Alix regulates egress of hepatitis B virus naked capsid particles in an ESCRT-independent manner

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Summary

Hepatitis B virus (HBV) is an enveloped DNA virus that exploits the endosomal sorting complexes required for transport (ESCRT) pathway for budding. In addition to infectious particles, HBV-replicating cells release non-enveloped (nucleo)capsids, but their functional implication and pathways of release are unclear. Here, we focused on the molecular mechanisms and found that the sole expression of the HBV core protein is sufficient for capsid release. Unexpectedly, released capsids are devoid of a detectable membrane bilayer, implicating a non-vesicular exocytosis process. Unlike virions, naked capsid budding does not require the ESCRT machinery. Rather, we identified Alix, a multifunctional protein, as a regulator of capsid budding. Ectopic overexpression of Alix enhanced capsid egress, while its depletion inhibited capsid release. Notably, the loss of Alix did not impair HBV production, furthermore indicating that virions and capsids use diverse export routes. By mapping of Alix domains responsible for its capsid release-mediating activity, its Bro1 domain was found to be required and sufficient. Alix binds to core via its Bro1 domain and retained its activity even if its ESCRT-III binding site is disrupted. Together, the boomerang-shaped Bro1 domain of Alix appears to escort capsids without ESCRT.

Introduction

Enveloped virus budding involves the distortion of a cellular membrane away from the cytoplasm, envelopment of the viral core by a lipid bilayer containing viral envelope proteins, and subsequent membrane fission that releases the particle from the cell. Virus budding was generally thought to depend on the interaction between the viral envelope proteins and the inner viral core or matrix components. However, some viruses, such as retroviruses and rhabdoviruses, are able to release membrane-coated, virus-like particles (VLPs) even in the absence of viral envelope proteins. Other viruses, including coronaviruses and, in particular, hepadnaviruses, release empty subviral envelope particles that are devoid of nucleocapsids, in addition to infectious particles (Bruss, 2007; Welsch et al., 2007; Patient et al., 2009; Pincetic and Leis, 2009).

The prototype member of the hepadnavirus family is the hepatitis B virus (HBV), a small enveloped, DNA-containing pararetrovirus causing acute and chronic liver infections. Worldwide an estimated 350 million individuals are chronically infected and potentially develop liver cirrhosis or liver cell carcinoma. The assembly of progeny HBV virions begins with the formation of icosahedral nucleocapsids that package the viral pregenomic RNA together with the viral polymerase. Inside the capsids, composed of 180 or 240 copies of the 21 kDa core protein and with about 30 nm in diameter, the partially double-stranded 3.2 kb DNA genome is synthesized through reverse transcription of the pregenomic RNA. 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 that originate at the endoplasmic reticulum (ER) membrane (Bruss, 2007; Nassal, 2008; Patient et al., 2009). Subsequent budding of HBV is supposed to occur at intracellular membranes (Roingeard and Sureau, 1998; Rost et al., 2006). The production of infectious particles is accompanied by the formation of empty envelope particles that greatly outnumber mature virions and presumably act as decoys for the immune system. In addition, envelope protein-independent core particle budding has been incidentally reported for HBV-replicating cells (Beterams and Nassal, 2001; Perlman and Hu, 2003; Sun and Nassal, 2006; Watanabe et al., 2007; Wittkop et al., 2010). The functional implications and pathways of release of naked nucleocapsids remain enigmatic.

Like many other enveloped viruses, HBV release requires the recruitment of a network of cellular proteins normally involved in two analogous cellular membrane fission events, the budding of cargo-containing vesicles

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Lambert et al overexpression of dominant-negative (DN) mutants functional inactivation of Nedd4, ESCRT-III or Vps4 by network. This is corroborated by the findings that the helps to sort ubiquitinated cargo proteins into MVB vesicles for either degradation, lysosomal functions or exosomal release (Morita et al., 2007; Piper and Katzmann, 2007). For budding and membrane fission, enveloped viruses recruit the ESCRT machinery through different prototype late domains (PT/SAP, PPxY, YPDL, FPIV) of their core and matrix proteins (Schmitt et al., 2006; Chen and Lamb, 2008; Pincetic and Leis, 2009). In case of HBV (genotype D), the core protein contains a late domain-like PPxY sequence that mediates a productive interaction with the Nedd4 ubiquitin ligase (Rost et al., 2006). Nedd4 normally helps to sort ubiquitinated cargo proteins into MVBs and in such may link the HBV budding complex to the ESCRT network. This is corroborated by the findings that the functional inactivation of Nedd4, ESCRT-III or Vps4 by overexpression of dominant-negative (DN) mutants potently inhibits HBV budding (Kian Chua et al., 2006; Lambert et al., 2007; Watanabe et al., 2007). Whether all HBV genotypes and isolates, however, depend on the action of Nedd4 remains to be established, since the second proline residue of the PPxY motif is not strictly conserved among the core proteins (Chain and Myers, 2005).

Hepatitis B virus release can be also blocked by the overexpression of a DN mutant of Alix [apoptosis-linked gene 2 (ALG-2)-interacting protein X, also called AIP1] (Watanabe et al., 2007). Human Alix is a multifunctional adaptor and plays key roles in membrane biology, cytokinesis, cell signalling and the ESCRT-dependent release of some enveloped viruses (Strack et al., 2003; Sakaguchi et al., 2005; Odorizzi, 2006; Raiborg and Stenmark, 2009). Alix consists of three distinct regions: the N-terminal boomerang-shaped Bro1 domain, the central V domain and the C-terminal proline-rich region (PRR). The Bro1 domain tightly interacts with the ESCRT-III component CHMP4, while the PRR region serves as a docking site for a number of proteins, including the ESCRT-I subunit Tsg101, endophilins, ALG-2 and the centrosome protein 55 (Odorizzi, 2006). The V domain of Alix can bind directly to the YPXL late domain type of viral proteins thereby serving as an adaptor to recruit the ESCRT machinery to virus budding sites (Martín-Serrano et al., 2003; Strack et al., 2003). In case of human immunodeficiency virus (HIV) type 1, the Gag nucleocapsid domain can additionally associate with the Alix Bro1 domain (Dussupt et al., 2009; Popov et al., 2009). Besides its functions in virus budding, Alix along with its Bro1 region has been implicated to function in membrane remodelling processes (Kim et al., 2005; Fisher et al., 2007; Falguieres et al., 2008; Popov et al., 2009).

