Clathrin-mediated Endocytosis and Lysosomal Cleavage of Hepatitis B Virus Capsid-like Core Particles*

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The hepatitis B virus (HBV)2 is a small, enveloped DNA virus that infects hepatocytes and replicates through reverse transcription. The viral capsid also termed the core particle is a 30-nm diameter icosahedral structure assembling from 180 or 240 virally encoded core antigen (HBcAg) monomers (1). HBcAg is a 185-amino acid protein that harbors two distinct domains, namely an assembly domain (residues 1–144) that drives particle formation and an arginine-rich domain (residues 150–185) that binds nucleic acids (2). The particle lattice is generated by repetitive 1.5-nm diameter channels that allow partial exposure of the arginine-rich domains of HBcAg on the shell surface (3). The core particle is extremely immunogenic, eliciting strong and long-lasting B and T cell immune responses during infection (4). T cell responses provoked by core particles are essential for keeping HBV replication under control (5).

When expressed in various heterologous systems, HBcAg efficiently self-assembles into capsid-like core particles indistinguishable from native capsid shells (6–8). Particle assembly tolerates fusion of foreign proteins to the HBcAg monomer, making it an attractive carrier of B and T cell epitopes for vaccination purposes (4). Consistently, T cell epitopes carried by capsid-like particles are efficiently presented on class I and class II MHC molecules of antigen-presenting cells (APCs) (9). Also, the capacity of the arginine-rich domain of HBcAg to bind nucleic acids has been utilized by us and by others to incorporate oligodeoxynucleotides (ODN) into the lumen of recombinant core particles (10, 11). The encapsulated ODN are delivered into cultured cells with high efficiency (10). Furthermore, ODN encapsulated in core particles or conjugated to an arginine-rich peptide derived from the C terminus of HBcAg exert enhanced immunostimulatory effects in vivo, and these effects have been suggested to result from their efficient endosomal delivery and activation of toll-like receptor 9 (TLR9) signaling (11–13). These findings led researchers to propose that the core particles are efficiently endocytosed by cells, yet this possibility and the underlying mechanism have not been investigated.

Recently we and others have shown that capsid-like core particles efficiently bind a wide variety of cell types including macrophages and B cells (14, 15). Particle attachment requires the arginine-rich domain of HBcAg and sulfated heparan sulfate proteoglycans on the target cell membrane (14, 15). We further showed that interaction with membrane heparan sulfate facilitates cytokine induction by the core particle in macrophages. Here we investigated core particle cell entry following heparan sulfate binding and the underlying mechanism. We found that the particles are internalized via clathrin-mediated endocytosis involving Ep15 and Rab5. Endocytosed particles are trafficked to lysosomes, where HBcAg is endoproteolytically cleaved into its distinct domains by cathepsin B and disassembles. These findings illuminate the molecular mechanism underlying core particle-mediated delivery of macromolecules for potentially therapeutic purposes and may have implications for HBcAg immunogenicity.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—Sodium azide (NaN3), 2-deoxy-D-glucose, chlorpromazine, nystatin, chloroquine, aprotinin, pepstatin A, E64d, and Z-Phe-Ala fluoromethylketone (Z-FA-fmk) were from Sigma. Heparin was from Calbiochem. LysoTracker Red was from Molecular Probes. The eGFP-N1 plasmid was from Clontech. The plasmid encoding GFP-tagged Ep15 D95/295 was kindly provided by Prof. Dautry-Varsat (Pasteur Institute, Paris, France) and was previously described (16).

