations transiently rendered the cells fragile, thus differentially affecting the proteins already in later parts of the Golgi. Regardless of the explanation for our results in vitro, the finding in vivo that basolateral secretion of the recombinant s-proteins was >200-fold over that at the apical front (Table 1) strongly supports our conclusion that direct delivery of single TMD apical PM proteins does not occur in polarized hepatocytes.

Does Hepatic Apical PM Protein Sorting Differ from Other Epithelial Cells in Location or Mechanism?

The correct targeting of resident PM proteins is achieved by a variety of mechanisms in different epithelia cell types. Apical proteins are sorted from basolateral ones, based on specific targeting signals encoded in their amino acid sequence and on the cellular machinery of signal recognition and transport operating in a particular cell type.

Identification of apical PM protein recognition mechanisms has been difficult, because, with one exception, identification of the signals has been elusive. A GPI anchor is the clearest apical sorting signal, because its transfer onto a randomly secreted protein is sufficient to redirect the protein into the apical pathway of most cells (Lisanti *et al.*, 1989). Aside from this class, the signals on integral apical PM proteins seem to be located either in the ectodomain or in the

TMD. The recognition mechanisms have been postulated to be lectins that bind to *N*-glycan signals, cholesterol-rich lipid "rafts," and/or a membrane proteolipid with which TMD signals interact. However, none of these mechanisms appears to be utilized universally, because exceptions to each have been reported (see Aroeti *et al.*, 1998, for references).

A tetra-spanning TMD protein of ~17 kDa, first identified in *m*yelin *a*nd *l*ymphocytes, hence the name MAL, has been implicated in the apical sorting of HA by Puertollano *et al.* (1999). These investigators used antisense to deplete MDCK cells of endogenous MAL and found that HA's transport was less efficient and less polarized. Ectopic expression of human MAL rescued the defect. MAL is expressed in many epithelial cells, where it is concentrated in the TGN region (Puertollano and Alonso, 1999). Thus, it is intriguing that liver does not express MAL, a finding consistent with the absence of a direct apical delivery mechanism for the single TMD class of apical PM proteins in hepatocytes.

Membrane domains, termed rafts, that are enriched in glycolipids and cholesterol have been proposed as "platforms" upon which apical PM protein sorting occurs in MDCK cells (Harder and Simons, 1997). The rafts are defined operationally as detergent-insoluble, lipid-containing complexes that float in sucrose density gradients. Some newly synthesized single TMD and GPI-anchored PM proteins appear in these complexes as they move through the Golgi (Brown and London, 1998). This behavior correlates with the efficiency of their subsequent delivery to the apical PM. Lowering cholesterol levels through use of metabolic inhibitors and/or reagents that extract cholesterol acutely from living cells, disrupts the rafts (i.e., decreases the detergent insolubility of the proteins) and also reduces the specificity of apical delivery of some proteins (Keller and Simons, 1998, but see Hannan and Edidin, 1996). The existence of rafts in hepatocyte PM, Golgi, or endosomes is an open question.

How Are PM Proteins Sorted in Polarized Hepatic Cells?

A current view of PM protein sorting is that nonpolarized cells use the same TGN machinery that sorts apical from basolaterally destined PM proteins in polarized cells (Musch *et al.*, 1996; Yoshimori *et al.*, 1996). Thus, all cells are "polarized cells waiting to happen" (Mellman, *et al.*, 1983). That is, external cues (ie, other cells with appropriate adhesion molecules, the extracellular matrix) drive reorganization of the cell's cytoskeleton and elaboration of junctional elements, establishing an apical-basal axis, which then reveals the *pre-existing* polarized traffic. However, this view ignores the fact that polarized hepatocytes deliver at least two classes of their apical PM proteins indirectly through the basolateral surface. Furthermore, so few apical and basolateral PM proteins have been examined together in any detail (e.g., HA and vesicular stomatitis virus glycoprotein), that we question whether TGN-based segregation is a fundamental feature of all cells and all apical versus basolateral PM proteins.

What Is the Nature of Post-TGN Carriers in Hepatic Cells?

Do newly synthesized apical and basolateral PM proteins travel to the basolateral surface together and/or with soluble proteins? The most obvious hepatocyte secretory cargo destined for the basolateral surface are very low density lipoproteins and albumin; in vivo, they are easily detected in large, distinctive vesicles ~200–400 nm in diameter. Few newly synthesized endogenous PM proteins (other than the pIgA-R) have been seen inside hepatocytes in situ, because most are relatively long-lived, their rates of synthesis are consequently very low, and they are transported continuously to the basolateral surface without storage. Although some evidence suggests that PM and secretory proteins are sorted into separate classes of basolaterally destined vesicles, presumably at the TGN (Saucan and Palade, 1994), other evidence suggests the opposite (Salamero *et al.*, 1990). Furthermore, two secretory molecules (albumin and ^{35}S-heparan sulfate proteoglycans) were found in different vesicle carriers isolated from primary hepatocytes (Nickel *et al.*, 1994). Unfortunately, integral PM proteins were not examined in this study. In summary, it appears that at least two, and perhaps three, carriers with distinct basolateral cargoes exit the hepatocyte TGN. The successful development and utilization of reagents and techniques reported in this study should allow us to resolve this and other important questions about membrane trafficking in hepatic cells.

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