In this study, we investigated the role of Alix in HBV virus and virus-like particle budding. We find that the release of naked (nucleo)capsids is not a by-product of HBV replicating liver cell budding. Alix alone can be mimicked in cells expressing core alone. While Alix is dispensable for HBV production, it is essential for naked capsid egress. The Alix-assisted exocytosis of non-enveloped capsids may account for the hitherto less understood phenomenon why the HBV capsid is a potent B-cell immunogen.

**Results**

**Naked capsid release from HBV-replicating and -non-replicating cells**

Previous studies have implicated that HBV-replicating liver cell lines not only secrete virions but also non- or partially enveloped nucleocapsids (Beterams and Nassal, 2001; Perlman and Hu, 2003; Sun and Nassal, 2006; Watanabe et al., 2007; Wittkop et al., 2010). The origin of these capsids has been frequently attributed to ‘exhausted/frustrated’ or lysed cells that were programmed to replicate HBV at high levels. Here we addressed the process of naked capsid release and investigated whether it depends on an ongoing replication. Therefore, a comparative analysis of core synthesis and capsid release was performed in the human hepatoma cell line HuH-7 transfected with the following HBV constructs: (i) pHBV, a replication-competent construct in which the viral core/polymerase promoter was replaced by the human metallothionein IIa (hMTIIa) promoter, (ii) pCore, a plasmid harbouring the core open reading frame without the N-terminal precore signal sequence under the control of the hMTIIa promoter, and (iii) pPDAF.Core, a construct identical to pCore except for an N-terminal insertion of a DPAF-epitope tag, which is recognized by the monoclonal antibody MA18/7 (Heermann et al., 1984). After transient expression for 3 days, cellular lysates and supernatants were harvested and (sub)viral particles present in the media were collected by ultracentrifugation. Lysates were examined by Western blotting using an antiserum against denatured core particles (K45) that specifically recognized the 21 kDa core protein (Fig. 1A). Because this antiserum often yields unspecific background staining, core was tagged with the DPAF-epitope that led to a slightly decreased electrophoretic mobility of this construct. To probe for the release
of non-enveloped capsids into cellular supernatants, a capsid-specific dot-blot analysis was performed. Since the media of cells transfected with the replication-competent HBV construct may contain complete virions plus non-enveloped capsids, the analysis of this material under conditions of denaturing anti-core Western blotting would not be meaningful. Therefore, the supernatants were examined with a capsid-specific antibody (3HB17) under non-denaturing conditions in order to solely detect non-enveloped capsids. As shown in Fig. 1A, non-enveloped capsids could be detected in either case. The evaluation of supernatants for lactate dehydrogenase (LDH) activity resulted in no indications for cytotoxicity. To monitor the sensitivity of the LDH assay, we used a plasmid expressing the vesicular stomatitis virus G glycoprotein (VSV-G) that causes direct toxicity for a broad cell range (Licata and Harty, 2003). HuH-7 cells were transfected with control, pCore or VSV-G constructs, transfection efficiencies were determined by immunofluorescence analysis using core- and VSV-G-specific antibodies. LDH levels are shown in % amount relative to 3 × 10⁶ cells expressing the constructs.

C. Released core particles resemble authentic capsids. Concentrated culture medium of pCore-transfected cells was applied to isopycnic caesium chloride gradient centrifugation and fractions were assayed by a core-specific (HBcAg) ELISA. D and E. Released capsids do not contain a lipid envelope. (D) Concentrated culture medium of control- or core-transfected cells were mock-treated or treated with NP-40 prior to the capsid-specific dot-blot analysis. (E) Extracellular capsids were left untreated or treated with proteinase K (PK) in the absence or presence of NP-40, and samples were analysed by core-specific immunoblotting (K46).
The reactivity of the core proteins in the dot-blot assay suggested that they might resemble capsid particles. To address this point more precisely, supernatants of pCore-transfected cells were subjected to caesium chloride density centrifugation. As diagrammed in Fig. 1C, the core proteins banded at density of 1.33 g ml⁻¹, typical for native capsids (Beterams and Nassal, 2001). Next, antibody and protease accessibility studies of these capsids were performed. Because capsids reacted equally with capsid-specific antibodies both in the presence and in the absence of detergent (Fig. 1D), and since they were sensitive to digestion with proteinase K even in the absence of detergents (Fig. 1E), the capsids are devoid of an evident lipid layer. Notably, this is in striking contrast to retro- and rhabdoviral VLPs that are enwrapped by a membrane during budding from cells (Welsch et al., 2007).

**HBV naked capsid release does not require ESCRT-I, ESCRT-III and Vps4**

Previous works demonstrated that HBV budding requires key components of the ESCRT machinery, like ESCRT-III and Vps4 (Kian Chua et al., 2006; Lambert et al., 2007; Watanabe et al., 2007). To analyse whether naked capsid release may have similar requirements, we perturbed the ESCRT network in cells expressing only the core protein. For interference with ESCRT-I functions, we depleted Tsg101 and Vps28, two subunits of the heterotetrameric ESCRT-I complex. To impair ESCRT-III and Vps4, ectopic overexpression of their DN mutant forms was used. ESCRT-III subunits, like CHMP3, can be converted to a potent DN form by fusion with the yellow fluorescent protein (Babst et al., 1997). In case of Vps4, its two isoforms, Vps4A and Vps4B, can be blocked by overexpression of ATP hydrolysis-deficient mutants (Babst et al., 1997; Yoshimori et al., 2000; Bishop and Woodman, 2001; Scheurig et al., 2001; Martin-Serrano et al., 2003; Strack et al., 2003).

