Capsid Phosphorylation State and Hepadnavirus Virion Secretion

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ABSTRACT The C-terminal domain (CTD) of hepadnavirus core protein is involved in multiple steps of viral replication. In particular, the CTD is initially phosphorylated at multiple sites to facilitate viral RNA packaging into immature nucleocapsids (NCs) and the early stage of viral DNA synthesis. For the avian hepadnavirus duck hepatitis B virus (DHBV), CTD is dephosphorylated subsequently to facilitate the late stage of viral DNA synthesis and to stabilize NCs containing mature viral DNA. The role of CTD phosphorylation in virion secretion, if any, has remained unclear. Here, the CTD from the human hepatitis B virus (HBV) was found to be dephosphorylated in association with NC maturation and secretion of DNA-containing virions, as in DHBV. In contrast, the CTD in empty HBV virions (i.e., enveloped capsids with no RNA or DNA) was found to be phosphorylated. The potential role of CTD dephosphorylation in virion secretion was analyzed through mutagenesis. For secretion of empty HBV virions, which is independent of either viral RNA packaging or DNA synthesis, multiple substitutions in the CTD to mimic either phosphorylation or dephosphorylation showed little detrimental effect. Similarly, phospho-mimetic substitutions in the DHBV CTD did not block the secretion of DNA-containing virions. These results indicate that CTD dephosphorylation, though associated with NC maturation in both HBV and DHBV, is not essential for the subsequent NC-envelope interaction to secrete DNA-containing virions, and the CTD state of phosphorylation also does not play an essential role in the interaction between empty capsids and the envelope for secretion of empty virions.

IMPORTANCE The phosphorylation state of the C-terminal domain (CTD) of hepatitis B virus (HBV) core or capsid protein is highly dynamic and plays multiple roles in the viral life cycle. To study the potential role of the state of phosphorylation of CTD in virion secretion, we have analyzed the CTD phosphorylation state in complete (containing the genomic DNA) versus empty (genome-free) HBV virions. Whereas CTD is unphosphorylated in complete virions, it is phosphorylated in empty virions. Mutational analyses indicate that neither phosphorylation nor dephosphorylation of CTD is required for virion secretion. These results demonstrate that while CTD dephosphorylation is associated with HBV DNA synthesis, the CTD state of phosphorylation may not regulate virion secretion.

KEYWORDS C-terminal domain, capsid protein, dephosphorylation, duck hepatitis B virus, hepadnavirus, hepatitis B virus, maturation, phosphorylation, virion secretion

Hepadnaviruses are small DNA viruses with an icosahedral capsid and an outer envelope (1). This family of viruses includes the human hepatitis B virus (HBV) and the widely used animal model virus, the duck hepatitis B virus (DHBV). Hundreds of millions of people are chronically infected with HBV worldwide and left untreated;

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nearly a quarter of them will die prematurely due to HBV-induced liver diseases, including cirrhosis and hepatocellular carcinoma (2). Hepadnavirus assembly starts with the packaging of the viral pregenomic RNA (pgRNA), a precursor to the genomic DNA, into a replication-competent nucleocapsid (NC). Packaging of pgRNA into NCs is dependent on the viral polymerase protein, a specialized reverse transcriptase (RT), which specifically recognizes the pgRNA and is packaged together with the pgRNA as a ribonucleoprotein complex by the assembling capsid protein subunits (1). Following NC assembly, the RT protein converts the pgRNA first to the single-stranded (minus-strand) DNA and then to a partially double-stranded, relaxed circular (RC) DNA (3). Both the pgRNA- and single-stranded DNA (ssDNA)-containing NCs are considered immature, as they are not enveloped for secretion as virions extracellularly. Only the dsDNA-containing NC, considered mature, is selectively recognized by the viral envelope or surface proteins for envelopment and secretion as complete virions (1, 4). This requirement of dsDNA synthesis for NC envelopment and virion formation is thus a major characteristic of hepadnaviruses (DNA viruses) that distinguishes them from conventional retroviruses (RNA viruses).

