Hepatocytes traffic and export hepatitis B virus basolaterally by polarity-dependent mechanisms.

Purnima Bhat¹²³*, Michelle J. Snooks¹ & David A. Anderson¹³⁴

Key words: cell polarity, trans-Golgi network, endosome, liver, hepadnavirus, virus export.
Abbreviations

DHBV, duck hepatitis B virus; PDH, primary duck hepatocytes; SAC, sub-apical compartment; TGN, trans-Golgi network.
Abstract

Viruses commonly utilize the cellular trafficking machinery of polarized cells to effect viral export. Hepatocytes are polarized in vivo, but most in vitro hepatocyte models are either non-polarized, or have morphology unsuitable for the study of viral export. Here, we investigate the mechanisms of trafficking and export for the hepadnaviruses, hepatitis B virus (HBV) and duck hepatitis B virus (DHBV), in polarized hepatocyte-derived cell lines and primary duck hepatocytes. DHBV export, but not replication, was dependent on the development of hepatocyte polarity, with export significantly abrogated over time as primary hepatocytes lost polarity. Using Transwell cultures of polarized N6 cells and Adenovirus-based transduction, we observed that export of both HBV and DHBV was vectorially regulated and predominantly basolateral. Monitoring of polarized N6 cells and non-polarized C11 cells during persistent, long-term DHBV infection demonstrated that newly synthesized sphingolipid and virus displayed significant co-localization and FRET, implying co-transportation from the Golgi to the plasma membrane. Notably, 15% of virus was released apically from polarized cells, corresponding to secretion into the bile duct in vivo, also in association with sphingolipids. We conclude that DHBV, and probably HBV, is reliant upon hepatocyte polarity to be efficiently exported, and this export is in association with sphingolipid structures, possibly lipid rafts. This study provides novel insights regarding the mechanisms of hepadnavirus trafficking in hepatocytes, with potential relevance to pathogenesis and immune tolerance.
Introduction

Hepatocytes are polarized epithelial cells with distinct apical and basolateral domains facing the bile canaliculi and the hepatic sinusoid respectively. Tight junctions between cells form a barrier between blood and bile. Direct apical transport of synthesized proteins from the trans-Golgi network (TGN) plays a minor role in hepatocytes, and similarly in hepatocyte-derived HepG2 cells (16, 39). Instead, proteins destined for the apical membrane are usually transported to the basolateral membrane, and sorted in basolateral early endosomes, before transport to the apical surface (21, 41). Sphingolipid sorting in epithelial cells results in both apical and basolateral transport by differentially regulated mechanisms (42).

The role of epithelial cell polarity in viral entry and export has been extensively investigated for a broad number of pathogens, using cell culture models of columnar epithelia. These studies point to cell-specific, virus-specific transportation mechanisms that have a significant relationship with clinical pathophysiology. Epstein Barr virus, for example, is exported non-vectorially in mucosal epithelial cells (30), correlating with its local and systemic infection, while Rotavirus infects intestinal cells apically and is released via the same domain into the gastrointestinal lumen, largely limiting its serious clinical effects to the gastrointestinal tract(9). Recently, we showed that hepatitis A virus (HAV) was exported from the basolateral domain of polarized hepatocytes (26), contrary to the expected biliary export of the virus, and in contrast to what was seen previously in similar studies using enterocyte-derived cells (3), demonstrating the importance of using relevant hepatocyte-derived cell lines to study interactions for hepatotropic viruses.
Persistent hepatitis B virus (HBV) infection causes liver cirrhosis and hepatocellular carcinoma. Hepadnaviruses, including both HBV and duck hepatitis B virus (DHBV), share many aspects of their replication and infectivity behaviour: they reproduce exclusively in hepatocytes, are assembled in the Golgi and exported to re-enter the bloodstream. It is therefore probable that the virus is exported predominantly via the basolateral domain for release into the viral sinusoid, as markers of viral replication in hepatocytes correlate with viraemia. Localization of DHBV at the basolateral surface of duck hepatocytes also suggests that the virus is exported from this plasma membrane.(6)

It has also been suggested that HBV budding is reliant on the host ESCRT machinery, which regulates trafficking of cargo into vesicular bodies for transportation through the cell(12), although this was in a non-hepatocyte, non-human cell line, and in a cell transfection, not infection, system. The mechanism by which hepatocytes transport hepadnaviruses and whether they vectorially sort viruses before export is unknown.