To inactivate ESCRT-I, cells were treated with siRNAs to reduce endogenous levels of Tsg101 or Vps28 prior to transfection with the DPAF.Core construct. As shown in Fig. 2A, both siRNAs effectively reduced the expression of their targets as compared with control siRNA-treated cells. Moreover, consistent with published data (Stuffers et al., 2009), depletion of Tsg101 decreases the level of Vps28 and vice versa, likely because the ESCRT-I complex loses its integrity upon depletion of one subunit. Under these conditions, the intracellular level of DPAF-tagged core was unaffected. However, when culture media were analysed for capsid release, the loss of Tsg101 and Vps28 substantially increased capsid export (Fig. 2A). To account for the unaltered intracellular core levels, it seems conceivable that the loss of ESCRT-I may have affected core protein expression. This would be consistent with the observation that Tsg101 has been implicated as a positive and negative regulator of gene transcription (Feng et al., 2000, and references therein).

Similar results were obtained upon functional ablation of ESCRT-III and Vps4. Here, cells were co-transfected with the PDAF.Core construct plus expression vectors for wild-type (WT) and DN forms of CHMP3 and Vps4A. In case of CHMP3, a FLAG-tagged WT version and a YFP-tagged DN construct were used, while Vps4A WT and its ATPase-defective E228Q DN mutant were tagged with green fluorescent protein (GFP). As shown by tag-specific immunoblotting of cell lysates, all constructs were efficiently synthesized (Fig. 2B and C). Neither CHMP3.wt nor Vps4A.wt affected the intra- and extracellular level of DPAF.Core, as compared with control-transfected cells. In contrast, the expression of their DN versions reduced the intracellular amounts of DPAF.Core concomitant with an increase of extracellular capsids (Fig. 2B and C). Identical results were observed with the untagged core protein (data not shown), thus excluding a possible interference of the DPAF-tag. Collectively, these data indicate that naked capsid budding does not require a functional ESCRT cascade. Rather, ablation of ESCRT activities markedly enhances this process.

Given that the budding processes of HBV virions and naked capsids differ in their requirement for ESCRT, we next analysed the intracellular distribution of core synthesized in the presence or absence of an ongoing virus production. HuH-7 cells were transfected with the HBV replicon (pHBV) or the core vector (pCore) and subjected to deconvolution immunofluorescence. For co-staining, cells were labelled with antibodies against capsids (K45) and lysobiphosphatidic acid (LBPA), a marker for late endosomes and MVBs (Kobayashi et al., 1998). In either setting, core was dispersed throughout most of the cytoplasm, although it displayed a more pronounced perinuclear distribution when expressed alone (Fig. 2D). Importantly, core did not colocalize with LBPA when synthesized on its own. In contrast, in HBV-replicating cells a substantial colocalization of core and LPBA was observed along with some dispersion of LBPA-positive structures to the cell periphery (Fig. 2D). Combining the biochemical and imaging data, we conclude that the budding process of HBV naked capsids does not involve ESCRT/MVB functions and structures.

**HBV budding does not require Alix**

Besides ESCRT-III and Vps4, the ESCRT-associated protein Alix had been implicated to assist in HBV budding. Watanabe et al. (2007) reported that an overexpression of a dsRed-tagged Alix derivative in HBV-replicating cells blocked virus formation. To re-evaluate this observation,
we first performed a similar experiment. In difference, we took use of an Alix mutant in which the C-terminal PRR domain was replaced by GFP (Alix.GFP). As references, empty plasmid DNA or a construct encoding a haemagglutinin (HA)-tagged version of WT Alix were included. After transient expression of these constructs with the HBV replicon, cell lysates and supernatants were assayed by immunocapture with anti-capsid (K45) or anti-envelope antibodies, respectively, followed by particle disruption and real-time PCR measurement of the number of HBV genomes. Consistent with the earlier study (Watanabe et al., 2007), the exogenous expression of Alix.GFP substantially suppressed the production of intracellular nucleocapsids and extracellular virions, while WT

Fig. 2. HBV naked capsid release does not require ESCRT functions.

A. ESCRT-I-independent capsid budding. HuH-7 cells were treated with siRNAs targeting Tsg101, Vps28 or control siRNA. Two days later, cells were transfected with the DPAF.Core construct. After an additional 2 days, cells were lysed with SDS buffer and extracellular capsids were recovered by ultracentrifugation. Lysates were subjected to Tsg101- and Vps28-specific Western blotting to demonstrate depletion. For core detection, lysates and supernatants were analysed by anti-DPAF immunoblotting. To confirm equal loading of cell lysates, we took advantage that the anti-DPAF antibody specifically cross-reacts with an endogenous protein of HuH-7 cells of unknown nature (Load).

B and C. ESCRT-III- and Vps4-independent capsid budding. DPAF.Core was transfected into HuH-7 cells with either empty plasmid DNA (Control) or WT or DN versions of CHMP3 and Vps4A, as indicated above each lane. Co-transfections were performed, respectively, at a 3:1 DNA ratio. Three days post transfection, SDS lysates were prepared and analysed by FLAG- or GFP-specific Western blotting to demonstrate expression of CHMP3.wt or CHMP3.dn, Vps4A.wt and Vps4A.dn respectively. Cell-associated core and capsids released into the media were probed by anti-DPAF immunoblotting.

