Hepatitis B virus (HBV) budding from infected cells is a tightly regulated process that requires both core and envelope structures. Here we report that HBV uses cellular γ2-adaptin and Nedd4, possibly in conjunction with ubiquitin, to coordinate its assembly and release. In search of interaction partners of the viral L envelope protein, we previously discovered γ2-adaptin, a putative endosomal sorting and trafficking adaptor of the adaptor protein complex family. We now demonstrate that the viral core interacts with the same γ2-adaptor, and that disruption of the HBV/γ2-adaptin interactions inhibits virus production. Mutational analyses revealed a hitherto unknown ubiquitin-binding activity of γ2-adaptin, specified by an ubiquitin-interacting motif, which contributes to its interaction with core. For core, the lysine residue at position 96, a potential target for ubiquitination, was identified to be essential for both γ2-adaptin-recognition and virus production. The participation of the cellular ubiquitin system in HBV assembly was further suggested by our finding that core interacts with the endosomal ubiquitin ligase Nedd4, partly via its late domain-like PPAY sequence. Overexpression of a catalytically inactive Nedd4 mutant diminished HBV egress, indicating that protein ubiquitination is functionally involved in virus production. Additional evidence for a link of HBV assembly to the endosomal machinery was provided by immunolabeling studies that demonstrated colocalization of core and L with γ2-adaptin in compartments positive for the late endosomal marker CD63. Together, these data indicate that an enveloped DNA virus exploits a new ubiquitin receptor together with endosomal pathway functions for egress from hepatocytes.

The production of enveloped virus particles requires the spatially and temporally coordinated interactions of the components that make up an infectious virion. For many viruses, the assembling particle then buds through a cellular membrane, away from the cytoplasm, to acquire its own membrane. Intense research has begun to uncover that enveloped RNA viruses like retro-, rhabdo- and filoviruses use components of endocytic trafficking, ubiquitination and vacuolar protein sorting pathways to coordinate their assembly and release (1-3). In most cases, the cellular functions coopted by these viruses are activated by so called late domains of their Gag and matrix proteins (4). To date, three prototype late domains have been identified that act to recruit the cellular machinery necessary for viral budding and membrane fission. The PTAP late motif interacts with Tsg101, an ubiquitin-binding protein that is an essential component of the ESCRT-I complex (endosomal sorting complex required for transport) responsible for multivesicular body (MVB) formation (5-7). The PPXY motif can recruit ubiquitin E3 ligases of the Nedd4 family that normally help to sort ubiquitinated cargo proteins into internal vesicles of the MVB (8-11), while the YPDL
late domain appears to serve as docking site for early and late endocytic proteins (12,13). By means of such interactions, late domain-containing RNA viruses gain access to the inward MVB budding machinery of the host.

By contrast, assembly and budding mechanisms in families of enveloped DNA viruses, like the hepadnavirus family, are still poorly understood. The hepatitis B virus (HBV) is the prototype member of this virus family, comprising small enveloped DNA viruses that replicate via reverse transcription and supposedly bud at intracellular membranes (14,15). Similar to retroviruses, hepadnaviruses assembly begins with the formation of icosahedral nucleocapsids that package the viral pregenomic RNA together with the viral polymerase. Inside the capsids, formed by 120 dimers of the single 21-kDa core protein, the partially double-stranded 3.2-kb DNA genome is synthesized through reverse transcription of the pregenomic RNA (16). Mature nucleocapsids, formed in the cytoplasm, can then be enclosed by the viral envelope composed of cellular lipids and three viral glycoproteins, the small S, middle M and large L envelope protein. The envelope proteins originate at the endoplasmic reticulum (ER) membrane and share the transmembrane region of S. The M and L proteins differ from S by their N-terminal extensions, termed preS, which contribute to their functional differences. Virus assembly strictly depends on the L protein, whereas the S protein is required but not sufficient and the M protein is dispensable (15).

In contrast to retroviruses, HBV budding and egress is dependent on both envelope and core structural proteins. Genetic approaches led to the identification of two short envelope regions within L and S that are essential for virus formation (17,18), possibly by providing scaffolding function for the interaction with the nucleocapsid. For core, mutational analyses have mapped eleven single amino acids that cluster at the capsid surface and are required for nucleocapsid envelopment (19). It has been unclear, however, whether a direct envelope-core interaction initiates and drives the assembly process. Similarly, the cellular membrane compartments used during HBV budding remain largely undefined. As solitary expression of the S protein leads to the assembly of empty subviral particles that bud at post-ER/pre-medial-Golgi membranes and leave the cell via the constitutive secretory pathway, it is hypothesized that viral particles may take the same export route (14).

Despite the previously reported investigation of the role of chaperones (20,21), little is known about the general network of host proteins needed for HBV morphogenesis. One candidate molecule involved in viral trafficking and budding may be γ2-adaptin that we discovered as an L-specific interaction partner in a yeast two-hybrid screen using the assembly-promoting region of L as bait (22). Adaptins are subunits of adaptor protein complexes involved in the sorting of cargo proteins to specific membrane compartments within the cell. Four heterotetrameric adaptor protein (AP) complexes have been described in mammalian cells, designated AP-1 through AP-4. They concentrate the appropriate cargo into the nascent vesicle, generate membrane curvature and allow vesicles to pinch off the donor membrane towards the cytoplasm (23). γ2-Adaptin has similarities to γ1-adaptin, one large subunit of the trans-Golgi network (TGN)/endosome adaptor AP-1, in both primary sequence and domain structure consisting of a N-terminal head and a C-terminal ear domain that are linked by a hinge region (24,25). Despite their relatedness, γ2-adaptin appears to serve a separate, yet unknown function distinct from that of AP-1 (26). Consistent with this, we previously demonstrated that L recruits γ2-adaptin, but not γ1-adaptin in vivo (22).

In this study, we investigated the potential role of γ2-adaptin played during virus production in HBV-replicating liver cells. By analyzing its mode of action in virus morphogenesis, we were expecting to elucidate physiological function(s) of γ2-adaptin. In parallel, we searched for intracellular compartments and additional host factors participating in HBV maturation with special emphasis on the viral core particle that contains a surface-exposed sequence matching the known PPXY late domain.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs** - For replication of HBV in permissive liver cell lines, plasmid pHBV was used which carries an 1.1-mer of the HBV DNA genome under the transcriptional control of the human metallothionein IIa (hMTIIa) promoter (27). The expression vector pNi2.S contains the HBV S envelope gene with a C-terminally tagged influenza virus hemagglutinin (HA) epitope driven by the hMTIIa promoter (28).
For expression of the HBV core gene in mammalian cells (pC), the S-encoding region was excised from pNI2.S and replaced by the core gene. Plasmid pHBV.C is identical to pHBV with the exception that a stop codon was created at triplet 38 of the core gene to ablate core expression from the HBV replicon. For trans-complementation, pHBV.C was coexpressed with vectors encoding either the wild-type (wt) or mutant core genes. All mutations were introduced with the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene) and verified by sequencing. The expression vector for γ2-adaptin containing the human γ2-adaptin cDNA derived from hepatoma HepG2 cells with a N-terminal HA-tag (25) was a gift from K. Nakayama, Tsukuba University, Japan. The deletion mutant γ2HAΔEar lacking the C-terminal ear domain and parts of the hinge domain (amino acids 528 to 785) has been described previously (22). An oppositional mutant (γ2HAΔHead) devoid of the N-terminal head domain (amino acids 2 to 522) was constructed by cloning, and γ2-adaptin mutants with lesions in the ubiquitin-interacting motif were generated by site-directed mutagenesis. For expression in E. coli, plasmid pGEX-γ2.14-533 was constructed which carries the amino acid sequence 14 to 533 of γ2-adaptin fused in-frame to the C-terminus of glutathione S-transferase (GST) encoded by plasmid pGEX-3X (Amersham Biosciences). The human hemopexin gene was PCR-amplified and cloned from a liver cDNA library into the vector pCMV-HA (BD Biosciences; cloning details will be available on request). The plasmid pGADNOT carrying the human Nedd4.1 gene (8) was kindly provided by F. Bouamr (Howard Hughes Medical Institute, New York, USA). For ectopic expression in N-terminally, FLAG-tagged form, the Nedd4 gene was subcloned into the expression vector γ3×FLAG (a gift from E. Gottwein, University of Heidelberg, Germany).