The viral capsid is composed of 180 or 240 copies of a single protein, the viral capsid (or core) protein, which is also intimately involved in multiple stages of viral replication in addition to capsid assembly (5). The N-terminal two-thirds (the N-terminal domain [NTD], the assembly domain) of the relatively small core protein, approximately 21 kDa (for HBV) or 32 kDa (for DHBV), forms the capsid shell (5–8), while its highly basic C-terminal domain (CTD) plays essential roles in viral pgRNA packaging and DNA synthesis (9–12). While traditionally thought to be dispensable for capsid assembly, the CTD has also been shown recently to be required to facilitate capsid assembly under physiological conditions (13).

The sequential execution of the different capsid functions in viral assembly and replication is regulated by the dynamic phosphorylation of the CTD (12, 14–20). Both the HBV and DHBV core proteins (HBc and DHbC, respectively) harvested from intracellular NCs are phosphorylated at multiple sites within the CTD (3 major sites plus 3 to 4 minor sites in HBc and six in DHbC) (12, 15, 19, 21–24). However, DHbC in extracellular virions was found to be hypo- or nonphosphorylated (22), although the phosphorylation state of HBc in virions has not been carefully characterized. Detailed mass spectrometry analyses of DHBV NCs harvested from different stages of maturation allowed us to reveal that intracellular mature NCs are already dephosphorylated before envelopment such that mature NCs inside cells as well as in extracellular DHBV virions contain completely dephosphorylated CTD (15). Moreover, genetic analyses of DHbC led us to propose a dynamic CTD phosphorylation model whereby CTD phosphorylation facilitates the early step of pgRNA packaging and/or first (minus)-strand DNA synthesis but its subsequent dephosphorylation is required for the second (plus)-strand DNA elongation and stabilization of mature NCs containing dsDNA (14).

We have recently found that HBV also secretes genome-free virions, which contain the envelope and capsid but no DNA or RNA, *in vitro* and *in vivo* and that these are in fact 100-fold more abundant than the DNA-containing (or complete) virions. The secretion of these empty HBV virions is completely independent of viral RNA packaging or DNA synthesis (25, 26). To support empty virion formation, abundant levels of empty HBV capsids are assembled in human cells, in addition to the aforementioned NCs. In contrast to capsid assembly in bacteria, which leads to packaging of nonspecific RNAs such as the cellular tRNAs in the absence of the RT protein or pgRNA, HBV capsid assembly in human cells does not lead to nonspecific RNA packaging, likely as a result of HBc CTD phosphorylation, which on one hand helps to facilitate specific packaging of the RT-pgRNA complex into NCs and on the other blocks nonspecific RNA packaging when the RT-pgRNA complex is absent or limiting (13, 25). To reconcile the envelopment of mature, dsDNA-containing NCs as well as empty capsids, but not immature pgRNA- or ssDNA-containing NCs, we proposed a single-strand blocking model to explain selective HBV virion morphogenesis, whereby a negative signal is triggered by the presence of the (single-stranded) pgRNA or ssDNA in immature NCs to inhibit their

envelopment (25). In contrast, the absence of any RNA or DNA in empty capsids and the conversion of the ssDNA to dsDNA in mature NCs allow these capsids to be enveloped and secreted as either empty or complete virions. This is in contrast to the classical "maturation signal" hypothesis (4, 27), which suggests that a positive signal emerges on mature NCs as a result of dsDNA synthesis and triggers envelopment of mature NCs selectively, which, of course, does not explain the secretion of empty virions.

To date, the actual signal on capsids that specifies the selective envelopment and secretion of either empty or dsDNA-containing capsids, but not immature NCs, remains to be clearly defined (4, 15, 25, 27, 28). As genome maturation occurs within the NC and envelopment occurs from without, the capsid shell would seem to be ideally situated to transmit the genome information from its interior to the exterior for recognition by the envelope proteins. Mutational analyses of HBc have indeed uncovered a critical role of the NTD specifically in mature NC envelopment and virion secretion. In particular, substitutions of selected residues on the surface of HBV capsids do not impair viral DNA synthesis but block the secretion of DNA-containing virions (29). On the other hand, the role of CTD in virion secretion has been difficult to analyze due to its multiple roles in pgRNA packaging and viral DNA synthesis, which precedes, and is a prerequisite for, the secretion of DNA-containing virions. In the case of DHBV, it is tempting to suggest that CTD dephosphorylation, which is associated with and required for NC maturation as described above, may signal mature NC envelopment; however, there is as yet no evidence in support of this notion.