D. HuH-7 cells were transfected with the core expression vector (Core) or the HBV replicon (HBV) and immunostained with rabbit anti-capsid (K45) and mouse anti-LBPA antibodies. After staining with AlexaFluor 546-conjugated anti-rabbit and AlexaFluor 488-conjugated anti-mouse antibodies, cells were visualized by deconvolution fluorescence microscopy. The overlays of the fluorescences are shown in the right column with yellow colour indicating colocalization. DNA staining of the nuclei is in blue. Bar, 10 μm.
Alix mediates HBV capsid release

In the course of these experiments, we surprisingly observed that the overexpression of WT Alix increased the release of non-enveloped nucleocapsids (see also below). To test whether Alix may directly contribute to naked capsid egress, we investigated the synthesis and release of core under conditions of overexpressing WT Alix. The WT and DPAF-tagged core constructs were transfected either alone or together with the HA-tagged Alix construct into HuH-7 cells. Cell lysates and supernatants were probed by HA-, core- and DPAF-specific Western blotting to show synthesis of Alix and core (Fig. 4A). In fact, the extracellular levels of untagged and tagged cores were significantly higher when ectopically expressed Alix was present. Quantification analysis revealed that upregulated Alix enhanced core release about 10- to 12-fold. Because high levels of Alix had been implicated to potentially promote apoptosis (Mahul-Mellier et al., 2006), we performed cytotoxicity assays of supernatants that did not reveal any indications of cell damage and lysis. Thus, Alix overexpression profoundly increased the yield of extracellular core protein in a non-lytic manner. Similar results were obtained upon Alix overexpression in HBV-replicating cells. Here, supernatants of co-transfected cells were subjected to a capsid-specific immunoprecipitation (IP) followed by PCR analysis of the viral DNA. As shown in Fig. 4B, upregulated Alix stimulated naked nucleocapsid release about eightfold. By using this experimental approach, we also estimated the ratio between virions (via envelope-specific IP) and naked nucleocapsids (via capsid-specific IP) released from cells.
transfected with the HBV replicon alone and calculated a ratio 10:6 respectively.

Because the extracellular core pool assayed in Fig. 4A was collected by ultracentrifugation, it likely presented as assembled capsids rather than mono/dimeric core proteins, as they would not sediment at the applied centrifugation force. To substantiate this point, we analysed the fate of two core mutants, Core.K96A and Core.DPPAY, under conditions of excess Alix. A previous report had shown that both mutants are defective in capsid envelopment and virion formation (Ponsel and Bruss, 2003). However, Core.K96A is still able to form capsids, while mutations of the PPAY sequence blocked capsid assembly. As shown in Fig. 4C, the overexpression of Alix stimulated the release of Core.K96A but had no effect on Core.DPPAY. Of note, in this experiment the supernatants were concentrated by either ultracentrifugation or trichloroacetic acid (TCA) precipitation in order to screen for capsids or capsids plus core proteins respectively. From these data we conclude that Alix specifically increased the release of (nucleo)capsid particles rather than of core proteins.

Besides naked (nucleo)capsids, HBV morphogenesis is accompanied by the formation of subviral empty envelope particles that are mainly disposed of the small S envelope protein. These particles have been shown to mature by budding into intraluminal cisternae of post-ER/pre-Golgi compartments and to leave the cells via secretion (Bruss, 2003).

Fig. 4. Excess Alix enhances HBV naked (nucleo)capsid release.
A. Core (lanes 1 and 2) or DPAF-tagged core (lanes 3 and 4) was co-transfected with control DNA or HA-tagged Alix at a 1:3 DNA ratio respectively. Cell extracts were prepared with SDS and analysed by HA-specific immunoblotting to monitor expression of Alix. For detection of core, the core antiserum (K46) (lanes 1 and 2) or the DPAF antibody (lanes 3 and 4) was used. In either case, non-specifically stained bands served as a control for gel loading (Load). Capsids harvested from the culture media were analysed in the same manner. The experiments were repeated three times, and capsids released into the supernatants were quantified and demonstrated in per cent amount relative to control cells. To probe for cell lysis, supernatants were assayed for LDH activity.
B. The pHBV replicon was co-transfected with Alix or control plasmid DNA. Cell supernatants were immunoprecipitated with the capsid-specific antiserum (K45) prior to PCR measurement of the viral DNA. Mean PCR results are demonstrated in per cent amount relative to control-transfected cells.
C. The core mutants Core.K96A (lanes 1 and 2) and Core.DPPAY (lanes 3 and 4) were subjected to the co-transfection assay exactly as in (A). Cell lysates and media concentrated by either ultracentrifugation (UC) or TCA precipitation (TCA) were immunoblotted with the core antiserum (K46).
D. An HA-tagged version of the HBV small envelope protein (S.HA) was transfected into HuH-7 cells together with Alix or a control construct at a 1:3 ratio respectively. Amounts of S were examined by ELISA and are expressed as mean units of optical density at 492 nm (n = 3).
Alix mediates HBV capsid release

HBV naked capsid release requires Alix

We next asked whether Alix is essential for capsid release and analysed core budding in Alix-deficient cells. HuH-7 cells were transfected with control- or Alix-specific siRNA duplexes prior to transfection with the DPAF.Core construct. Western blot analysis of cell lysates showed that Alix was almost completely down-regulated as compared with control siRNA-treated cells (Fig. 5A). While the knock-down of Alix had no effect on the expression and stability profile of DPAF.Core, it decreased the release of capsids by about 80% (Fig. 5A). To study the intracellular distribution profile of core, transfected cells were immunostained with capsid-specific antibodies (K45). In control and Alix-depleted cells, core dispersed throughout the cytoplasm (Fig. 5B). Notably, however, the staining pattern was not fully identical, as core was not evenly distributed in Alix-depleted cells. Rather, we reproducibly observed ‘core-free’, vacuole-like areas in these cells, indicating that the loss of Alix led to a spatial mis/redistribution of core concomitant with its budding arrest.

Mapping of Alix domains responsible for its HBV capsid release-enhancing activity

To specify the Alix domain(s) responsible for the enhancement of naked capsid release, we constructed different Alix mutants. Human Alix consists of three regions: the Bro1 domain named for the apparent yeast homologue Bro1p [amino acid (aa) 1–358], the V domain that is formed by two three-helix bundles and folded in the shape of the letter V (aa 362–702), and the PRR region (aa 703–868) that serves as a docking site for a number of proteins, including Tsg101 (Strack et al., 2003; Sakaguchi et al., 2005; Odorizzi, 2006; Raiborg and Stenmark, 2009). Accordingly, deletion mutants were generated that lack either one or two of these domains (Fig. 6A). Alix also binds CHMP4 (ESCRT-III) proteins through an exposed hydrophobic patch in the Bro1 domain with Ile212 and Leu216 being important (Fisher et al., 2007; Usami et al., 2007). Since the substitution of these residues by Asp inhibited CHMP4 binding, a correspondingly point mutant was made (Fig. 6A; Alix.DD). Next, co-transfection studies using the DPAF-tagged core were performed as above. As shown in Fig. 6B, the WT and mutant Alix proteins were expressed at equal levels and could be identified by their predicted molecular weights. By inspecting the amounts of PDAF.Core in lysates and capsids in supernatants, excess WT Alix accelerated capsid release as above. A comparable enhancing activity was observed for the Alix.DD, the CHMP4 interactor sites of Alix, did not affect its capsid release-promoting activity. Rather, as evidenced by the phenotypes of the Alix.DD and Bro1 mutants, the stimulation of HBV capsid egress strictly depends on the Bro1 domain. While the loss of the Bro1 domain abrogated enhanced capsid export, its sole overexpression is sufficient to enhance capsid release (Fig. 6B).