HBV establishes persistent infection in hepatocytes, driven from a nuclear pool of covalently closed circular DNA. Cultured cell lines are generally refractory to HBV infection, but replication can be established in hepatocyte-derived lines via transient or stable transfection or transduction with vectors. High viral replications levels have been achieved with recent models using novel dual-transfection systems(14). These models have revealed many details of hepadnavirus replication, assembly and release, but most hepatocyte-derived cell lines are non-polarized. Previously, we have developed a HepG2-derived cell line (N6) that retains characteristic features of polarized hepatocytes but
displays the morphology of simple columnar epithelia, allowing conventional studies of virus trafficking and release (26). Here we have used N6 cells transfected or transduced with HBV or DHBV to reveal the first details of hepadnavirus trafficking and export in relevant polarized cells. Current hepatocyte culture models cannot be infected with hepatitis virus. We have therefore used DHBV as a representative hepadnavirus in many of the experiments described here, allowing us to examine the export of infectious virions from physiologically relevant, polarized hepatocyte-derived cell lines, by subsequent titration of infectious virus on primary duck hepatocytes in culture.

Materials and Methods

Materials Brefeldin A and human albumin were from Sigma-Aldrich, USA: goat anti-rabbit Alexa 488, goat anti-mouse Alexa 586, Alexa 568 holo-transferrin, BODIPY-TR-ceramide complexed to bovine serum albumin, and TOTO-3 were from Molecular Probes, USA. PCR was performed using iQ SYBR Green Supermix (Bio-Rad, USA), in a Bio-Rad iQ lightcycler, and with primers supplied by Geneworks (Australia).

Cell lines HepG2 subclones N6 and C11 (26) were maintained in MEM with 1% penicillin and streptomycin, 1% glutamine and 10% FBS. Cells were seeded onto (0.4 μm pore) 25 μm Transwell collagen-coated inserts (Costar, USA) and used 14 days later. N6 and C11 cells were also used to establish the DHBV-infected cell lines DVP and DVNP, respectively using a strategy of transient transfection and short-term drug treatment to eliminate non-transfected cells without necessarily selecting for stable transfection. A linear 1.3 genome length of DHBV was amplified from pUC119-DHBV (kind gift, Ulla Protzer) using primers flanking the full 1.3 length viral genome and ligated into pCI-Neo.
(New England Biolabs) before transformation in *E. coli*. The resultant plasmid, pCI-Neo.DHBV was transfected into N6 and C11 cells using Lipofectamine (Invitrogen), and cells treated with 800 µg/ml G418 for 10 days. Microcolonies of cells were trypsinized using cloning cylinders, and seeded into 96-well plates in G418-containing medium for 48 h, representing about five population doubling times with drug selection. Cells were subsequently maintained and passaged every 14 days without G418 selection, and clones DVP and DVNP were selected that maintained the morphology of N6 or C11 cells and exhibited persistent production of DHBV.

**Vectors and transduction** Defective adenovirus vectors allow the efficient transduction of HepG2 cells with transient production of infectious DHBV or HBV(27). Adeno-(GFP)-DHBV and Adeno-(GFP)-HBV were kindly provided by Ulla Protzer (Germany) and Joe Torresi (Australia), respectively. Transwell membrane- cultured N6 and C11 cells were transduced with 10^6 infectious particles of Adeno-GFP-HBV or Adeno-GFP-DHBV via the basolateral domain. Transwells were washed, and cells incubated at 37ºC until GFP expression was first detectable by immunofluorescence microscopy.