To determine the functional significance of NC dephosphorylation in virion secretion, we have characterized the state of HBc CTD phosphorylation in empty and complete virions from both cell culture and human blood. We have also determined the capacity for virion secretion by HBV and DHBV mutants in which the CTD phosphorylation sites were replaced by Asp (D) or Glu (E) to mimic constitutive phosphorylation or by Ala (A) to mimic the nonphosphorylated state. Taking advantage of empty HBV virion secretion, we have determined the role of HBc CTD phosphorylation in empty virion formation, independent of its role in viral pgRNA packaging and DNA synthesis. Also, by taking advantage of the fact that DHBc CTD phosphomimetic mutant allows the synthesis and accumulation of low levels of mature dsDNA, we have determined the effects of these mutations on the secretion of DNA-containing DHBV virions.

RESULTS

Development of antibodies that selectively recognized the HBc CTD state of phosphorylation. HBc CTD harbors three major S phosphorylation sites (S155, S162, and S170, all within an SP motif) and four potential minor S/T phosphorylation sites (T160, S168, S176, and S178) (Fig. 1A) (12, 21, 24). In order to define the dynamic state of CTD phosphorylation during the different stages of HBV replication, we have been developing antibodies that can recognize the CTD in a phosphorylation state-dependent manner. We reported recently that one of the CTD-specific monoclonal antibodies (MAbs), 25-7, selectively recognizes nonphosphorylated CTD (13). The 25-7 epitope is not yet mapped precisely but include sequences from position 165 to 182 based on enzyme-linked immunosorbent assay (ELISA) mapping using overlapping peptides as well as Western blot analysis of CTD deletion mutants (13), which includes the major CTD phosphorylation site S170 and three minor sites, S168, S176, and S178 (Fig. 1A). In addition, the CTD antibody C170, generated using an unmodified CTD peptide (positions 170 to 183), harboring also the major S170 site and the two minor sites S176 and S178 (Fig. 1A), preferentially recognizes nonphosphorylated CTD also (13, 30).

We report here two additional CTD MAbs, A701 and B701, that could recognize the HBc CTD in a phosphorylation state-dependent manner (Fig. 1A). A701, a mouse MAb raised against the HBc CTD sequence from position 156 to 176, nonphosphorylated, recognized an epitope within the CTD sequence from position 156 to 164, as determined by ELISA peptide mapping and Western blot analysis of CTD deletion mutants. This region harbors the major phosphorylation site S162 and the minor site T160

FIG 1 Selectivity of HBc antibodies for the phosphorylated or nonphosphorylated CTD. (A) Schematic diagram of the HBc domain structure. The NTD, linker, and CTD are indicated. The CTD sequence (positions 150 to 183) is shown below, with the major and minor sites of phosphorylation highlighted. The epitopes/regions of CTD recognized by the indicated antibodies are also shown. The short dashed horizontal line denotes the uncertain N-terminal boundary of the MAb 6-1. (B) Recognition of CTD phosphorylation state by the CTD antibodies. HBc expressed in the human hepatoma line HepG2 cells (lanes 1 and 3; phosphorylated) or in *E. coli* (lanes 2 and 4; nonphosphorylated) was resolved by SDS-PAGE and detected by Western blotting using the CTD MAbs (B701 [lanes 1 and 2, top] and A701 [lanes 3 and 4, top]), the NTD-specific MAb (lanes 3 and 4, bottom), or the polyclonal HBV antibody (Dako; lanes 1 and 2, bottom). pCTD, phosphorylated CTD. (C) Competition ELISA to determine the epitope of MAb B701. The ELISA plate was coated with the peptide used as the immunogen to generate MAb B701 (HBc positions 155 to 176 [panel A]; all S phosphorylated). The indicated peptides, as competing peptides, were added before MAb B701: P10, pSPRRRTpSPRRRR (HBc positions 155 to 167, S155 and S162 both phosphorylated); P13, pSPRRRTpSPRRRR (HBc positions 155 to 167, S155 phosphorylated); P14, SPRRRTPpSPRRRR (HBc positions 155 to 167, S162 phosphorylated); P15, SPRRRTPSPRRRR (HBc positions 155 to 167, nonphosphorylated). (–), control, peptide corresponding to HBc positions 162 to 175, nonphosphorylated; (+), control, the immunogen peptide; blank, 1% BSA, instead of the primary antibody or competing peptide, was used to coat the wells. Concentrations of the competing peptides are indicated on the right. The concentration of MAb B701 used in the assay was 0.5 μ g/ml.