Although these results pointed to an important role of the Bro1 domain of Alix in HBV capsid egress, there remained the possibility that the non-stimulating activity of Alix.DD was simply due to a DN inhibition of its endogenous counterpart. To address this, we studied the budding of two retroviral Gags, HIV.Gag and equine infectious anaemia virus (EIAV) Gag, in the presence of excess Alix.DD. Both Gags require the basic ESCRT...
machinery for budding, but only EIAV.Gag needs Alix (Garrus et al., 2001; Martin-Serrano et al., 2003; Strack et al., 2003). GFP-tagged HIV.Gag or EIAV.Gag were co-transfected with the HA-tagged AlixΔBro1 construct, and cell lysates and supernatants were analysed by tag-specific immunoblotting. As shown in Fig. 6C, AlixΔBro1 did not compromise either Gag release, indicating that it does not act in a DN manner, neither on endogenous Alix in particular nor on the ESCRT cascade in general. Together, these results indicate that HBV capsids engage the Bro1 domain of Alix for ESCRT-independent exit from cells.

HBV core interacts and colocalizes with the Bro1 domain of Alix

These findings raised the question how core may engage Alix. To address this issue, we analysed whether core may physically interact with Alix. Core was co-transfected with HA-tagged Alix, AlixΔBro1 or Bro1 at a 2:1 DNA ratio into HuH-7 cells, respectively, and cell extracts were probed with epitope-specific antibodies to confirm synthesis of core and the Alix constructs (Fig. 7A). Lysates were next subjected to immunoprecipitation with anti-capsid antibodies (K45), and the immune complexes were examined

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**Fig. 6.** Mapping of Alix domains responsible for its HBV capsid release-mediating activity.

A. Schematic representation of WT Alix and its mutants. The domain architecture of Alix with the Bro1, V and PRR domains is depicted. Numbers below refer to aa positions, and the two stars in Alix.DD denote point mutations introduced at aa positions 212 and 216.

B. DPAF.Core was co-transfected with HA-tagged WT or the indicated Alix mutants at a 1:3 ratio. Following transient expression, cells were lysed with SDS and supernatants were subjected to ultracentrifugation. Synthesis of wt Alix and its mutants is shown by HA-specific immunoblotting. Protein levels of intracellular cores and extracellular capsids were examined by DPAF-specific Western blotting. Uniformity of sample loading is shown by a band cross-reacting with the DPAF antibody (Load). Numbers to the left of the top panel refer to molecular weight standards in kDa.

C. EGFP-tagged EIAV.Gag or HIV.Gag were co-transfected with control DNA or HA-tagged AlixΔBro1 at a 1:3 DNA ratio respectively. Gag expression and VLP release efficiency were analysed by GFP-specific immunoblotting, while synthesis of AlixΔBro1 was determined by anti-HA Western blotting.
by HA-specific immunoblotting. As shown in Fig. 7A, core could indeed co-precipitate full-length Alix and its Bro1 domain, but not AlixΔBro1. Combining these results with the mapping analysis (Fig. 6), the Bro1/core interaction appears to be essential and sufficient to assist in naked capsid egress.

To corroborate these findings, we performed deconvolution immunofluorescence microscopy to visualize the distribution of core and Alix, AlixΔBro1 or Bro1 in co-transfected cells. In either setting, core yielded its typical cytoplasmic staining (Fig. 7B). Alix and its Bro1 domain were found mostly dispersed throughout the cytoplasm and, importantly, an overlay of the fluorescence patterns revealed a substantial degree of colocalization between core and Alix or Bro1 (Fig. 7B). Upon deletion of the Bro1 domain, the staining pattern completely changed, as AlixΔBro1 appeared in aberrant structures that looked like large vesicles or aggregates. Consistent
with the co-precipitation studies, no colocalization could be detected for Alix/Br1 and core. To exclude the possibility that core colocalized with Alix and Br1 simply due to their overall cytoplasmic dispersion, we analysed the degree of colocalization between core and a cytosolic marker protein. For this, an EGFP-tagged version of the microtubule-associated protein 1 light chain 3 (LC3B) was used that normally resides in the cytoplasm unless cells are induced for autophagy (Tanida et al., 2004). Although EGFP-LC3B was distributed throughout the cytoplasm, we found only a marginal colocalization with core (Fig. 7B). Accordingly, the merged signals obtained for Alix/core and Br1/core were likely to present at least partial colocalization of the proteins. We conclude from the strict correlation between capsid binding and capsid release stimulation and vice versa that the core/Alix interaction may be functionally important.

Alix is not required for HBV capsid assembly and membrane association of core

To gain insights into the mechanistic action of Alix, we first focused on the core assembly reaction. During this process, monomeric core rapidly forms dimers that assemble into icosahedral capsids containing 90 or 120 dimers. HuH-7 cells were treated with control or Alix-specific siRNAs prior to transfection with DPAF.Core, and lysates were probed for intracellular assembled capsids by native agarose gel electrophoresis and Western blotting with anti-capsid antibodies (K45). Besides caesium chloride density analysis, this technique has been approved as a reliable method to detect assembled capsids within the cell (Pairan and Bruss, 2009, and references therein). The analysis revealed no difference in the signal intensities (Fig. 8A), indicating that Alix is dispensable for HBV naked capsid formation.