**Primary duck hepatocytes** Animal experimentation was approved by the AMREP Animal Ethics Committee. DHBV-positive primary duck hepatocytes (PDH) were obtained by infecting day-old Pekin-Aylesbury ducklings with DHBV (strain D16). Seven days later, duckling livers were collagenase-perfused as previously described (31). PDH were maintained in Williams E media with 10 mM Tris pH 7.6, 6 mM HEPES, 0.02% sodium bicarbonate, 1% penicillin, 1% streptomycin, 1% glutamine, 0.02% glucose, 10 µM hydrocortisone 21-hemisuccinate, 0.01% insulin and 1.5% DMSO. Supernatants from Adeno-(GFP)-DHBV transduced, or persistently infected cell lines...
were applied to DHBV-negative PDH in serial dilution (1) to determine infectious virus titer. Cells were checked for GFP expression after 3 days to confirm the absence of carry-over Adeno-(GFP)-DHBV particles. Coverslips were fixed after 7 days. DHBV-positive foci were quantified by indirect immunofluorescence.

**Quantitative PCR** Quantitative PCR reactions were performed using primers DHBV 03 (ACTAGAAAACCTCGTGGACT) and DHBV 04 (GGGAGAGGGAGCCCGCACG), or HBV PC1 (GGGAGGAGATTAGTTAA) and HBV PC2 (GGCAAAAACGAGAGTAACTC)(15, 37).

**Southern blotting** Briefly, cells were lysed by freeze-thaw 3 times in PBS. Nuclei were pelleted out. Samples were treated DNase-1 treated to remove non-encapsidated chromosomal DNA. Proteinase K was used to degrade capsid proteins and release viral DNA. Samples were sequentially phenol and chloroform extracted and viral DNA was ethanol precipitated overnight. Samples were RNase treated and electrophoresed on a 1% agarose gel. The gel was washed in 0.25 M HCl, then sequentially washed in buffers (1.5M CaCl2, 0.5M NaOH followed by 1.5M NaCl, 0.25M NaOH). The DNA was transferred to positively-charged nylon membrane by capillary transfer overnight in SSC. The membrane was baked at 120ºC for 20 min and hybridized with a full-length 32P dCTP labelled DNA probe (Perkin Elmer, Australia) using a random priming kit (Amersham). Hybridized blots were exposed to Hyperfilm-ER (Amersham).

**Western blotting** Intracellular proteins Intracellular protein analysis was performed for the presence of L and S protein, which reside in the endoplasmic reticular membranes of the cell. Cell lysate was fractionated to isolate the membrane fraction. Samples were electrophoresed through a 13 % SDS-page gel in a Mini Protean II apparatus (Bio-Rad).
Proteins were transferred onto Hybond-C nitrocellulose membrane (Amersham) using a Trans Blot semi-dry transfer cell (Bio-Rad). Membranes were blocked in 3% skim milk powder in PBS followed by probing using mouse anti-DHBV pre-S primary antibody (1H1) in 1% skim milk powder in PBST for 60 min. Alexa 680 anti-mouse secondary antibody was added and membranes visualized on an Odyssey fluorescence plate reader (Li-Cor Biosciences).

**Indirect immunofluorescence** Fixed cells were treated with 1H1 antibody, and rabbit anti-ZO-1 antibody, and counterstained with Alexa 568 goat anti-mouse and Alexa 488 goat anti-rabbit secondary antibodies; nuclei were stained with TOTO-3.

**Transferrin studies** DHBV-infected monolayers of PDH, DVP and DVNP cells were pulsed with 0.05 mg/ml Alexa 568-labeled transferrin at 4ºC for 15 min, then warmed to 37ºC for 2 hours. Cells were then washed and cooled rapidly to 18ºC for 60 min to hold all endosomal and Golgi-related traffic (32). Brefeldin A 1 μg/ml, or vehicle (equivalent units ethanol) as "no drug" control was added, and cells were further incubated at 18ºC for 15 min. Coverslips were collected at time 0, and the cells warmed to 37ºC. Coverslips were collected and fixed in 4% paraformaldehyde with 1% triton-X 100 for immunofluorescence.

**Sphingomyelin studies** PDH (DHBV infected) were cultured on coverslips, incubated with 5 μM BODIPY TR C5-ceramide for 30 min at 4ºC, and rinsed in ice-cold medium before incubation at 18ºC for a further 30 min to hold all newly manufactured sphingolipids at the Golgi. 5% BSA was added to the cells to capture any basolaterally appearing sphingolipid. Brefeldin A 1 μg/ml, or vehicle, was added, and cells were further incubated at 18ºC for 15 min. Cells were then warmed rapidly to 37ºC to release
Golgi traffic, and coverslips collected at 0, and 30 min, fixed with paraformaldehyde and viral antigen visualized with 1H1 and Alexa 488 goat anti-mouse antibody.