Antibodies - For immunoprecipitation of enveloped HBV particles, a mixture of L- and S-specific rabbit antibodies was used as described previously (18). To generate core-specific antibodies, a C-terminally truncated core gene encoded by ptacVR4 (a gift from H. Meisel, Charité, Berlin, Germany) was expressed in E. coli, and capsids were purified by sucrose gradient centrifugation, according to a protocol of Maassen et al. (29). Polyclonal antisera against recombinant core particles (αK45) were raised in rabbits and proved to be suitable for immunoprecipitation of capsids. In parallel, rabbits were immunized with core particles that were denatured by boiling in 1% SDS/100 mM DTT. This antiserum (αK46) recognized core in Western blotting analyses. For detection of γ2-adaptin, a rabbit antiserum (αK8330) generated against its C-terminal 19 amino acids was used (22). GST-specific antibodies were raised in rabbits by immunization of animals with recombinant purified GST. Mouse or rat monoclonal antibodies against the HA epitope were purchased from BabCO or Roche Applied Science, respectively. An anti-FLAG antibody was obtained from Sigma, a mouse antibody recognizing human heat shock protein Hsc70 was obtained from StressGen Biotechnologies and a mouse antibody against human CD63 was purchased from Santa Cruz. Peroxidase-labeled, secondary antibodies were obtained from Dianova and used in dilutions recommended by the supplier. The fluorophor-labeled antibodies AlexaFluor 488-conjugated goat anti-mouse immunoglobulin G, AlexaFluor 546-conjugated goat anti-rat immunoglobulin G and AlexaFluor 647-conjugated goat anti-rabbit immunoglobulin G were purchased from Molecular Probes.

Cell Culture, Transfection and RNA Interference - The human hepatoma cell line HuH-7 was used throughout. Transfections with plasmid DNAs were performed with Lipofectamine Plus (Invitrogen) as recommended by the manufacturer. The amounts of plasmids used in cotransfection experiments are indicated in the figure legends. Unless otherwise indicated, the cells were analyzed 72 h after transfection. For (co)transfection of cells with small interfering RNA (siRNA) or siRNA plus plasmid DNA, the TransMessenger™ transfection reagent from Qiagen was used. Briefly, 5 x 10^5 cells per well of a 6-well plate were transfected with 250 ng siRNA either alone or in combination with 500 ng RNase-free plasmid DNA according to the protocol of the supplier. For silencing in HBV-replicating HuH-7 cells, the cells were first transfected with siRNAs and pHBV for 2 d, and the transfection with siRNAs was repeated for another 2 d. Four siRNAs directed against γ2-adaptin were designed by and obtained from Qiagen (4-for-Silencing kit) with the following sequences: #121 corresponds to the γ2-adaptin target at nucleotide positions 359 to 379 (AAGGCCTGGCCTTGTGCACCTT), #287 is directed against positions 832 to 852 (AACACGACACCCACGCAG), #689 corresponds to positions 2064 to 2084.
(AAAACCCCTGGTTGCTGTTA), and #690 targets nucleotide positions 2065 to 2085 (AAAACCTGTTGCTGTTAAT). A non-sense control siRNA (Qiagen) with no known homology to mammalian genes was used for control transfection experiments.

Cell Lysis and Immunoprecipitation Procedures - To probe for protein expression and release into the extracellular medium, transfected cells were lysed in Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.5% Nonidet P-40 for 20 min on ice. Lysates were centrifuged for 5 min at 15,000g and 4°C and subjected to SDS-PAGE and Western blotting using standard procedures. Where indicated, the culture supernatants of transfected cells were analyzed in addition.

For γ2-adaptin-specific coimmunoprecipitation studies, cells were lysed with a 2% solution of the non-denaturating detergent CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}-HBS (50 mM Heps-KCl, pH 7.5, 200 mM NaCl), supplemented with 1 x protease inhibitor mixture (Serva) for 20 min on ice. After centrifugation, lysates were precleared with a 10% suspension of protein A- sepharose (Amersham Biosciences) for 1 h at 4°C with rocking. The lysates were then subjected to immunoprecipitation using 50 µl of a 10% protein A- sepharose slurry that had been precoated with 10 µl of the core particle-specific αK45 antiserum. After incubation for 4 h at 4°C, immune complexes were washed three times with 0.5% CHAPS/HBS and once with 125 mM Tris-HCl, pH 6.8, prior to SDS-PAGE and immunoblotting. Total protein extracts were included on the gels as controls.

To probe for Nedd4/core-complex formation, cells were broken by dounce homogenization (30 strokes) in hypotonic buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, plus protease inhibitors). Extracts were adjusted to 50 mM NaCl and subjected to centrifugation at 1,000g for 10 min at 4°C to sediment nuclei, followed by centrifugation at 10,000g for 10 min at 4°C. The soluble fraction was then precleared with protein A- sepharose and subjected to a core-specific immunoprecipitation exactly as described above.

Detection of Intracellular HBV Nucleocapsids and Extracellular Virions - For replication of HBV in HuH-7 cells, plasmid pHBV was used. Three to four days after transfection, intracellular nucleocapsids and extracellular virions were isolated by capsid- or envelope-specific immunoprecipitations, respectively, prior to detection of the encapsidated viral progeny DNA by radioactive labeling of the partially double-stranded genome with [α-32P]dCTP (Amersham Biosciences) by the endogeneous polymerase as described previously (18). After extraction of the labeled DNA genomes from the immunoprecipitated samples, they were resolved by agarose gel electrophoresis and visualized by PhosphorImaging (Molecular Dynamics). Images were quantitated with ImageQuant software (Molecular Dynamics). Alternatively, the viral progeny DNA was detected on dot blots. To this aim, an EcoRI-linearized unit-length HBV genome was labeled with digoxigenin-dUTP by random priming as instructed by the manufacturer (Roche). After extraction of the DNA genomes from the immunoprecipitated samples as described above, they were denatured by boiling in 0.4 M NaOH, 10 mM EDTA and filtered with a dot-blot manifold (Schleicher & Schüll) onto nylon membranes. The membrane was baked at 120°C for 30 min and hybridized with the labeled probe according to the Roche DIG DNA labeling and detection kit.