Next, we assessed whether Alix might affect the membrane association of core. For the related duck HBV core protein, it had been shown to partition between the soluble and membrane fraction even in the absence of the viral envelope (Mabit and Schaller, 2000). To evaluate whether the HBV core shares a similar intrinsic membrane binding capacity, membrane flotation analyses were performed (Fig. 8B). For control purposes, GFP was used as a soluble protein, while the Rab7 GTPase served as a reference for a soluble and membrane-associated protein. Transfected cells were subjected to membrane flotation and flotation fractions were analysed by immuno blotting. As expected, the GFP signal was found exclusively in the high-density phase of the gradient. Rab7 was detected in both high-and low-density phases consistent with its soluble and membrane-associated forms. A similar distribution phenotype was observed for the core protein, indicating that a fraction of core is targeted to cellular membranes. Importantly, upon depletion of Alix core is also associated with membranes, implicating that Alix is per se not essential for the core/capsid membrane targeting. A close inspection and comparison of the core distribution profile in siControl- and siAlix-treated cells, however, revealed that the pool of membrane-associated

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core increased when Alix was depleted, suggesting that Alix may affect the portion of membrane-bound core (Fig. 8B). We conclude that Alix does not function in capsid assembly but may rather direct following steps in HBV capsid egress.

Discussion

Previous works have shown that supernatants of HBV-producing hepatoma cell lines contain besides infectious virions also non-enveloped DNA-containing core particles (Beterams and Nassal, 2001; Perlman and Hu, 2003; Sun and Nassal, 2006; Watanabe et al., 2007; Wittkop et al., 2010). This phenomenon, however, has been largely ignored, since it has been assumed that these core particles may be a by-product of efficient HBV replication in vitro, thereby being released by default and/or from damaged cells. In this work, we have challenged the underlying mechanism(s) and find that naked core particles are efficiently exported in vitro even in the absence of a replicating HBV genome. In addition, we show that capsid release from cells proceeds in a non-lytic manner and renders extracellular particles devoid of a membrane coat. Moreover, the pathway of HBV capsid release is unconventional in that it requires Alix without ESCRT support.

Intense research in the last years revealed that many enveloped viruses rely on the ESCRT machinery for budding. Regardless of which late domain they encode, these viruses in common depend on the late acting ESCRT components, ESCRT-III and Vps4, which drive the fission reaction (Chen and Lamb, 2008; Pincetic and Leis, 2009; Raiborg and Stenmark, 2009). However, a functional inactivation of Alix in MVB sorting is less clear, Alix is known to interact with Tsg101 and CHMP4 (Strack et al., 2003; Fisher et al., 2007; Usami et al., 2007; McCullough et al., 2008). The loss of these interactors by either RNA silencing or overexpression of DN ESCRT-III/Vps4 mutants (Fig. 2) concomitant with a sequestration of the entire ESCRT machinery at aberrant MVBs (Garrus et al., 2001; Strack et al., 2003) could increase the availability of Alix, a situation reminiscent to overexpressed Alix.

By analysing the effects of excess and deficit Alix, we find that it is specifically required to facilitate HBV capsid egress. To account for the incomplete inhibition of capsid release in Alix-depleted cells, we assume that other Bro1 domain-containing proteins may somehow compensate for the loss of Alix. In humans, the Bro1 domain is a common element in several proteins, like His domain phosphotyrosine phosphatase, the Bro1 domain-only protein Brox and rhophilins (Doyotte et al., 2008; Popov et al., 2009). Bro1 domains are expected to have a similar boomerang shape and some Bro1 domains are functionally exchangeable, as it was shown in a minimal HIV-1 Gag release assay in which both, the Bro1 domain of Alix and Brox, stimulated VLP release (Popov et al., 2009).

Our mapping analysis indicates that the Bro1 domain is required and sufficient for the HBV capsid release-promoting activity of excess Alix. The dispensability of the PRR region of Alix is of particular interest, as it indicates that Alix binding to the ESCRT-I subunit Tsg101, endophilins, Cep55 and ALG-2 are all not required. Because the Alix/Cep55 interaction is essential for abscission during cytokinesis (Carlton and Martin-Serrano, 2007; Morita et al., 2007), whereas the Alix/ALG-2 interaction is a prerequisite for induction of apoptosis (Mahul-Mellier et al., 2006), we infer that neither of these mechanisms contributes to the enhancement of capsid release by upregulated Alix. The CHMP4 binding site in Alix is also not required for HBV capsid budding. Structural and functional studies have revealed that L212D and I216D point mutations of Alix abolished its interaction with CHMP4 and its function as a quality control system and sorts unneeded cargo into MVBs for lysosomal destruction (Piper and Katzmann, 2007; Raiborg and Stenmark, 2009). Core proteins synthesized in the absence of an ongoing HBV replication may present waste material, destined for degradation in lysosomes. Hence, if ESCRT-mediated protein sorting is blocked, core may escape lysosomal breakdown with the consequence that more core is available for ESCRT-independent release pathways. However, according to our immunofluorescence studies that did not reveal any colocalization between core (if synthesized alone) and the MVB/lysosome marker LPBA, we do not favour this interpretation. Rather, the inactivation of the ESCRT cascade may render more functional Alix available to guide capsid egress. Although the genuine function of Alix in MVB sorting is less clear, Alix is known to interact with Tsg101 and CHMP4 (Strack et al., 2003; Fisher et al., 2007; Usami et al., 2007; McCullough et al., 2008). The loss of these interactors by either RNA silencing or overexpression of DN ESCRT-III/Vps4 mutants (Fig. 2) concomitant with a sequestration of the entire ESCRT machinery at aberrant MVBs (Garrus et al., 2001; Strack et al., 2003) could increase the availability of Alix, a situation reminiscent to overexpressed Alix.

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in ESCRT-dependent virus budding and cytokinesis (Carlton and Martin-Serrano, 2007; Fisher et al., 2007; Morita et al., 2007; Usami et al., 2007). Given that Alix can mediate HBV capsid budding irrespective of its interactions with Tsg101 and CHMP4, it appears to function independently of ESCRT.