**Microscopy** We used a Zeiss LSM 510 META microscope, with a 60x oil-immersion objective lens. FRET images between Alexa 488 and Alexa 568 were obtained by excitation at 488 nm with an HFT 488 dichroic mirror, and an LP 560 emission filter. In this pair, 488-stimulated molecules (donor) will emit sufficient light to excite a 568 (acceptor) molecule, which will in turn emit light in the 568+ spectrum. To ensure we minimized any bleed-through effect, we analyzed images in the 610 nm emission spectrum. Additionally, single-stained control images were taken to confirm minimal bleed-through. Raw images were analyzed with ImageJ. Images were processed with Adobe Photoshop.

**Statistical analysis** Mean values with SEM are given. Significance of differences was determined by un-paired two-tailed Students t-test. We accepted values of p < 0.05 as significant.

**Results**

**Virus export is both dependent on cell polarity and vectorial.** We have observed that a significant proportion of primary duck hepatocytes (PDH) will re-establish polarity for a variable time in culture following preparation by collagenase perfusion. We examined the distribution of virus, and ZO-1 as a marker of cell polarity, in clusters of congenitally DHBV-infected PDH in culture. Figure 1A shows a primary hepatocyte couplet in culture demonstrating the formation of the apical surface ( bile ductule) and tight junction contact points between cells. Figure 1B shows a hepatocyte cell cluster with evidence of early
development of polarity in the centre, demonstrated by extensive tight junction formation (ZO-1), while peripheral cells have not established polarity due to lack of cell contacts at the edge. Congenital DHBV infection leads to ubiquitous infection throughout the liver but the non-polarized peripheral cells show considerably more staining for virus-specific antigen than the central, polarized cells. By contrast, Figure 1C, a cluster of infected PDH without evidence of cell polarity shows DHBV staining in all cells, suggesting position in the cluster and intercellular contacts per se do not inhibit viral replication. From these observations we postulated that polarized hepatocytes may export virus efficiently, while non-polarized cells retain virus.

We investigated the kinetic relationship between cell polarity and export of infectious virus from DHBV-infected PDH over 30 days in culture (Fig. 2). Intracellular virus was present at high levels on days 1-6, but exported virus was not detected until day 9 (Fig. 2A). From days 12-18 the level of virus export increased dramatically, accompanied by a decline in intracellular virus. This period coincides with development of polarity in the PDH culture demonstrated by extensive tight junction formation (Fig. 2B). At 18 days the total amount of infectious virus recovered from the culture was somewhat reduced, but the large majority of this was still exported from the cells, which remained polarized as demonstrated by ongoing vectorial export of albumin. From 24 days polarity was progressively lost, with a coincident decline in viral export and a proportional increase in intracellular virus, with high yields of infectious virus almost exclusively retained within the cells at 30 days. This data strongly suggested that in primary infected hepatocytes, establishment and maintenance of cell polarity was associated with efficient export of
PDH do not establish continuous cell monolayers in Transwell inserts. In contrast, N6 cells can be successfully grown in this manner, and the vectorial export of albumin at their basolateral domain provides a functional measurement of cell polarity (26). N6 cells were transduced with Adeno-GFP.DHBV at 6 days after seeding, and supernatant and cell lysates examined for infectious virus over 30 days post transduction (Fig. 2C). By day 12 (6 days post-transduction), cells were highly polarized and also showed high levels of viral export, with minimal accumulation of intracellular virus. The degree of cell polarity and virus export declined thereafter, with progeny virus almost exclusively retained within the cells by day 30, supporting the strong relationship between cell polarity and the efficiency of DHBV export as seen in PDH.

We then examined vectorial release of virus from polarized and non-polarized cells transduced with Adeno-GFP.DHBV, or with Adeno-GFP.HBV. Polarized N6 cells exported more than 75% of progeny HBV (measured by real-time PCR) and infectious DHBV into the basolateral domain, whereas equal proportions were exported from non-polarized cells to the apical and basolateral compartments of the Transwell apparatus (Fig. 3A).