Pull-down Assays – For ubiquitin pull-down assays, HuH-7 cells were lysed in assay buffer (25 mM Heps-KCl, pH 7.4, 75 mM NaCl, 0.25% Triton X-100, 2.5 mM Mg acetate supplemented with protease inhibitor cocktail) three days posttransfection. Cleared lysates were immediately incubated with 50 µl aliquots of protein A-agarose (Sigma) or ubiquitin-agarose (Sigma) that had been prewashed 3 x with assay buffer. Samples were reacted for 4 h at 4°C with rocking in the presence of 0.1% BSA. Precipitates were then washed 3 x with 25 mM Heps-KCl, pH 7.4, 75 mM NaCl, 0.25% Triton X-100, once with 125 mM Tris-HCl, pH 6.8, and subjected to HA-specific immunoblotting.

For GST pull-down experiments, wildtype GST and the GST fusion of γ2-adaptin.14-533 (GST-γ2.14-533) were expressed in transformed E. coli DH5α cells. Protein expression was induced with 1 mM IPTG for 4 h at 30°C, and cells were lysed by sonification in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.2 % Triton X-100, 1 mg/ml lysozyme supplemented...
with protease inhibitor cocktail. The GST proteins were purified on glutathione-sepharose 4B beads (Amersham Biosciences), according to the manufacturer's instructions. Beads loaded with 20 µg recombinant proteins were equilibrated in assay buffer (25 mM Hepes-KCl, pH 7.4, 75 mM NaCl, 0.25% Triton X-100, 2.5 mM Mg acetate) and incubated with 20 µg recombinant core particles that were produced in E. coli as described above (see Antibodies). After rotation at 4°C for 4 h, the beads were washed 3 x with 25 mM Hepes-KCl, pH 7.4, 75 mM NaCl, 0.25% Triton X-100, once with 125 mM Tris-HCl, pH 6.8, and subjected to core-specific immunoblotting.

Immunofluorescence - For immunofluorescence microscopy, cells grown on cover-slips were fixed and permeabilized with ice-cold methanol containing 2 mM EGTA for 15 min at –20°C. After washing and blocking for 30 min in PBS containing 2% goat serum, cells were incubated with primary antibodies (anti-L or anti-core particle rabbit antiserum, anti-HA rat antibody, anti-CD63 mouse antibody) for 1 h at 37°C, rinsed with PBS and then incubated with AlexaFluor-tagged secondary antibodies for 1 h at 37°C. Following washing and mounting of the cells, images were captured on a Zeiss deconvolution microscopy system operated by Axiovision software (Zeiss) using a 100 x (NA 1.25) oil immersion objective. The system included an AxioCamHR digital camera mounted on an Axiovert 200M fluorescence microscope. Seven optical sections along the Z axis were acquired with increments of 0.35 µm. The fluorescent data sets were processed by 3D deconvolution with the InverseFilter method to generate optical sections of the data sets. Adobe Photoshop CS software was used for additional processing of the images.

RESULTS

Depletion of γ2-Adaptin Inhibits HBV Viral Particle Release - Previous experiments in our laboratory demonstrated that the HBV L envelope protein specifically interacts with γ2-adaptin by complex formation depending on the ear domain of γ2-adaptin and a preS-specific subdomain of L, known to be essential for capsid envelopment (22). To probe whether γ2-adaptin actually plays a role in virus replication, we sought to deplete it from HBV-producing cells. By testing four different small interfering RNAs for their capacity to reduce the expression of γ2-adaptin, two molecules directed against very C-terminal sequences of γ2-adaptin (#689, #690) approved to be effective while the two other siRNAs with targets in the N-terminal protein domain (#121, #287) did not deplete γ2-adaptin (Supplemental Fig. 1). Because the #690 molecule turned out to be the most effective siRNA, it was chosen for the silencing analyses. Human hepatoma HuH-7 cells were transiently cotransfected with an expression vector carrying a cloned, replication-competent HBV genome (pHBV, HBV replicon) together with the siRNA #690 or a non-sense control siRNA (control). Of note, transfected liver cell lines support the production of infectious virions, but are refractory to the uptake of progeny HBV particles (30). Western blot analysis of the cells showed that the 90-kDa γ2-adaptin protein had been down-regulated more than 70% in siRNA #690-transfected cells as compared to mock-treated cells (Fig. 1A). Under these conditions, neither the intracellular level of the viral L envelope protein nor that of the core protein was affected (Fig. 1A). This indicated that the synthesis, stability and/or half-lives of the viral envelope and capsid substructures were independent of γ2-adaptin function. We next assayed the depleted cells for the production and release of virions using immunoprecipitation of cellular supernatants with envelope-specific antisera, radioactive labeling of the partially double-stranded HBV genome by the viral endogenous polymerase and detection of the genome by gel electrophoresis. In parallel, intracellular nucleocapsid assembly was investigated using core particle-specific antibodies for immunoprecipitation of cellular lysates prior to the endogenous polymerase reaction (EPR). As shown in Fig. 1A, nucleocapsid assembly within the cells was virtually unaffected, irrespective of whether γ2-adaptin was knocked down or not. By contrast, the degree of virion release into the medium of silenced cells was significantly reduced. Triplicate analyses and quantification determinations revealed a close correlation between the level of γ2-adaptin inhibition (74 ± 3.4%) and the suppression of HBV export (49 ± 5.2%) in such that an enhanced depletion of γ2-adaptin corresponded to a progressive depletion of virus release. The analysis of the two non-silencing siRNAs #121 and #287 and the other silencing molecule #689 supported this notion, as it established an excellent correlation between depletion of γ2-adaptin and HBV production (Supplemental Fig. 1). These results indicate that γ2-adaptin mediates one or more productive
steps in the late stages of HBV assembly and/or egress.

Depletion of γ2-Adaptin Does Not Inhibit Protein Secretion - The route of HBV egress is unclear as yet, but has been suggested to involve the constitutive pathway of secretion (14). Because the physiological function of γ2-adaptin is likewise unknown, the observed block to HBV release could simply be due to a general distortion of the cell secretory apparatus imposed by the absence of γ2-adaptin. To address this point, we assessed the secretion competence of γ2-adaptin-depleted HuH-7 cells. As a reporter, we used hemopexin (Hxp), a soluble secretory protein, and coexpressed it in HA-tagged form together with the silencing or non-sense siRNAs. As shown by specific immunoblotting, the knockdown again reduced γ2-adaptin by about 70%, but had virtually no effects on the secretion of hemopexin (Fig. 1B). Similar results were obtained when the fate of the HBV small S envelope protein was investigated in such depleted cells. The S protein is known to self-assemble into empty envelope particles that mature by budding into intralumenal cisternae of post-ER/pre-medial-Golgi compartments and to exit the cell by the constitutive secretory pathway (14). On cotransfection of the HA-tagged S protein with the inhibitory siRNA, the level of S secretion was identical to that obtained with control-treated cells (Fig. 1B). Together, these results show that γ2-adaptin did not play a role in the constitutive pathway of secretion. Moreover, these data also implicate that the assembly and budding of HBV empty envelope S particles did not depend on the action of γ2-adaptin. Hence, the mechanisms of HBV subviral and viral particle production appear to differ in their requirement of cell function(s).