Our data also reveal that Alix is not essential for budding of infectious HBV particles. The different use of cell functions by HBV viral and capsid particles is puzzling and poses the question how the virus may orchestrate these pathways. One critical determinant assigning the budding pathway may be the stage of capsid maturation. During virus budding, only mature nucleocapsids containing the DNA genome can be packaged by the viral envelope proteins while immature (nucleo)capsids with pregenomic RNA, nucleic acid intermediate forms or no nucleic acid are excluded from envelopment (Gerel-saikhan et al., 1996; Pairan and Bruss, 2009). The nature of the maturation signal has not been unequivocally identified, but appears to involve the phosphorylation degree and conformation state of the capsid (for review, Bruss, 2007). Hence, mature nucleocapsids may gain access to ESCRT-dependent HBV budding either directly, via capsid/envelope interactions, or indirectly with the help of cellular factors. Such factors could be γ2-adaptin and Nedd4, two ESCRT-associated proteins that interact with the core protein and guide HBV formation (Rost et al., 2006; Lambert et al., 2007). Immature capsids may fail to enter this pathway and may rather be recognized by Alix in order to be shunted into an ESCRT-independent exit route.

So far, we do not know the precise mechanistic action of Alix. We show that Alix contributes neither to membrane targeting of the HBV core protein nor to capsid assembly. Hence, Alix appears to act at late steps of naked capsid budding, possibly when premade capsids are tethered to membranes. Our observation that the Bro1 domain of Alix is sufficient for both binding to the core protein and enhancing capsid release, it may be recruited by core in order to remodel membranes. In view of the crystal structures of the Bro1 domain of Alix and its yeast homologue Bro1 (Kim et al., 2005; Fisher et al., 2007), the Bro1 domain is an attractive candidate for a component of a negative curvature sensing system. By binding to membranes with its convex surface, the boomerang-shaped Bro1 domain of Alix could contribute directly to the generation of negative curvature required for budding away from the cytosol. An inherent membrane-deforming ability of isolated Bro1 domains was recently suggested by Popov et al. (2009) who showed that these domains can promote VLP release of a minimal HIV-1 Gag construct, even when their CHMP4 binding sites – and hence their access to the MVB machinery – were disrupted. By deforming membranes at HBV capsid budding sites, Alix may simultaneously function as an adaptor recruiting components that are needed for the final liberation of the capsids from the cell. Based on the close relationship between Alix and lipids (Odorizzi, 2006; Falguieres et al., 2008), candidates for such components could be lipids with fusogenic properties, lipid modulators and lipases. Because extracellular capsids are seemingly coatless, their egress may involve processes of membrane rupture. Such mechanisms have been described to accompany the morphogenesis of vaccinia virus (Chlanda et al., 2009). Understanding how Alix mechanistically operates during HBV capsid budding will be an important next step forward.

Our observations also raise the question whether the release of HBV naked capsids is beneficial for the infected host or the virus or both. Naked capsids are hardly found in the blood of infected patients or chimpanzees (Possehl et al., 1992). Noteworthy, however, the (nucleo)capsid or core antigen [also referred to hepatitis B core antigen (HBcAg)] is extremely immunogenic and high titres of anti-HBc antibodies are produced in virtually all patients who have been exposed to HBV. The potent immunogenicity of HBcAg is due, at least in part, to the fact that it can function as a T-cell-independent antigen and directly binds and activates B-cells to produce antibodies (Milich and McLachlan, 1986; Milich et al., 1997). Accordingly, naked (nucleo)capsids must exist in vivo, but may be rapidly cleared from extracellular fluids by phagocytosis, endocytosis and/or degradation. The origin of free extracellular HBcAg/core particles is not yet clear. They may originate from HBV-infected cells killed by cytotoxic T lymphocytes (CTL). In transgenic mice and cell culture systems, it has been shown that cytoplasmic nucleocapsids can survive apoptosis, induced by CTLs or artificial stimuli, and persist in the extracellular milieu (Pasquetto et al., 2000; Arzberger et al., 2010). However, as shown in this study, it seems equally possible that extracellular capsids originate from core-producing cells by a non-lytic, Alix-mediated exocytosis mechanism. In either case, extracellular core particles are competent to prime B- and T-cells (Milich and McLachlan, 1986) and in such advantageous for the infected host. Accordingly, it is tempting to speculate that the Alix-assisted capsid release may resemble a host cellular defence mechanism.

For HBV, naked nucleocapsid release could be instrumental in spreading infection. Upon protein transfection of HBV-derived nucleocapsids into cell lines, the capsids proved to be able to initiate productive infection, indicating that they are ‘infectious’ if delivered into cells (Rabe et al., 2006). In vivo, such delivery mechanisms could involve endocytosis processes, since capsids have been shown to bind to cell surface-exposed heparan sulfate proteoglycans that trigger clathrin-dependent uptake of naked capsids (Vanlandschoot et al., 2005; Cooper and Shaul, 2006).
Alix mediates HBV capsid release

To conclude, our observations identify Alix as a component of the HBV VLP but not virus budding machinery, implicating that HBV has evolved divergent strategies to bud from infected cells. The unconventional exit of naked capsids may provide a model system to study how Alix promotes particle budding without an ESCRT.

Experimental procedures

Plasmids

For HBV replication in liver cell lines, plasmid pHBV was used (Radziwill et al., 1990). It carries a 1.1-mer of the HBV DNA genome in which the viral core/polymerase promoter is preceded by the hMTIIa promoter. The vector pNi2.C (pCore) contains the HBV core gene [nucleotide (nt) 1905–2454; numbering as referred to the plus strand of the HBV DNA genome, genotype D; GenBank™ Accession No. J022203] under the control of the hMTIIa promoter. The HBV DNA fragment from nt 827 to 1986 containing a post-transcriptional regulatory element for mRNA export and the polyadenylation signal were fused downstream of nt 2454. To tag the core gene with a foreign epitope, the tetrapeptide sequence DPAF was fused to the N-terminus of core (DPAF.Core) using the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene). Mutant core genes carrying a lysine-to-alanine substitution at aa position 96 (Core.K96A) or a deletion of aa 129–132 (Core.APAY) were purchased from Molecular Probes.