**Hepatocyte export pathways.** We disrupted intracellular hepatocyte transport routes to map viral export pathways in hepatocytes. Infected primary hepatocytes were treated with Brefeldin A, and replicate six-hour collections of supernatant were collected before and
after drug treatment and analyzed by real-time PCR. Brefeldin A treatment of infected PDH resulted in a marked (∼2.5-fold) reduction of total virus export, suggesting that active Golgi and endosomal viral transport is required for efficient hepadnaviral release from primary liver cells (Fig. 3B).

We noted above (Fig. 3A) that while the majority of virus export from polarized HepG2 cells was basolateral, a small proportion of the virus (about 12%) was released at the apical domain. Interestingly, when we applied Brefeldin A to polarized HepG2 N6 Transwell cultures that had been transduced with Adeno-HBV (m.o.i. 1), there was a significant increase in the proportion of virus exported to the basolateral domain, from 78% to over 90% (p = 0.023) (Fig. 3C). As Brefeldin A disrupts the Golgi complex and inhibits TGN-related sorting(5, 35), these data suggest that while most substrates are preferentially transported by Golgi-dependent and Golgi-independent means to the basolateral surface in hepatocytes (Fig. 3C), active sorting via a Brefeldin A-sensitive pathway directs some virus to the apical surface.

**Development of persistently infected cells.** In order to more closely model viral trafficking within polarized hepatocytes persistently infected with hepadnaviruses, we developed DVP and DVNP cells that are, respectively, polarized N6 and non-polarized C11 cells persistently infected with DHBV, without the presence of an Adenoviral vector which interferes with cell integrity over the course of longer experiments. In these DVP and DVNP cells, DHBV production was established by transient transfection and short-term G418 treatment to eliminate non-transfected cells, but without long-term drug
treatment to select stably transfected cells. Both cell lines secreted high titers of DHBV, peaking at over $5 \times 10^8$ copies/ml/day from DVP and over $2 \times 10^8$ copies/ml/day from DVNP detected using real-time PCR, with 10-fold lower titres of infectious virus detected by infection of PDH cells. These lines retained the polarity of the parent cells, with DVP cells showing apical ZO-1 staining in a honeycomb pattern (Fig. 4A) and vectorial albumin export to the basolateral domain, and virus export basolaterally (Fig. 4B), while the DVNP cells showed no organized ZO-1 staining and exported albumin equally to both apical and basolateral Transwell compartments. DVP cell lysate contained double-stranded, and single-stranded DHBV DNA (Fig. 4C). Western blot of lysates shows production of viral L and S proteins (Fig. 4D), confirming intracellular viral replication of DHBV is occurring through viral replicative intermediates and via viral protein production.

**Virus traffic is independent of transcytosis.** Hepatocytes manufacture vast quantities of substrates which are export from the cell, predominantly into the sinusoid via the basolateral domain. Synthesized protein is transported directly to the basolateral domain, with any apically-directed molecules then subsequently transcytosing to the apical membrane (23). Like other epithelial cells, they also transcytose extracellular proteins. Unique among epithelial cells, hepatocytes both transcytose transferrin from the extracellular environment, and synthesize it de novo. The bulk of the transcytosed transferrin returns to the basolateral plasma membrane, but a small, slow pathway has been shown to take some of it to the apical surface in HepG2 cells(38). The well-defined transferrin transcytosis pathway intersects with its biosynthetic pathway at the TGN (4,
PDH and DVP (polarized), and DVNP (non-polarized), cells were pulsed with Alexa 314
568-labeled transferrin and examined by confocal microscopy. At 37°C, colocalization of
transferrin and DHBV was significantly higher in non-polarized cells than polarized cells
(Fig. 5). Inhibition of transport from the Golgi with brefeldin A resulted in perinuclear
Golgi localization of both virus and transferrin (Fig. 5B, D, F). These data imply that
transferrin and DHBV share only a part of the transportation pathways in the hepatocyte,
and that this differential transportation is polarity dependent.