γ2-Adaptin Interacts with HBV Core Particles - Due to their modular domain organization, large adaptin molecules of AP complexes are known to interact with several protein partners thereby assembling adaptor-specific protein networks (23,31). In analogy, we reasoned that γ2-adaptin could act to link the viral envelope and nucleocapsid at the HBV assembly site. In order to detect an interaction between the HBV core and γ2-adaptin in living cells, we performed coimmunoprecipitation experiments. HuH-7 cells were cotransfected with expression vectors encoding the core gene either alone (Core) or in the context of the HBV replicon (HBV) together with a HA-tagged version of γ2-adaptin. Three days after transfection, lysates were prepared with a mild detergent and analyzed for protein expression by core- and HA-specific Western blotting. γ2-Adaptin appeared in the expected position of 90 kDa, and the core protein was obtained as a 21-kDa species (Fig. 2A). Precleared lysates were next subjected to immunoprecipitation with antisera specific for core particles (αK45), and the immune complexes were examined by HA-specific immunoblotting. As shown in Fig. 2A, γ2-adaptin was indeed efficiently coprecipitated with core. Complex formation between core and γ2-adaptin was observed when core alone was expressed and in the context of productive virus formation. Because γ2-adaptin interacted with core even in the absence of the other viral gene products, the interaction approved to be direct rather than being indirectly mediated by the L envelope protein. Similarly, our previous results (22) had demonstrated a physical L/γ2-adaptin-interaction that does not require the presence of core. Together, these findings indicate that the HBV core particle and the envelope interact on the same adaptor protein.

To corroborate this finding we analyzed whether core also interacts with the endogeneously expressed γ2-adaptin as is the case for L (22). Lysates of cells transfected with the core gene were immunoprecipitated with core-specific rabbit antiserum, and the precipitates were immunostained with a rabbit antiserum against γ2-adaptin (22). Although the overall background labeling was higher in this assay, likely due to the utilization of antisera from the same species, complex formation between core and the endogeneously synthesized γ2-adaptin could be detected (Fig. 2B).

Given the shared γ2-adaptin-interacting properties of core and L, we next asked whether they use a common interaction surface of γ2-adaptin. By using a C-terminally truncated mutant of γ2-adaptin (γ2HAAEar), we previously mapped its ear domain as the binding site for L (22) and now analyzed whether the same may be the case for core. In addition, a reciprocal mutant depleted for the N-terminal head domain (γ2HAAΔHead) was constructed and assessed for an interaction with core using the cotransfection and coimmunoprecipitation assay as described in Fig. 2A. Although both mutants were stably synthesized, the γ2HAAHead mutant clearly failed to interact with core, whereas core binding of the γ2HAAΔEar mutant was unaffected (Fig.
The combined results indicate a bipartite interaction mechanism in such that the head domain of γ2-adaptin is necessary for the association with core, whereas its ear domain mediates binding to L.

**γ2-Adaptin is an Ubiquitin-binding Protein and Interacts with HBV Core Particles in an Ubiquitin-dependent Manner** - To further define the role of γ2-adaptin in HBV maturation, we tried to identify the structural determinants of the interaction between γ2-adaptin and core in more detail. Inspecting the primary sequence of γ2-adaptin, we noted the existence of a putative ubiquitin-interacting motif (UIM) located in its head domain at amino acid positions 369 to 377. The UIM motif was originally identified by database searches for sequences similar to a 20-amino acid motif in the S5a (Rpn10) subunit of the proteasome that interacts with ubiquitin (32). It comprises a highly conserved core sequence ΦXXAXXXSXXe, in which Φ denotes a large hydrophobic residue, X is any amino acid, and e denotes an acidic residue, which is, however, the less conserved residue within UIMs. As γ2-adaptin contains such a motif except for the terminating acidic residue (Fig. 3A), we analyzed whether it could interact with ubiquitin. By performing pull-down analyses, we indeed observed binding of HA-tagged γ2-adaptin from a HuH-7 lysate to ubiquitin-agarose, but not protein A-agarose (Fig. 3B). To further characterize the interaction between γ2-adaptin and ubiquitin, we generated γ2-adaptin mutants with either point mutations in the conserved U I M or alanine and serine residues (γ2.m1, γ2.m2) or a precise deletion of the UIM (γ2.m3) (Fig. 3A). All three mutants were expressed at the same level as wt γ2-adaptin in transfected cells, but their ability to bind to ubiquitin-agarose was severely diminished. While the γ2.m1 and γ2.m2 mutants carrying inactivating mutations still displayed a weak interaction with ubiquitin, the γ2.m3 mutant devoid of the UIM was completely blocked in ubiquitin binding (Fig. 3C). To our knowledge, this is the first demonstration that a putative member of the “classic” adaptin protein family exhibits an ubiquitin-interacting ability.

As there is evidence that ubiquitin participates in the budding processes of several enveloped RNA viruses (1-4), we next asked whether the UIM of γ2-adaptin contributes to the interaction with the HBV core particle. Therefore, HuH-7 cells were cotransfected with core and the UIM-deficient γ2.m3 mutant, and lysates were probed by core-specific immunoprecipitation followed by HA-specific immunoblotting for γ2-adaptin as described above. In fact, the γ2.m3 mutant demonstrated a strong inhibition of core binding compared to that of the wt protein (Fig. 3D). We took the UIM-dependent interaction between core and γ2-adaptin as a first hint that ubiquitin recognition by γ2-adaptin may be important for HBV assembly. Given the failure of the γ2.m3 mutant to productively associate with core, we considered that its overexpression might act in a *trans*-dominant negative manner thereby interfering with HBV release. However, we observed that heavily overexpression of even the wild-type γ2-adaptin alters cell physiology and, surprisingly, leads to a specific posttranslational down-regulation of the HBV gene products (data not shown).

**HBV Core Lysine Residue 96 is Essential for Virus Release and Interaction with γ2-Adaptin** - The known sites of ubiquitination are free amino groups on lysine side chains of a target protein to which ubiquitin becomes covalently attached. The HBV core protein contains two lysine residues at amino acid positions 7 and 96 that are conserved among all HBV genotypes (Fig. 4A). To investigate the impact of these residues on virus formation, they were individually substituted by alanines. Because an introduction of these mutations into the HBV replicon would simultaneously affect the overlapping polymerase gene, the mutations were created in the pC expression plasmid. These plasmids were then used to trans-complement a core-negative HBV replicon (pHBV.C) that is blocked in core expression due to a stop codon introduced in the N-terminus of the core gene. Upon cotransfection of HuH-7 cells, the C.K7A and C.K96A mutants were stably expressed as shown by core-specific immunoblotting (Fig. 4B). In addition, both mutants formed intracellular nucleocapsids as efficiently as wt core, thus indicating that the mutations neither affected capsid assembly nor packaging of the viral genome. However, when cellular supernatants were assayed for the production and release of virions, the C.K96A mutant was completely blocked, whereas the C.K7A mutant was not (Fig. 4B).