To probe for protein expression, cells were lysed with either the non-denaturing detergent Nonidet P-40 or the denaturing reagent sodium dodecylsulfate (SDS). NP-40 lysates were prepared by incubating the cells with Tris-buffered saline (TBS, 50 mM Tris-HCl pH 7.5/150 mM NaCl) containing 0.5% NP-40 for 20 min on ice. Thereafter, lysates were centrifuged for 5 min at 15 000 g and 4°C. For lysis with SDS, the cells were scraped and 4°C. For lysis with SDS, the cells were scraped

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from the plates using 1× Laemmli buffer, and cell suspensions were boiled for 10 min prior to centrifugation. Cell extracts were subjected to SDS-PAGE and Western blotting analyses using standard procedures. To analyse the assembly and release of capsids/retroviral VLPs from transfected cells, clarified culture medium was concentrated by ultracentrifugation through a 20% (w/v) sucrose cushion (4 h at 100 000 g and 4°C). Pellets were suspended in TBS and mixed with Laemmli buffer. Alternatively, proteins in the supernatants were precipitated with TCA. Unless otherwise indicated, 25% of cell lysates and 50% of supernatants were recovered in Western blots. In addition, native viral particles recovered from cell supernatants were subjected to dot-blot analyses using non-denaturing conditions. To evaluate the presence of damage and toxicity of transfected cells, LDH activity was quantified in culture media using a colorimetric LDH quantification assay (Roche).

Detection of intracellular HBV nucleocapsids and extracellular virions

For HBV replication in HuH-7 cells, plasmid pHBV was used. After transfection, cellular supernatants were harvested and cell lysates were prepared with Triton X-100. Intracellular nucleocapsids and extracellular virions were isolated by capsid- or envelope-specific immunoprecipitations, respectively, as described (Löffler-Mary et al., 2000). In difference, magnetic Protein G beads (Millipore) were used for immunoprecipitations. Thereafter, viral DNA was isolated from the immunoprecipitated samples using High Pure Viral Nucleic Acid Kit™ (Roche). Total HBV DNA was determined by multiplex real-time PCR (Applied Biosystems) in two runs using duplicates. Primer sequences are available on request.

Density gradient analyses and ELISA

For caesium chloride gradient analysis of capsids, the material recovered by ultracentrifugation was suspended in TNE (10 mM Tris-HCl pH 7.5/150 mM NaCl/10 mM EDTA) and separated by isopycnic gradient centrifugation through 10–50% (w/v) caesium chloride in TNE. After centrifugation for 20 h at 100 000 g and 4°C, fractions were collected and screened for capsids using an HBV core-specific ELISA (HBeEIA, Abbott; no longer available in Europe). HBsAg reactivity was determined with the Auszyme Monoclonal Kit (Abbott).

For the density flotation assay, 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 centrifuged at 100 000 g for 10 min at 4°C to sediment nuclei and debris. The resulting post-nuclear supernatant was made to 40% Optiprep (w/w) (Sigma-Aldrich), placed on the bottom of a centrifuge tube and overlaid with 28% Optiprep. The step gradient was centrifuged for 3 h at 100 000 g and 4°C, and fractions were collected from the top.

Protease protection assay

For proteinase K protection analysis of capsids, concentrated cell culture media were suspended in TBS, divided into three aliquots which were either left untreated or treated with proteinase K (0.5 μg μl⁻¹) in the presence or absence of 0.5% NP-40. After incubation on ice for 60 min, proteinase K was inactivated by the addition of 10 μl ml⁻¹ phenylmethylsulfonyl fluoride, and samples were mixed with Laemmli buffer.

Polyethylene glycol (PEG) precipitation and Western blotting of cytoplasmic capsids

For concentration of intracellular capsids, they were precipitated with polyethylene glycol (PEG) exactly as described in Pairan and Bruss (2009). Precipitates were then separated by native 1% (w/v) agarose-Tris-acetate-EDTA gel electrophoresis. The gel was blotted by capillary transfer, using a nitrocellulose membrane and 10× SSC buffer (1.5 M NaCl/150 mM sodium citrate pH 7.0), as described (Pairan and Bruss, 2009). The membrane was reacted with anti-capsid antibodies (K45) using standard techniques.

Co-immunoprecipitation assay

To probe for complex formation, 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), 20 mM N-ethylmaleimide, supplemented with 1× protease inhibitor mixture (Serva) for 20 min on ice. After centrifugation, lysates were immediately subjected to immunoprecipitation using tosyl-activated, superparamagnetic polystyrene beads (Dynabeads Sheep anti-rabbit IgG; DYNAL) that had been pre-coated with the anti-capsid antibody K45, as described (Rost et al., 2008). After incubation for 3 h at 4°C with agitation, the immune complexes were washed three times with 0.5% CHAPS/HBS, and once with phosphate-buffered saline (PBS) prior to SDS-PAGE and immuno blotting. Total protein extracts corresponding to 10% of the lysates were included on the gel as controls.

Fluorescence microscopy

For protein immunostaining, cells grown on coverslips were fixed and permeabilized with ice-cold methanol containing 2 mM EGTA. Cells were blocked in PBS containing 2% animal serum, incubated with the indicated primary antibodies for 1 h at 37°C, rinsed with PBS, and then incubated with AlexaFluor-tagged secondary antibodies for 1 h at 37°C. DNA was stained with Hoechst 33342 (Sigma-Aldrich). For LPBA plus protein labelling, a fixation protocol using 3% paraformaldehyde was employed, as described (Rost et al., 2008). Z-stack images were acquired separately for each channel using a Zeiss Axiovert 200 M microscope equipped with a Plan-Apochromat 100× (1.4 NA) and a Zeiss Axiocam digital camera. Axiovision software 4.7.1 was used for merging pictures. Z-stack images were optically deconvoluted using the software supplied by Zeiss. Tiffs were assembled into figures using Photoshop CS2 (Adobe).

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