**Hepatocytes export DHBV with sphingomyelin.** Sphingomyelin and glucosylceramide
are synthesized from ceramide in the Golgi. Sphingomyelin is transported to the
basolateral cell surface, while glucosylceramide is transported first to the apical surface
before transcytosis (22). These sphingolipids are associated with rafts and trafficking in
polarized cells including hepatocytes (8, 21, 25). As we had observed that a proportion of
DHBV was directly released at the apical domain (Fig. 3A, 3C), the association of
sphingolipids with viral transport was investigated.

In the Golgi of HepG2 cells, labeled ceramide forms labeled sphingomyelin and
glucosylceramide, which are vectorially transported to the plasma membrane(21, 32-34,
42). Infected PDH were pulsed with labeled ceramide and stained for virus as described,
with BSA added to media to capture exported sphingolipids and prevent recycling. There
was marked co-localization of sphingolipid and viral envelope antigen in primary
hepatocytes (Fig. 6A). Virus and sphingolipid remained colocalized at the Golgi in the
presence of brefeldin A, suggesting co-transportation and shared mechanisms of sorting
We further investigated the colocalization of sphingolipid and hepadnavirus by analyzing FRET between these molecules in primary hepatocytes. Figure 6B shows high-signal FRET between labeled sphingolipid and virus in primary hepatocytes. DVP and DVNP cells pulsed with BODIPY-ceramide, showed synthesized sphingolipid and DHBV colocalized, and demonstrated FRET signal regardless of cell polarity. Photobleaching of acceptor molecules showed marked donor fluorescence recovery, confirming FRET interaction between DHBV and sphingolipid (Fig. 7). These data strongly suggest that progeny hepadnaviruses and endogenous sphingolipid are co-trafficked in hepatocytes, resulting in predominant basolateral release but with significant levels of direct apical release.

Discussion

Hepatocyte polarity is intrinsic to the synthetic and metabolic functions of the liver. The formation of plasma membrane polarity is influenced by the asymmetric distribution of post-Golgi transport proteins, implicating a fundamental relationship between Golgi transport and membrane polarity formation (10, 36). Studies of HAV in the N6 cell line revealed unexpected basolateral export of this enterically transmitted virus (26), underlying the importance of using polarized hepatocyte-derived cells to study trafficking of hepatotropic viruses. Conversely, HBsAg has been used as a protein to model cell-specific mechanisms of vectorial sorting (18), but the apical sorting of HBsAg observed in MDCK (canine kidney) cells is unlikely to be relevant to HBV replication in the liver.
since massive quantities of HBsAg are found in the plasma, reflecting predominant basolateral sorting in hepatocytes, as we have observed here, and previously with HAV (26).

Hepatitis B virus, along with other hepadnaviruses, is non-cytopathic and strictly hepatotropic. The small viral genome requires that it utilizes cellular machinery for many of its functions, a factor that contributes to its exclusive hepatotropism. It is known that viral envelope proteins are assembled and modified in the ER and associate with the genome-containing core on passage through the ER-Golgi complex before export(13). Additionally, HBV export pathways involving regulated endosomal structures, and even some independent of the endosomal machinery have been described, albeit in non-polarized hepatocyte models(2, 12). The trafficking and export of hepatitis C virus and its core protein have been shown to depend on endosomal structures in HuH7 hepatocyte-derived cells(11). The export mechanisms for hepadnaviruses in physiologically relevant, polarized hepatocyte-derived cells have not been previously addressed. Here we have used predominantly DHBV to model HBV egress so we could utilize a primary cell culture model where possible, especially to examine viral trafficking and infectivity.

We observed that cell polarity was necessary for the efficient export of DHBV from primary hepatocytes, but not required for viral replication, as evidenced by intracellular accumulation of infectious particles over time with diminishing cell polarity (Fig. 2). This was also seen in persistently infected human hepatocyte-derived cells, which maintained a steady rate of virus excretion between 18 and 30 days in culture but demonstrated more than 10,000-fold increase in intracellular virus during that time. Since the export pathways of Adenovirus-transduced HBV and DHBV were the same (Fig. 3), it is likely
that HBV export is also vectorial. Such dependence on cell polarity for virus export has
significant implications for pathogenesis. Loss of cell polarity is characteristic of
cholestatic liver disease, and recent studies have also demonstrated that hepatitis C virus
(HCV) can directly down-modulate hepatocyte polarity(19). It is therefore likely that
cholestasis and/or HCV co-infection could lead to increased intracellular retention of
progeny HBV and viral antigen, and thus increased pathological consequences.