As the C.K96A-mutation induced a specific loss of nucleocapsid envelopment, and hence virion egress, we next asked whether this defect might be correlated to non-recognition by
γ2-adaptin. Indeed, by performing the cotransfection and coprecipitation assay as outlined above, we found that the C.K96A mutant failed to interact with γ2-adaptin (Fig. 4C). In contrast, the lysine residue at position 7 that is dispensable for virus production proved to be equally dispensable for interacting with γ2-adaptin (Fig. 4C). Together, the strict correlation between virus production and core/γ2-adaptin complex formation and vice versa confirmed the pivotal role of γ2-adaptin in HBV assembly. Furthermore, these results indicate that both processes critically depend on lysine-96 of core.

In combining our data, the simplest causal link could be that lysine-96 of core might be ubiquitinated, and that such modified core particles might be recognized by γ2-adaptin in an UIM-dependent manner. We made several attempts to detect ubiquitinated core products, but all results were uniformly negative. By using different ubiquitin-reactive antibodies, ectopic expression of an epitope-tagged ubiquitin and/or cell lysis conditions with strong denaturants in the presence of N-ethylmaleimide to prevent postlysis removal of ubiquitin from core by deubiquitinating enzymes, modified core molecules could not be observed.

To obtain further, albeit indirect evidence for a role of ubiquitin in core/γ2-adaptin-complex formation, we performed in vitro binding experiments with recombinant core and γ2-adaptin produced in bacteria. Because prokaryotes lack the ubiquitin modification system, we were expecting that in the absence of γ2-adaptin (Fig. 4C), together, the strict correlation between virus production and core/γ2-adaptin complex formation and vice versa confirmed the pivotal role of γ2-adaptin in HBV assembly. Furthermore, these results indicate that both processes critically depend on lysine-96 of core.

Nedd4 Interacts with HBV Core Particles - Recent evidence suggests that ubiquitin-conjugating activities induced by viral late domains are part of retroviral budding strategies (4,9,10). Intriguingly, the HBV core contains a PPXY-like motif (Fig. 4A) that is exposed on the capsid surface according to a cryoelectron microscopy map (33). Based on findings for budding RNA viruses, this motif could be a binding site for Nedd4 proteins, a family of ubiquitin-ligating enzymes with N-terminal lipid–binding domains, central proline-recognition WW domains and C-terminal E3 ubiquitin ligase domains (HECT) (34). To test this hypothesis, the human Nedd4.1 gene was expressed in N-terminally FLAG-tagged form in HBV-replicating cells. Probing cell extracts with anti-FLAG and anti-core antibodies demonstrated efficient ectopic expression of the 120 kDa-Nedd4 protein as well as stable synthesis of core (Fig. 5). Importantly, when immune complexes were isolated with antibodies against core particles and examined by FLAG-specific Western blotting, we could easily identify coprecipitated Nedd4. As only a faint background band was visible in samples prepared from mock-transfected cells, the association proved to be specific. To analyze whether the late domain-like sequence of core is involved in binding to Nedd4, two mutants were generated in which the first proline (C.P129A) or the tyrosine (C.Y132A) of the PPAY motif were replaced by alanines. Western blotting of lysates of cotransfected cells showed stable synthesis of both core mutant proteins and confirmed equal levels of Nedd4 expression and (Fig. 5). The subsequent coimmunoprecipitation reproducibly revealed that the C.Y132A mutant was completely devoid of Nedd4-binding activity. Surprisingly, however, the C.P129A mutant still associated with Nedd4 (Fig. 5). Similar results have been reported by Martin-Serrano et al. (11) who analyzed the sequence requirements of the PPXY late domain of murine leukemia virus (MLV) Gag for HECT ubiquitin ligase binding and found that a mutation of the first proline residue was less detrimental as compared to that of the tyrosine.

Nedd4 is Required for HBV Release - To address whether the Nedd4/core-interaction is functionally relevant for HBV production, we turned to strategies that used dominant-negative (dn) mutants of this enzyme. For this purpose, we created a point mutant of Nedd4 carrying a cysteine-to-serine substitution in the active site of its HECT domain (Nedd4.C894S, briefly Nedd4.dn). Several studies have documented that such a mutant is unable to conjugate ubiquitin in a trans-dn manner (9-11). Moreover,
Segura-Morales et al. (35) have recently shown that the use of a Nedd4.dn mutant more potently inactivates endogenous Nedd4 as compared to siRNA-mediated depletion of the enzyme. The effects of overexpression of Nedd4 and the dn mutant on HBV maturation were tested by cotransfecting HuH-7 cells at a Nedd4/HBV DNA ratio of 4:1. Immunoblotting analyses revealed stable synthesis of wt and mutant Nedd4 (Fig. 6A). None of the two proteins had any detectable effect on the steady state level of the HBV L envelope protein or the core, thus indicating that their overexpression did not nonspecifically affect general cell function (Fig. 6A). Moreover, nucleocapsids were formed within these cells as efficiently as in control cells (Fig. 6B). In contrast, the amount of virions released into the medium was greatly reduced in cells overexpressing Nedd4.dn. Quantitation of this effect showed that in the presence of Nedd4.dn the export of HBV was inhibited by 49 ± 5.8% relative to mock-transfected cells (Fig. 6B). To account for the incomplete block to release, some redundancy known between the cellular Nedd4 isoforms could be considered. We conclude from these data that core interacts with Nedd4, partly via its PPAY motif, in a productive manner.

**HBV Core and Envelope Colocalize with γ2-Adaptin in the Endocytic Pathway** - The aforementioned data collectively suggest that γ2-adaptin and Nedd4 (co)operate at late stages of HBV assembly and release, possibly at the site of virus budding. Because both proteins have been implicated to localize and/or to act at endosomal compartments (25,34), we hypothesized that HBV might utilize these structures during maturation. To test this hypothesis, we performed indirect immunofluorescence analyses of HBV-producing HuH-7 cells that were cotransfected with HA-tagged γ2-adaptin. In the first set of triple labeling experiments, shown in Fig. 7, we focused on the L envelope protein that had been shown to leave the ER after its synthesis (28). Concordantly, L revealed a faint perinuclear ER-like staining, but was enriched in a juxtanuclear punctuate structure at steady state (blue). γ2-Adaptin was localized to paranuclear structures (red) and, consistent with our previous results (22), a significant portion colocalized with the L-specific juxtanuclear region as indicated by the purple fluorescence in the merged image. To examine the subcellular localization of the L/γ2-adaptin-complex, we costained cells with antibodies against CD63, a tetraspanin found in late endosomes and MVBs (green). Remarkably, we observed substantial colocalization of L and CD63 (turquoise), as well as of γ2-adaptin and CD63 (yellow). Accordingly, the spatial proximity of L, γ2-adaptin and CD63 resulted in a white fluorescence in the merged image. This indicates that, at a steady state, L and γ2-adaptin colocalize on a subset of CD63-positive structures.