Recombinant HBV Core Particles—The coding regions for the full-length (amino acids 1–185) or the C-terminally deleted versions of HBcAg were PCR-amplified from a HBV genome template (subtype adw) using the appropriate primers and cloned into pRSET B (Invitrogen). DNA constructs were analyzed by DNA sequencing. The particles were prepared in Escherichia coli and purified as previously described (10). For analysis of recombinant capsid-like particles on sucrose gradients, samples from the core particle preparations were layered onto step gradients with six 330-μl steps of 10, 20, 30, 40, 50, and 60% sucrose...
Clathrin-mediated Endocytosis of HBV Core Particles

**FIGURE 1.** HBc core particles are endocytosed following cell attachment. COS-1 (upper panels) and HepG2 (lower panels) cells were incubated with HBc core particles (1 mg) with or without heparin (0.3 mg/ml) or with medium containing 0.3 mg/ml heparin (C, E) prior to cell fixation. Immunofluorescence was performed using polyclonal anti-HBcAg antibodies (red) and nuclei were stained with Hoechst 33342 (blue) as described under “Experimental Procedures.” The cells were visualized by confocal microscopy, and representative optical fields are shown.

(w/v) in PBS and centrifuged for 45 min at 4°C and 55,000 rpm in a TLS-55 rotor (Beckman) using the TL-100 ultracentrifuge as previously described (17). All experiments using recombinant full-length (HBc) core particles were reproducible using bacterially prepared HBV capsids (subtype ayw) generously provided by Prof. Paul Pumpens (Biomedical Research and Study Center, University of Latvia, Riga, Latvia).

**Antibodies**—Monoclonal anti-β-tubulin was from Sigma. Polyclonal rabbit antibodies against HBcAg (pac74) were generated by repeated injections with bacterially expressed, purified HBcAg capsids, followed by selection of hybridoma-secreting anti-core antibodies. The epitope recognized by mac22 lies between residues 110–130 of HBcAg.

**Cell Culture**—HepG2 and COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum and antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin). For immunohistochemistry analyses, the cells were seeded in 13 mm coverslips in 24-well plates (Nunc) 1 day before the experiment following cell attachment. Immunofluorescent microscopy analyses were obtained using a Bio-Rad MRC-1024 confocal system, utilizing an argon-krypton mixed gas laser and mounted on a Zeiss Axiovert microscope. Representative fields of one of three experiments with similar results are shown. In each experiment the presented images were acquired using identical laser intensities.

**Immunoblotting**—Total cell extracts made in radioimmune precipitation assay lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40 (v/v), 0.5% AB-deoxycholate (v/v), 0.1% SDS (v/v), 1 mM dithiothreitol, and a protease inhibitors mixture (Sigma)) were subjected to SDS-PAGE followed by Western blot analysis according to standard protocols. For detection of particles under native conditions, samples from core particle preparations or cell extracts made by three cycles of freezing and thawing in PBS containing protease inhibitors were subjected to electrophoresis through 1% agarose gel in TAE (40 mM Tris acetate, 1 mM EDTA). The proteins were either stained with GelCode (Pierce) or capillary-transferred to a nitrocellulose membrane followed by Western blot analysis according to standard protocols.

**RESULTS**

HBc Core Particles Are Internalized via Clathrin-mediated Endocytosis—In this study we used COS-1 and HepG2 cells to study HBV core particle entry into cells and the possible underlying mechanism. Incubation of cells with full-length HBc core particles followed by cell fixation and immunofluorescent microscopy revealed HBcAg-positive staining on the surface and possibly in the cytoplasm of cells but not in the cell nucleus (Fig. 1A). HBcAg-positive staining was observed for all cells examined in at least 10 different optical fields (data not shown), demonstrating efficient particle cell attachment. No signal was detected in mock-treated cells, demonstrating the specificity of the anti-HBcAg antibodies used for the immunofluorescent analysis (data not shown). Particle cell attachment was abolished when soluble heparin was included during the incubation period (Fig. 1B), in accordance with the fact that particle binding to cells is mediated by heparan sulfate (14, 15). Interestingly, when the cells were incubated with particles in the absence of heparin but washed with medium containing heparin to remove externally bound particles at the end of the incubation period, punctate HBcAg-positive staining was observed reminiscent of endosomal or lysosomal vesicles (Fig. 1C). This result suggested that the particles become endocytosed. To test this possibility further, the incubations were preformed in the presence of energy inhibitors (azide and 2-deoxy-D-glucose) that block endocytosis. Depletion of cellular energy had no substantial effect on particle binding to cells.