The observed predominance of basolateral viral export is not surprising, consistent with
the default pathway for export of most substrates from hepatocytes and allowing direct
export of infectious virus into the bloodstream. Indeed, we have previously shown that
hepatitis A virus is almost exclusively exported from hepatocytes via the basolateral
domain(26), even though its enteric transmission requires that HAV eventually be
released into the bile and feces. Surprisingly, we observed apical release for a significant
proportion of progeny DHBV from polarized N6 or DVP cells, which would be
consistent with excretion of virus and antigen in bile. If also true of HBV, then it is
interesting to note that antigenicity of HBsAg has been shown to be preserved in the
gastrointestinal tract (29). Although it appears unlikely that the enveloped DHBV (or
HBV) infectious virus would survive exposure to bile salts, excretion of large amounts of
viral antigen into bile and the enterohepatic circulation could have a significant role in
promoting the development of immunological tolerance to HBV.

The transferrin trafficking pathway in hepatocytes is distinct from its pathway in other
cells. In HepG2 cells, it has been elegantly shown to follow entry and bulk-flow traffic to
the subapical compartment for sorting, and recycling to the basolateral membrane(4, 40). In this study, although virus and transferrin co-localized in non-polarized cells, DHBV did not appear to traffic with transferrin when the transportation of the two substrates was vectorially regulated, implying a differential transportation system, albeit still dependent on cell polarity.

Sphingolipid trafficking in polarized hepatocytes is distinct from protein trafficking and is differentially regulated(7, 40), but is also polarity dependent (7, 33). We saw a close physical association between sphingolipid and virus in hepatocytes, strongly implying a co-transportation mechanism. The nature of this virus-sphingolipid interaction remains conjecture. Enveloped viruses, being coated with lipoproteins, lend themselves to transport by sphingolipid rafts, as has been suggested for measles virus, and HIV (17, 22, 24, 38). Additionally, the behavior of DHBV export from non-polarized cells is consistent with the behavior of raft-dependent sorting described as similar in both polarized and non-polarized hepatocytes (21).

We note that the antibody we used to identify DHBV will not distinguish between subviral particles, which have identical envelope protein composition (containing both S and L proteins). As such we cannot exclude the possibility that our colocalization studies reflect the fate of the excess of subviral particles, rather than the minor proportion of intact virions, however we believe that it is unlikely that these particles have different pathways. While it has been suggested that subviral particles in non-hepatocyte (MDCK) cells traffic by different routes to HBV particles (18), this would appear to be a function
of the cells rather than the antigen because it is contrary to the mass export of both HBsAg and HBV virions into plasma from hepatocytes. In hepatocytes, synthesized DHBV and subviral particles have been shown to enter the same type of vesicular bodies post endoplasmic reticulum (20), which reinforces the need to use hepatocyte-derived cells for the current studies.

It has been presumed that viruses hitch a ride on pre-existing cellular mechanisms for transport within the cell and export from the cell, particularly non-cytopathic viruses such as DHBV and HBV. The determinants of this interaction, however, had not been studied. It is possible that similar sphingolipid-associated mechanisms may be utilized by other hepatotropic viruses, including hepatitis C virus. The findings of this study broaden our understanding of the mechanisms of pathogen export from hepatocytes.

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Figures

Figure 1: DHBV export is dependent on cell polarity in primary hepatocytes. Primary duck hepatocytes were prepared from a duckling congenitally infected with DHBV, and cultured for 6 days. A. A polarized couplet of hepatocytes in culture form apical surfaces with tight junctions, resembling a bile ductule (arrow). DHBV(green), nuclei (red). B & C. Colonies may be polarized (B) showing differential intracellular viral staining (red) between central polarized cells (ZO-1, green), and peripheral non-polarized cells, suggesting preferential viral export from polarized cells. In contrast, non-polarized colonies (C) show no ZO-1 staining and marked intracellular viral accumulation.