Similar results were obtained when we costained these cells with antibodies against core particles, γ2-adaptin and CD63 (Fig. 8). In agreement with previous studies, core was distributed in both the cytoplasm and the nucleus (blue). Its nuclear localization is likely based upon the reimport of nucleocapsids into the nucleus for amplification of the viral genome (36). Consistent with our coprecipitation analyses, costaining for γ2-adaptin (red) showed an extensive colocalization with core, particularly in a perinuclear circular region (purple). As was the case for L, a fraction of the cytoplasmic core pool was also found in CD63-positive structures (yellow), thus pointing to an endosomal distribution of core particles. An overlay of the three fluorescence patterns yielded a striking degree of colocalization of core, γ2-adaptin and the MVB marker (white). We conclude from our imaging data that a significant proportion of both L/γ2-adaptin and core/γ2-adaptin complexes are present in the endosomal pathway of virus-replicating cells.

**DISCUSSION**

Our experiments demonstrate that productive HBV particle formation and egress utilize γ2-adaptin, a candidate cellular trafficking adaptor. In our previous studies we found that the viral L envelope protein interacts directly with γ2-adaptin (22). Here we show that the viral core interacts on the same adaptor. Disruption of these interactions by specific siRNA depletion of γ2-adaptin inhibits HBV release without compromising the cell secretory apparatus. As nucleocapsid assembly and envelope maturation were unimpaired in depleted cells, γ2-adaptin apparently acts late in the HBV assembly pathway, being responsible for one or more steps involving (i) the intracellular trafficking of the viral components, (ii) their linking and concentrating at the budding site in order to promote the pinching-off reaction, (iii) and/or the routing of virus exit from the cells. Our data demonstrating a dual, yet independent
interaction of γ2-adaptin with both the envelope and the core particle implicate that γ2-adaptin primarily functions to sort and tether the viral substructures. By exploiting the same adaptor protein, HBV has seemingly adopted a novel strategy that ensures the meeting and coupling of the viral subunits during assembly. Adaptor protein members have also been implicated to facilitate the production of enveloped RNA viruses, in particular retroviruses. For the human immunodeficiency virus type-1 (HIV-1), the AP-3 complex mediates the correct trafficking of the Gag protein to the budding site (37). The Gag proteins of equine infectious anemia virus and MLV utilize the endocytic AP-2 complex or AP-2-associated endophilins, respectively, during the assembly of virus-like particles (12,13,38). However, because retroviral Gags can bud in the absence of any other viral proteins, these studies did not include the viral envelopes. It would therefore be interesting to examine whether the adaptors engaged by these viruses may simultaneously target the envelope, as is the case for HBV.

A tethering role of γ2-adaptin played during HBV production would be compatible with known functions of adaptor protein (AP) complex subunits that were originally termed “assembly polypeptides”, as they can form interconnected networks to select different types of cargo proteins destined for inclusion into coated vesicles (23,31,39). Incompatibly, however, AP-made vesicles bud into the cytoplasm which is the topological reverse of HBV budding into the lumen of intracellular compartments. This raises the possibility that γ2-adaptin may not operate as a typical vesicle-forming adaptor. In support of this notion, γ2-adaptin-containing AP complexes have not been identified so far. Moreover, γ2-adaptin is unable to functionally substitute for the closely related γ1-adaptin, one large subunit of AP-1, as shown by embryonic lethality in mice upon homozygous disruption of the γ1-adaptin gene (26). Our silencing experiments indicate that the loss of γ2-adaptin cannot be rescued by γ1-adaptin and add further evidence for γ2-adaptin being a unique entity distinct from γ1-adaptin. The unique function(s) of γ2-adaptin may rely on its ubiquitin-binding ability, specified by an UIM motif, that we uncovered in this work. To our knowledge, such ability has not been found for any member of the typical adaptor protein family. Rather, there is accumulating evidence that ubiquitin-interacting activity is a common feature of monomeric adaptors, like e.g., epsin and Hrs, that control ubiquitin-dependent endocytic sorting processes to the MVB/lysosome (40,41). Interestingly, recent studies have revealed that ubiquitin recognition is also employed by the monomeric Golgi-localizing, γ-adaptin ear domain homology, ADP-ribosylation factor-binding proteins (GGAs) that sort biosynthetic cargo from the TGN to the endosomal system without being major constituents of the transport vesicle (42,43). Because γ2-adaptin is clearly a new member of ubiquitin receptors, it is tempting to speculate that γ2-adaptin may also function as a monomeric adaptor for ubiquitin-mediated sorting steps within the endosomal system.

Over the past few years, numerous works have indicated that ubiquitin is part of enveloped RNA virus budding (9,10,44-46). Although the precise mode of ubiquitin operation is yet unclear, it is currently assumed that ubiquitin, may it be conjugated to a viral structural protein or a cellular bystander protein, allows these viruses to get access to the MVB machinery in order to bud away from the cytoplasm. Here, we provide first evidence that HBV, an enveloped DNA virus that replicates via an RNA intermediate, also exploits the cellular ubiquitin system for productive infection. First, we showed that complex formation between γ2-adaptin and core involved the UIM and, presumably therefore the ubiquitin-binding function of γ2-adaptin. Second, the other key finding of this study is that core particles stably interacted with Nedd4 ubiquitin ligase in vivo. The recruitment of Nedd4 involved the late domain-like PPAY sequence of core, as a single point mutation of the tyrosine disrupted Nedd4 binding. And third, ectopic expression of a catalytically inactive dn mutant of Nedd4 proved to be a potent inhibitor of HBV egress, indicating that the active site of the ligase and, hence ubiquitination of a substrate protein functionally contribute to virus production. Under these conditions, HBV release was substantially reduced but not totally blocked. Similar observations have been made for retroviral PPXY-containing Gag proteins of the Rous sarcoma virus and the human T-cell leukemia virus type-1 whose budding was not completely abrogated on overexpression of a dn Nedd4 mutant (9,10). Possibly, functionally redundant ubiquitin ligase isoforms (34) can partially substitute for the enzymatic inactive Nedd4.
The participation of both ubiquitin ligating and binding proteins in HBV maturation suggests that core may be a target for ubiquitination by Nedd4 and, subsequently, for UIM-dependent recognition by γ2-adaptin. Indeed, we found that mutating one of the two potential ubiquitin acceptor lysine residues of core (CK96A) induced a defective assembly phenotype, similar to those observed by interference with Nedd4 and γ2-adaptin functions, along with a loss of γ2-adaptin recruitment. However, our attempts to identify ubiquitination of core have been unsuccessful so far. One feasible explanation for this observation is that only few molecules of the core particle, composed of 240 monomers, may be transiently conjugated with ubiquitin, and in such hampers detection. Consistent with this, the amount of ubiquitin linked to PPXY-containing retroviral Gags has been reported to be very low (44). Another possible interpretation of this result is that the relevant target for ubiquitination by Nedd4 is not core itself, but is rather an undefined cellular factor. One likely binding site for such a factor could be a cluster of amino acids, including the critical lysine-96, that are exposed on the core particle surface and essential for envelopment (19). This factor could then be ubiquitinated by the nearby Nedd4, allowing ubiquitin-dependent recognition by γ2-adaptin. Similar conclusions have been drawn from studies with the retroviral HIV and MLV Gag proteins where experiments with proteasome inhibitors revealed that budding of these Gags requires a functional ubiquitin-proteasome system. However, mutational analyses of their target lysine residues showed that Gag ubiquitination per se is not required, suggesting ubiquitination of factors other than the viral proteins to promote budding (44-46).