3 I. Sominskaya, personal communication.
HBcAg uptake, we used a mutant form of Eps15 (Eps15
with clathrin-mediated endocytosis of the particles into cells. 

This led to the conclusion that HBc core particle entry requires clathrin-mediated endocytosis but blocked particle uptake (Fig. 1, D and E, respectively). Incubating the cells at 4 °C had a similar effect to energy inhibitors (data not shown). Together these results demonstrate that HBc particles are internalized by endocytosis.

Uptake of viruses, virus-like particles and exogenous proteins occurs through various endocytic machineries (19). Two major routes that have been implicated in cell entry of arginine-rich, heparan sulfate-binding proteins include clathrin-mediated endocytosis and cholesterol-rich raft endocytosis (20). To examine whether either of these pathways is involved in HBc core particle uptake, we used chlorpromazine and nystatin known to inhibit the formation of clathrin-coated pits and cholesterol-rich raft microdomains, respectively. HepG2 cells were untreated or treated with the drugs and incubated with HBc core particles. The cells were then washed with heparin, fixed, and immunostained using anti-HBcAg antibodies. Examination by confocal microscopy revealed that chlorpromazine completely inhibited particle uptake whereas nystatin had no substantial effect (Fig. 2A). Under the same conditions, nystatin substantially reduced uptake of cholera toxin subunit B known to penetrate cells through cholesterol-rich rafts (data not shown). These results suggest that HBc core particle entry requires clathrin-mediated endocytosis but not lipid raft microdomains.

It was previously shown that pre-treatment of cells with NH$_4$Cl followed by incubation of the cells in the presence of amiloride leads to cytosol acidification which results in abolishment of the signal representing internalized core (Fig. 2B). Under the same conditions, nystatin substantially reduced uptake of cholera toxin subunit B known to penetrate cells through cholesterol-rich rafts (data not shown). These results suggest that HBc core particle entry requires clathrin-mediated endocytosis but not lipid raft microdomains.

To further examine the role of clathrin-mediated endocytosis in HBcAg uptake, we used a mutant form of Eps15 (Eps15Δ95/295) that lacks the second and third of the three EH domains of the protein (16). Eps15 binds the AP2 adaptor and overexpression of Eps15Δ95/295 generates a dominant negative effect that blocks the formation of clathrin-coated pits without affecting other endocytic pathways. HepG2 and COS-1 cells were transfected with constructs encoding either Eps15Δ95/295 fused to GFP (GFP-Eps15Δ95/295) or GFP alone. 24 h later the cells were incubated with HBc core particles and assayed for their capacity to internalize the particles by immunofluorescence microscopy. Overexpression of GFP alone had no effect on particle uptake (Fig. 2, C and D). By contrast, in cells overexpressing GFP-Eps15Δ95/295, entry of core particles was diminished. The stronger effect of GFP-Eps15Δ95/295 expression on particle entry into HepG2 cells relative to COS-1 cells likely results from its lower expression level in the latter cell line (compare relative fluorescent intensities of GFP-Eps15Δ95/295 in Fig. 2, C and D). Collectively, these results demonstrate that HBc core particles enter cells via clathrin-mediated endocytosis.

Transport of HBc Core Particles from the Cell Surface Is Modulated by Rab5 GTPase—The small GTPase Rab5 controls vesicular transport of ligands from the plasma membrane to early endosomes and homotypic fusion between endosomes (21). To test the possible involvement of the small GT Pasadena Rab5 in HBc core particle uptake and trafficking, either wild-type (WT) Rab5 or different Rab5 mutants defective in the GTPase site were expressed in COS-1 cells. Rab5 S34N is a dominant negative GDP binding mutant that reduces the endocytosis efficiency of recycling and lysosomally destined ligands (22, 23). Rab5 Q79L is a GTPase-deficient, constitutively active mutant shown to increase ligand endocytosis (22). Expression of WT Rab5 did not affect HBc particle uptake and particles partially co-localized with Rab5 within cells (Fig. 3). In cells expressing the constitutively active Rab5 Q79L, markedly enlarged endosomes were generated in agreement with a previous report (24) and HBcAg strongly accumulated in the Rab5 Q79L-con-
Clathrin-mediated Endocytosis of HBV Core Particles