Figure 2: Kinetics of infectious viral export. A. Congenitally infected PDH were cultured for 30 days. Viral export (squares) significantly increased after day 12, as intracellular virus levels (circles) fell. After day 27, virus was retained intracellularly, as export fell. B. Viral secretion correlates with formation of tight junctions (ZO-1) and their subsequent loss (day 30). C. In Adeno-DHBV transduced polarized N6 cells, declining functional polarity as determined by vectorial albumin transport (square) was associated with a significant reduction in viral export (cross) and corresponding increase in intracellular virus accumulation (triangle).

Figure 3: Viral traffic in hepatocytes. A. N6 (black) cells grown on membranes and transduced with Adeno-GFP.HBV or Adeno-GFP.DHBV demonstrate vectorial export of both albumin and virus predominantly to the basolateral domain, while C11 cells (grey)
show non-vectorial secretion. Non-polarized cells secreted albumin and virus non-vectorially. B. Viral traffic is dependent on the TGN and endosomal system. Treatment (black) with Brefeldin A to disrupt the vesicular transport system results in marked reduction in total virus export from PDH (pre-treatment, grey) *p<0.05. C. Apical transportation of virus from the Golgi. Brefeldin A treatment resulted in an increase in viral export to the basolateral domain, implying a preferential inhibition of apical transportation with brefeldin A.

Figure 4: Cell lines persistently infected with DHBV A.(i.) DVP and DVNP cells express DHBV surface antigen (red) but ZO-1 (green) distribution indicates only DVP cells are polarized. Bar, 5 μm. (ii.)When grown on membranes, DVP cells export a much greater percentage (almost 100%) of the total virus (black bars) produced basolaterally, compared to the non-polarized DVNP cells, which parallels polarized albumin secretion (grey bars). B. These cell lines export virus efficiently (i.), with low intracellular levels (ii.) until 24 days of culture. C. (i.) Southern blot of lysates show the cell lines produce all intracellular DHBV viral replicative DNA intermediates: relaxed circular (RC), double stranded (DS) and single stranded (SS). (ii.) Western blot shows both L and S antigen in lysates.

Figure 5: Virus traffics with transferrin in polarized hepatocytes. A. DHBV infected polarized cells PDH (A, B), DVP (C, D), and non-polarized DVNP (E, F) cells were exposed to exogenous transferrin (red). There is minimal co-localisation of transferrin and virus throughout the cytoplasm in both hepatocytes and DVP cells (A,C), compared
to non-polarized cells (E). Brefeldin A treatment resulted in Golgi localization of
transferrin and virus, regardless of polarity (B,D,E). Bar, 5 μm. B. Colocalisation was
quantified over each field in treated and untreated cells. Averages of at least nine fields
over 3 experiments are given.

Figure 6: DHBV co-localizes with sphingolipid. A. DHBV-infected PDH were pulsed
with BODIPY-TR-ceramide, fixed and stained. DHBV (green) co-localized with all
species of sphingolipid (red) which persists with brefeldin treatment. Sphingolipid
accumulation in bile ducts (small arrows) and in lipid vacuoles (large arrows) can be
seen. B. Infected polarized cells PDH, DVP, and non-polarized DVNP cells show
ubiquitous virus-lipid co-localization. FRET between virus and newly synthesized
sphingolipid is marked in both polarized and non-polarized cells. The pixel intensity
along a line through the colocalized regions (orange) is shown in the graphs on the right.
Nuclei, TOTO-3, blue; Bar, 5 μm.

Figure 7. FRET between sphingolipids and virus A. DHBV-infected PDH were pulsed
with BODIPY-TR-ceramide, fixed and stained. The indicated square ROI was
photobleached over several cycles using the 543 nm laser line. Acceptor excitation-
emission and donor excitation-emission images were then taken of the whole field. Donor
fluorescence change in the ROI was corrected for background photobleaching. B. The
acceptor fluorophore (red) was bleached, resulting in a quantifiable increase in the donor
intensity (green) of over 15% above background in the ROI. C. Progressive bleaching of
the acceptor molecule over 5 cycles resulted in background bleaching (maroon), but in
the R.O.I. the donor fluorophore intensity (blue) increased, resulting in a significant
corrected fluorophore increase (green). Relative fluorescence intensity from initial image
with each bleach cycle is shown. Data shown is representative of at least 3 experiments
with five regions bleached in each experiment. Bar, 5 μm.
REFERENCES