Budding of HBV is suggested to take place at intracellular membranes because its envelope proteins have never been detected at the plasma membrane, and electron microscopic studies have documented large, dilated membranous compartments containing viral particles (47). Our immunofluorescence data implicate that HBV trafficking and budding involve endosomal organelles positive for CD63, a tetraspanin found in late endosomes and MVBs. The imaging data not only confirmed L/γ2-adaptin and core/γ2-adaptin complex formation in living cells, but also showed that both complexes colocalized with CD63-positive structures. The spatial proximity of the marker and the two complexes, together with the essential role of γ2-adaptin in late stages of virus replication suggest that γ2-adaptin may organize HBV assembly platforms on endosomal membranes.

Although the precise mechanisms by which γ2-adaptin and Nedd4 govern HBV maturation remain to be elucidated, the collective data led us to propose the following sequence of events accompanying virus assembly. First, according to immunolabeling and biochemical studies (22,28), the transmembrane envelope proteins are transported out of the ER along the secretory pathway to at least the medial Golgi stacks where their N-linked glycans acquire modifications (48). Based on our previous work, L accumulates at Golgi-like structures where it recruits γ2-adaptin via its cytoplasmically oriented preS domain (22). Simultaneously, nucleocapsids mature in the cytoplasm thereby recruiting Nedd4 by their surface-accessible late domain-like motif. Ubiquitin deposition, may it be on core or a bystander protein, would next be recognized by the UIM motif of γ2-adaptin bound to transmembrane L. This HBV assembly complex may then be sorted to the MVB by means of γ2-adaptin, similar to the action of the GGA ubiquitin receptors that can sort ubiquitinated membrane cargo from the TGN to MVB (42,43). Alternatively, Nedd4, associated with the assembly complex, may provide the link to the MVB, as a recent report has demonstrated an interaction between Nedd4 and Tsg101, the entry component of the MVB ESCRT-I complex (49). In this scenario, virions would bud into the MVB and exit the cell by the exosome pathway, as is the case for HIV-1 in infected macrophages (50). However, it is equally possible that the MVB machinery is recruited by virtue of γ2-adaptin and/or Nedd4 to the HBV assembly platform where it could catalyze the pinching-off reaction of viral particles into the lumen of compartments for subsequent vesicular transport out of the cell.

In summary, our observations that γ2-adaptin and Nedd4, likely in conjunction with ubiquitin, are part of the HBV assembly pathway highlight mechanism by which viral and cellular components interact to generate HBV particles. The full functional significance of these host factors in HBV replication is a focus of ongoing investigation. This study also demonstrates that HBV maturation shares considerable similarities with budding processes of enveloped RNA viruses, thus raising the possibility that different
classes of virus might exit the cell by appropriating functions of the endosomal sorting machinery. The identification of γ2-adaptin as an ubiquitin receptor is another aspect that deserves further study.

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FOOTNOTES

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1The abbreviations used are: ESCRT-I, endosomal sorting complex required for transport-I; MVB, multivesicular body; HBV, hepatitis B virus; ER, endoplasmic reticulum; AP, adaptor protein; TGN, trans-Golgi network; hMTIIa, human metallothionein IIa promoter; HA, hemagglutinin; wt, wild-type; siRNA, small interfering RNA; EPR, endogeneous polymerase reaction; Hxp, hemopexin; UIM, ubiquitin-interacting motif; MLV, murine leukemia virus; dn, dominant-negative; HIV-1, human immunodeficiency virus type-1; GST, glutathione S-transferase; GGA, Golgi-localizing, γ-adaptin ear domain homology, ADP-ribosylation factor-binding proteins.

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FIGURE LEGENDS

Fig. 1. A, Depletion of γ2-adaptin inhibits HBV production. HuH-7 cells were cotransfected with a replication-competent HBV vector (HBV) and either anti-γ2-adaptin siRNA duplexes (#690) or control siRNA duplexes (control). A second transfection with the siRNAs was performed 2 d later, and cellular supernatants and lysates were harvested after additional 2 d. Lysates were subjected to γ2-adaptin-specific Western blotting to demonstrate depletion of γ2-adaptin (top panel left). For control of
HBV expression, the same samples were analyzed by Western blots using antibodies against L (middle panel left) or core (bottom panel left). Numbers to the left show positions of molecular size standards in kDa. HBV assembly and release were assayed by EPR of the corresponding samples. Release of enveloped virions in the culture medium was detected by immunoprecipitation and radioactive labeling of the viral genome by the viral polymerase. The migration of the HBV DNA genome, as visualized by agarose gelelectrophoresis and phosphorImaging, is indicated (lanes 5 and 6). Nonenveloped cytoplasmic nucleocapsids were immunoprecipitated from cell lysates and processed by EPR (lanes 3 and 4). B, Depletion of γ2-adaptin does not inhibit protein secretion. HuH-7 cells were cotransfected with HA-tagged versions of either the hemopexin gene (Hxp.HA) or the HBV S envelope protein (S.HA) together with inhibitory RNA (#690) or control RNA (control) exactly as in A. The top panels demonstrate depletion of γ2-adaptin for the corresponding transfection experiments by Western blots of cell lysates with anti-γ2-adaptin antiserum. The bottom panels depict secretion of hemopexin (lanes 1 and 2) and S (lanes 3 and 4) into the culture medium of the cells as assayed by HA-specific immunoblotting.

Fig. 2. γ2-Adaptin interacts with core in vivo. A, HuH-7 cells were cotransfected with the HA-tagged γ2-adaptin gene together with the core expression plasmid (Core), the HBV replicon (HBV) or a GFP expression vector (mock), serving as a negative control. The ratio of γ2-adaptin:cotransfected DNAs was 3:7 (in µg). Stable synthesis of γ2-adaptin and core was verified by SDS-PAGE of lysates and immunoblotting with an anti-HA (top) or anti-core antiserum (middle). For coimmunoprecipitation (IP), lysates were reacted with antibodies against core particles before Western blotting (WB) of the precipitates with the HA-specific antibody (bottom). B, Endogeneous γ2-adaptin interacts with core. Lysates of cells transfected with the empty vector (mock) or with the core expression plasmid (Core) were immunoprecipitated with the core-specific antiserum. Precipitates were resolved by SDS-PAGE and probed with anti-γ2-adaptin antiserum. C, N-terminal head domain of γ2-adaptin mediates the interaction with core. HA-tagged γ2-adaptin mutants carrying deletions of either the N-terminal head domain (γ2HAΔHead) or C-terminal ear domain (γ2HAΔEar) were coexpressed with core as for A. Stable synthesis of both mutants is shown by HA-specific immunoblotting (left) and the subsequent coimmunoprecipitation assay (right) was done as above. Of note, the ~58 kDa γ2HAΔEar mutant
comigrates with the bulk of unspecifically stained IgG heavy chains (IgHC). Numbers to the left of the panels show positions of molecular size standards in kDa.