HBc Core Particles Are Transported to Lysosomes Where HBcAg Is Cleaved into Its Distinct Domains by Cathepsin B—Ligands internalized by clathrin-mediated endocytosis are either recycled to the plasma membrane or targeted to late endosomes and lysosomes. To test the possibility that the internalized particles are targeted to lysosomes, FITC-labeled HBc core particles were applied to the culture medium of COS-1 cells for 6 h and LysoTracker-Red, a dye known to label lysosomes was added before cell fixation. Fluorescent confocal microscopy visualization revealed extensive co-localization of FITC-labeled HBc core particles with LysoTracker-Red in lysosomes (Fig. 4, A and B). Similar co-localization was observed in HepG2 cells (not shown). These results indicate that following clathrin-mediated entry and transport to early endosomes, the particles are destined to lysosomes.

To examine the kinetics of core particle internalization and its lysosomal targeting. Cells were incubated with FITC-labeled HBc core particles for various times, and the lysosomes were stained with LysoTracker red. The analysis showed that particles were becoming internalized into cells after 1 h (Fig. 4C). However, at this time point the internalized particles did not co-localize with LysoTracker-red in lysosomes. Limited co-localization of the particles with the lysosomal marker was noted after 2 h whereas the particles extensively co-localized with LysoTracker red after 4 h. These results indicate that core particle routing to lysosomes is relatively slow, resembling the lysosomal trafficking kinetics of HIV-1 Tat in cells (25).

To further investigate the fate of internalized HBcAg, we prepared total extracts from COS-1 cells incubated with HBc particles in the absence or presence of energy inhibitors and washed with normal medium or heparin-containing medium as described in the legend to Fig. 1. The extracts were subjected to Western blot analysis using mac22, a monoclonal antibody that recognizes an epitope between residues 110 and 130 of HBcAg. A 21-kDa reactive band corresponding to HBcAg monomers was detected in the extracts prepared from cells incubated with HBc particles (Fig. 5A, lane 1). Interestingly, an additional band of 16 kDa was observed in the same total extract. No signal

FIGURE 3. HBc core particle uptake and transport to early endosomes are regulated by Rab5 GTPase. COS-1 cells were transfected with expression vectors encoding GFP-tagged WT Rab5, Rab5 Q79L or Rab5 S34N as indicated. 24 h after transfection the cells were incubated with HBc core particles (1 nM) for 4 h, washed with medium containing heparin and fixed. The cells were immunostained with anti-HBcAg antibodies (red) and nuclei were stained with Hoechst 33342 (blue). Green fields represent GFP autofluorescence. The cells were visualized by confocal microscopy, and representative images are presented.

FIGURE 4. Endocytosed HBc core particles are targeted to lysosomes. A, COS-1 cells were incubated with FITC-labeled HBc core particles (2 nM) for 6 h, and LysoTracker-Red (200 nM) was added for the last 30 min. The cells were washed with medium containing heparin and nuclei were stained with Hoechst 33342. The cells were fixed and visualized using confocal microscopy. Insets in A are enlarged in B, C, time course analysis of core particle uptake and lysosomal trafficking. Cells were incubated with FITC-labeled HBc core particles (2 nM) for the indicated times, and LysoTracker-Red (200 nM) was added for the last 30 min for each time point. The cells were processed as described in A.
was detected in cells incubated with HBc particle in the continuous presence of heparin (Fig. 5A, lane 2), consistent with the immunofluorescent analysis (Fig. 1B). In cells incubated with HBc particles and washed with medium containing heparin prior to cell harvest, the intensity of the 21-kDa band was abolished (Fig. 5A, lane 3). By contrast, the intensity of the 16-kDa band in the same cell extract was unaffected, suggesting it represents endocytosed HBcAg that underwent processing.

FIGURE 5. Endocytosed HBcAg is cleaved at a region linking its assembly and arginine-rich domains. A, COS-1 cells were incubated with HBc core particles (1 nM) with no additional drugs (lanes 1 and 3), in the presence of 20 μg/ml heparin (lane 2) or in the presence of 0.1% NaN3 and 50 mM 2 deoxy-D-glucose (lanes 4 and 5) for 4 h. The cells were extensively washed with normal medium (lanes 1, 2, and 4) or with medium containing 0.3 mg/ml heparin (lanes 3 and 5) before the cells were harvested. Total cell extracts were prepared as described under “Experimental Procedures.” Samples from the inputs and from the retrieved extracts were subjected to SDS-PAGE and Western blot analysis using mac22 and anti-β-tubulin antibodies. B, analysis of the different HBcAg truncation mutants and full-length HBc core particles following SDS-PAGE and Western blotting with mac22 (left panel) or following native agarose gel electrophoresis and protein staining (right panel). HBc-162 and HBc-172 correspond to core particles assembled from HBcAg monomers containing stop codons after positions 162 and 172, respectively. C, analysis of the different HBcAg truncation mutants and full-length HBc core particles by rate zonal ultracentrifugation. Particles were resolved on step gradient of 10 to 60% sucrose as described under “Experimental Procedures.” Seven fractions were collected from top and samples from each fraction were analyzed by SDS-PAGE and Western blotting with mac22. Gradient fractions are indicated from top (lane 1) to bottom (lane 7). D, HBc-172 and HBc-162 are endocytosed by cells. COS-1 cells were incubated with HBc, HBc-172 or HBc-162 core particles (1 nM each) for 4 h. The cells were washed with medium containing 0.3 mg/ml heparin and fixed. Immunofluorescence was performed using polyclonal anti-HBcAg antibodies (red), and nuclei were stained with Hoechst 33342 (blue) as described under “Experimental Procedures.” The cells were visualized by confocal microscopy and representative optical fields are shown. E, COS-1 cells were incubated with HBc, HBc-172, or HBc-162 core particles (1 nM each) for 4 h and harvested. Total cell extracts were prepared and analyzed by SDS-PAGE and Western blotting. F, same extracts as in A were subjected to electrophoresis through a Tris-Tricine gel and probed with mac22 (left panel) or pac74 (right panel). In an attempt to characterize the processed product of internalized HBcAg, two HBcAg mutants lacking the last 13 or 23 C-terminal residues of HBcAg (HBc-172 and HBc-162, respectively) were prepared in E. coli. Samples from the purified protein preparations were subjected to SDS-PAGE and Western blotting with mac22, revealing reactive bands of the expected sizes for both mutants (Fig. 5B, left panel). The mutant and full-length HBcAg proteins migrated similarly as sharp bands following electrophoresis on a native agarose gel (Fig. 5B, right panel) and had similar sedimentation velocity in rate zonal ultracentrifugation (Fig. 5C), confirming their particulate structure. Immunofluorescent microscopy analysis of COS-1 cells incubated with the different particles and washed with heparin showed that both mutants retained the capacity to become endocytosed by cells (Fig. 5D). Next, total cell extracts from COS-1 cells incubated with HBc-162, HBc-172 or full-length HBc core particles and washed with normal medium prior to cell
harvest were analyzed by Western blotting with mac22. The analysis showed bands at the expected sizes for the different particle monomers in the respective extracts (Fig. 5E). An additional 16-kDa reactive band was detected in all three extracts. These results indicate that the full-length and mutant particles were all processed intracellularly. The similar size for the processing products derived from the full-length and C-terminally truncated mutants indicated that these 16 kDa products do not contain the 23 C-terminal residues of HBcAg.