**Fig. 3.** Ubiquitin binding of γ2-adaptin via its UIM is critical for interaction with core. A. γ2-Adaptin contains a putative UIM. The upper line indicates the consensus sequence of the UIM domain with Φ representing a hydrophobic residue, x is any amino acid, e is a negatively charged residue, along with the nearly invariant Ala and Ser positions. Beneath the aligned sequence of γ2-adaptin is shown (γ2.wt), and the amino acid positions mutagenized in the γ2.m1 and γ2.m2 mutants are indicated. The γ2.m3 mutant lacks the entire UIM as depicted by Δ. B. γ2-Adaptin binds ubiquitin. HA-tagged γ2-adaptin was expressed in HuH-7 cells, and lysates were incubated with ubiquitin-agarose (Ub-Ag) or naked protein A-agarose (PA-Ag). Bound proteins were separated by SDS-PAGE and detected by HA-specific immunoblotting. The amount of γ2-adaptin bound to Ub-Ag corresponds to 16 % of the input amount. C. Mutations in the UIM of γ2-adaptin inhibit binding to ubiquitin. Lysates were prepared from HuH-7 cells expressing HA-tagged wt or mutant versions of γ2-adaptin, and the expression level (input) of the constructs was assessed by Western blotting with the anti-HA antibody. The ubiquitin pull-down assay of the lysates was done as in B and is shown in the bottom panel. D. Binding of γ2-adaptin to core requires its UIM. HuH-7 cells were transfected with core in 7:3 mixtures with control DNA (mock), HA-tagged wt γ2-adaptin or the γ2.m3 mutant lacking the UIM. Cell extracts were immunoprecipitated with antiserum against core particles and analyzed by HA-specific Western blotting as in Fig. 3.

**Fig. 4.** Lysine-96 of core is essential for HBV production and γ2-adaptin-interaction. A. The amino acid sequence of core (HBV genotype D) is shown with the two lysines and the PPAY late domain-like sequence highlighted in bold. B. HuH-7 cells were cotransfected with the core-defective replicon pHBV.C plus expression vectors encoding either the wt or mutant core proteins as indicated above each lane (DNA ratios in µg were 5:5). Stable expression of the core products was assessed by core-specific immunoblotting of cell lysates (top). Nucleocapsid formation within the cell (middle) and virion release in the cell medium (bottom) were probed by EPR as in Fig. 1A. C, Coprecipitation
analysis of interactions between γ2-adaptin and the core mutants bearing the indicated lysine mutations was done as in Fig. 2. Lysates of cotransfected cells were assayed by HA-specific (top) or core-specific (middle) Western blots to confirm that equivalent amounts of the proteins were analyzed in the coimmunoprecipitation assay (bottom). D,

Fig. 5. Nedd4 interacts with core in vivo. The human Nedd4.1 gene fused with an N-terminal FLAG-tag was cotransfected in HuH-7 cells with the core-negative pHBV.C- replicon at a DNA ratio of 7:3.5 (in µg), respectively. For trans-complementation, 3.5 µg plasmid DNAs encoding GFP (mock), core (C.wt) or core variants carrying the indicated mutations in the PPAY sequence motif were used. Three days after transfection, cytosolic extracts were prepared and assessed for the expression of Nedd4 and core proteins by Western blots using antibodies against the FLAG epitope (top) or core (middle), respectively. For coimmunoprecipitation, extracts were reacted with the core particle-specific antiserum before Western blotting with the anti-FLAG antibody (bottom). Positions of Nedd4 and core as well as positions of molecular size standards in kDa are indicated on the left of the panels.

Fig. 6. Nedd4 is functionally involved in HBV production. HuH-7 cells were cotransfected with the HBV replicon plus vectors encoding either the wt or dn mutant Nedd4 proteins in FLAG-tagged form. Empty plasmid was used as a negative control (mock), and each cotransfection was done at a 3.5:7 DNA ratio, respectively. A, Stable synthesis of Nedd4 and its dn mutant is shown by FLAG-specific immunoblotting of cellular lysates (top). As control of HBV expression, the same samples were analyzed by Western blots using antibodies against L (middle) or core (bottom). B, Virus production was monitored by EPR, and signals from three EPR reactions using virions harvested from the culture medium (black bars) and nucleocapsids harvested from cell lysates (white bars) were quantitated and demonstrated in % amount relative to mock-transfected cells in the bottom graph.

Fig. 7. γ2-Adaptin colocalizes with L in CD63-containing compartments. HuH-7 cells were transfected with the HBV replicon and HA-tagged γ2-adaptin at a 7:3 (in µg) plasmid DNA ratio, respectively. Cells were fixed two days posttransfection and immunostained for L, γ2-adaptin and CD63 using rabbit anti-L, rat anti-HA and mouse anti-CD63 antibodies, respectively. The primary antibodies were
followed by a triple staining with AlexaFluor 647-conjugated goat anti-rabbit immunoglobulin G, AlexaFluor 546-conjugated goat anti-rat immunoglobulin G and AlexaFluor 488-conjugated goat anti-mouse immunoglobulin G antibodies. The fluorescent signal of L (blue) is shown in the left column, the aside column presents the localization of γ2-adaptin (red) and the staining pattern of CD63 (green) is indicated in the middle right column. The overlays of the fluorescences are shown in the right column with purple (c), turquoise (f), yellow (i) or white (m) color indicating colocalization of L, γ2-adaptin and/or CD63. Bar, 20 µm.

Fig. 8. γ2-Adaptin colocalizes with core in CD63-containing compartments. HBV-replicating cells expressing HA-tagged γ2-adaptin were analyzed by indirect immunofluorescence microscopy as described in the Fig. 7 legend. Differently, cells were stained for core with the rabbit antibody αK45 along with immunolabeling for γ2-adaptin and CD63. As indicated above each column, core staining is blue, γ2-adaptin is red and CD63 is shown in green. Colocalization of core and γ2-adaptin is shown in purple (c), of core and CD63 in turquoise (f) and of γ2-adaptin and CD63 in yellow (i). Colocalization between all three proteins is indicated by the white color in the corresponding merged image (m). Bar, 20 µm.
Figure 2
Figure 4

A

MDIDPYKSFAGATVELLSFLPSDDFFPSVESDLDTASALYREALSPEHSCHPTALKAILC
WGELMTLATWGVNLDPASRGKLVSYVTMVGLKFRQLLWHRISCLTFGRETVIEYLVSF
GVVIRTTPPAYAPPNAPILSTLPETTVRRRGRSPRRRPSRGRQSRGRQSRGRQ

B

|        | HBV.C+ |     | Cell | Cell | Medium |
|--------|--------|-----|------|------|--------|
| C.wt  |        |     |      |      |        |
| C.K7A |        |     |      |      |        |
| C.K99A|        |     |      |      |        |

C

|        | γ2HA+ |     | Input | Input | IP:αCore WB:αHA |
|--------|-------|-----|-------|-------|-----------------|
| C.wt  |       |     |       |       |                 |
| C.K7A |       |     |       |       |                 |
| C.K99A|       |     |       |       |                 |
| mock  |       |     |       |       |                 |
Figure 5
Figure 6
Figure 8

Legend:
- Core
- γ2-adaptin
- CD63
- Merge
γ2-adaptin, a novel ubiquitin-interacting adaptor, and nedd4 ubiquitin ligase control hepatitis B virus maturation

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