To detect smaller HBcAg-derived products in the total cell extracts analyzed in Fig. 5A, the same extracts were subjected to electrophoresis through a Tris-Tricine gel. Western blotting with mac22 detected 21-kDa HBcAg monomers and the 16-kDa processing product as before (Fig. 5F, left panel). The blot was stripped and re-probed with pac74, a polyclonal anti-HBcAg antibody. Under these conditions, pac74 preferentially recognizes the arginine-rich domain of HBcAg (data not shown). pac74 reacted with the 21-kDa HBcAg monomers but not with the 16-kDa processed product (Fig. 5F, right panel). This result indicates that the 16-kDa product does not contain the arginine-rich domain of HBcAg and is consistent with the observations made with the HBcAg truncation mutants. Remarkably, pac74 reacted with an additional, smaller band of 5 kDa (Fig. 5F, right panel). The 5-kDa band was resistant to heparin wash and was absent in extracts retrieved from energy-deprived cells (Fig. 5F, lanes 3–5), indicating this product was generated following particle endocytosis. Together, these results indicate that the 21-kDa HBcAg monomer is cleaved into its 16-kDa assembly domain and 5-kDa arginine-rich domain following particle entry into cells.

The lysosome contains various proteases, including serine, aspartic acid, and cysteine proteases responsible for processing of exogenous proteins taken up by endocytosis. The lysosomal localization of the particle suggests that processing of HBcAg is generated by endoproteolytic cleavage in this compartment. To test this possibility, cells were incubated with HBc core particles in the absence or presence of different inhibitors of lysosomal proteases. The cells were washed with medium containing heparin prior to cell harvest and total cell extracts were analyzed by Western blotting. Chloroquine, a general inhibitor of lysosomal proteases prevented cleavage of HBcAg (Fig. 6A, lane 2). Aprotinin and pepstatin A known to inhibit serine and aspartic acid proteases, respectively, did not affect HBcAg processing (Fig. 6A, lanes 3 and 4). By contrast, in the presence of E64d which inhibits cysteine proteases HBcAg cleavage was inhibited (Fig. 6A, lane 5). Furthermore, Z-FA-fmk, a specific inhibitor of the cysteine protease cathepsin B prevented the cleavage (Fig. 6A, lane 6). Thus, we conclude that lysosomal cathepsin B is responsible for the endoproteolytic cleavage of HBcAg.

Finally, we also characterized the mobility of internalized particles on a native agarose gel. Particulate HBV core particles typically migrate as a distinctive band on native agarose gels and their migration is disturbed following particle disassembly (26). Particles taken from the culture media at the end of the incubation period migrated as a distinct band (Fig. 6B, lane 1), indicating the particularity of HBcAg was not disturbed prior to cell attachment and entry. By contrast, the migration of HBcAg present in the lysate of heparin-washed cells was smearable (Fig. 6B, lane 2). The distinct particle migration was largely restored for HBcAg present in the lysate of heparin-washed cells incubated with HBc particles in the presence of chloroquine (Fig. 6B, lane 3). Together these results suggest that HBcAg cleavage promotes particle disassembly following cell entry.

**DISCUSSION**

Previous studies by us and by others demonstrated that the arginine-rich domain of HBcAg interacts with sulfated heparan sulfate proteoglycans to mediate core particle attachment to a wide variety of cell types including macrophages and B cells (14, 15). We show here that the attachment step is followed by clathrin-mediated endocytosis of the particles, depending on Eps15 and the small GTPase Rab5. The internalized cores are transported via early endosomes to lysosomes, where HBcAg is cleaved into its distinct domains by cathepsin B and the particle dissociates.

The family of mammalian heparan sulfate proteoglycans includes six GPI-anchored glypicans and four transmembrane syndecans that bind a wide variety of ligands (27). The mechanisms for ligand internalization may vary depending on the nature of the ligand and the proteoglycan that binds it, as well as on additional receptors that may participate in ligand binding. Using chlorpromazine, cytosol acidification and a dominant negative form of Eps15 we were able to show that core particle internalization is specifically attained via clathrin-mediated endocytosis. Clathrin-mediated particle uptake was observed in various cell types, including human THP-1 macrophages (data not shown). Further, in all cell types examined the particles were transported to lysosomes, leading to cathepsin B-dependent cleavage and processing of HBcAg. These findings suggest that a common yet specific pathway exists for particle uptake and trafficking in cells. The specificity may result from the multimeric nature of the particle, clustering heparan sulfate proteoglycans upon binding. The clustering may in turn drive movement of heparan sulfate proteoglycans into clathrin-coated pits. Alternatively, uptake and trafficking of the core particle may follow a constitutive lysosomal turnover pathway of the heparan sulfate proteoglycan serving as its receptor. The latter possibility is in line with the lysosomal route responsible for cellular catabolism of heparan sulfate proteoglycans (28).

Lysosomal targeting of the particle is followed by cathepsin B-mediated HBcAg cleavage. The cleavage site resides in a region linking the assembly and arginine-rich domains of HBcAg. This finding is consistent with a previous in vitro study showing that a protease-sensitive hinge links the assembly and arginine-rich domains of particulate HBcAg (29). The mobility of endocytosed HBcAg through a native agarose gel suggests that the particle becomes destabilized following HBcAg cleavage. This suggestion is consistent with previous studies showing that core particles genetically or proteolytically deleted of the arginine-rich domain are substantially less stable than full-length particles (2, 30, 31). In addition, non-proteolytic changes in HBcAg may be
induced by the lysosomal environment (e.g. disulfide bond reduction) to further facilitate particle destabilization.

While attempting to map the cleavage site within HBcAg, we found that particles lacking the 23 C-terminal residues of the monomer (HBc-162) attach and enter cells with similar efficiency to full-length HBc particles. We further found that core particles completely deleted of the arginine-rich domain of HBcAg (residues 150–185) do not bind cells (data not shown), consistent with the role played by this region in capsid cell attachment (14, 15). Thus, residues 150–162 are necessary whereas residues 163–185 are dispensable for core particle cell attachment and entry. Arginine-rich sequences exposed on the shell surface have been suggested to interact with negatively charged heparan sulfate glycosaminoglycans to mediate particle cell attachment (14). Our finding that residues 150–162 of HBcAg are responsible for core particle cell attachment lends further support to this notion because residues 150–159 but not 165–175 of HBcAg were shown to be exposed on the surface of intact core particles (29).

Recombinant HBV core particles have been used as carriers of foreign epitopes and ODN for vaccination and delivery purposes (4, 10–13). Capture of core particles by heparan sulfate proteoglycans followed by their clathrin-mediated uptake and lysosomal trafficking is likely at the basis of these processes. Cathepsin B-mediated cleavage at a position linking the assembly and arginine-rich domains of HBcAg is expected to trigger the release of the encapsulated cargo in the lysosome in a stepwise manner. First, HBcAg cleavage causes detachment of cargo bound by the arginine-rich domain (e.g. ODN) from the particle lattice. Second, because HBcAg cleavage promotes particle dissociation it would eventually allow the physical escape of the encapsulated cargo from the capsid interior. Thus, HBcAg cleavage followed by particle dissociation can facilitate release of the antigenic peptides and encapsulated ODN in lysosomes, allowing peptide loading on class II MHC molecules and TLR9 signaling, respectively. Furthermore, the acidic environment in lysosomes could possibly permit translocation of HBcAg and the delivered cargo to the cytoplasm, as was recently demonstrated for HIV-1 Tat and cationic peptides (25, 32). Cytoplasmic translocation following lysosomal delivery could drive cross-presentation of peptides carried by the particle (9) and could allow delivery of encapsulated macromolecules into the cytosol and the nucleus.

Finally, the possibility that native HBV core particles undergo endocytosis and lysosomal cleavage may have implications for HBcAg immunogenicity during HBV infection. Exogenous HBcAg uptake and processing are important for presentation of HBcAg T cell epitopes on MHC class II molecules (33). Uptake and lysosomal processing by antigen-presenting cells is expected to trigger CD4+ T cell activation, whereas entry and processing by HBV-infected hepatocytes known to express MHC class II but no co-stimulatory molecules is expected to result in immune tolerance (33). Thus, particle uptake and processing during HBV infection is possibly involved in the intricate processes of immune activation and immune evasion by the virus.

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