(Fig. 1A). The selectivity of A701 for the nonphosphorylated CTD was further verified using the full-length HBc; it reacted strongly with HBc expressed in bacteria (nonphosphorylated) but not (or only very weakly) with HBc expressed in human hepatoma cells (mostly phosphorylated) (Fig. 1B, top, lanes 3 and 4) (Longer exposure showed weak A701 signals with HBc from hepatoma cells [see Fig. 3C].) On the other hand, B701 is a mouse MAb prepared using the phosphorylated HBc CTD sequence from position 155 to 176 as the immunogen, with all S phosphorylated (three major sites, S155, S162, and S170, and two minor sites S168 and 176, with T160 being unphosphorylated in the peptide immunogen) (Fig. 1A). ELISA peptide mapping and Western blot analysis using HBc CTD deletion mutants mapped the B701 epitope to within position 155 to 164 (harboring the major S155 and S162 sites and the minor T160 site). Interestingly, competition ELISA using CTD peptides with single (S155 or S162) or double (both S155 and S162) phosphorylation further demonstrated that efficient B701 recognition required simultaneous phosphorylation at both S155 and S162 (Fig. 1C). B701 selectivity for the phosphorylated CTD was further verified using the full-length HBc; it reacted strongly with HBc expressed in human hepatoma cells (phosphorylated) but not (or only very weakly) with HBc expressed in bacteria (nonphosphorylated) (Fig. 1B, top,

lanes 1 and 2). As control antibodies that react with HBc proteins irrespective their CTD state of phosphorylation, the mouse MAb against the N-terminal end of HBc (25, 31) (Fig. 1B, bottom, lanes 3 and 4) and the rabbit polyclonal anti-HBc antibody (Dako) (Fig. 1B, bottom, lanes 1 and 2) readily detected the HBc proteins expressed in either HepG2 cells or *Escherichia coli*.

The HBc CTD state of phosphorylation varied in complete versus empty virions that were released into cell culture supernatant. Taking advantage of the CTD phosphorylation state-selective antibodies, we analyzed the CTD phosphorylation state of capsids in either complete or empty HBV virions harvested from cell culture supernatant of HepAD38 cells (HepG2 cells inducibly replicating wild-type [WT] HBV), which secrete both complete (DNA-containing) and empty virions. WT HBV virions were first fractionated over an isopycnic CsCl density gradient by ultracentrifugation, which we have shown previously to separate, partially, the complete and empty virions based on their buoyant density difference (25). In addition, we transfected HepG2 and Huh7 cells with the Pol[−] HBV mutant, which can secrete only empty virions due to the lack of polymerase expression that is required for both pgRNA packaging and DNA synthesis (25). Empty virions harvested from supernatant of the Pol[−] mutant-transfected cells were also purified on the same CsCl gradient as WT HBV virions. CsCl gradient fractions around the WT virion peaks, as well as the empty virion peak from the mutant HBV-transfected cells, were resolved by native agarose gel electrophoresis followed by Southern and Western blot assays to analyze their DNA and HBc contents. Virion DNA (representing complete virions) peaked in fraction 14 (Fig. 2A [lane 4] and H), whereas total virion HBc detected by the HBc NTD antibody (including both phosphorylated and nonphosphorylated) peaked in the lighter fraction 13 (Fig. 2F [lane 3] and H and 3A [lane 3]), reflecting the lower density of empty virions than of complete virions, as we reported before (25). Fractions lighter than fraction 13 contained little to no DNA, but virion HBc in those fractions was readily detectable by the NTD MAb (and HBs) (Fig. 2F [lanes 1 and 2] and H and 3A [lanes 1 and 2]), representing the presence of mostly or exclusively empty virions with few to no complete virions. As expected, the Pol[−] mutant-transfected HepG2 or Huh7 cells did not secrete any DNA-containing virions but secreted abundant levels of empty virions, and the HBc levels detected by the NTD MAb in these mutant empty virions were similar to those detected by the same MAb in the peak WT HBV (mostly empty with some complete) virions (Fig. 2F [lanes 8 and 9 versus 3] and 3A [lane 8 versus 3]).

The virion HBc profile on the density gradient as detected by the A701, C170, or 25-7 antibody, all selective for nonphosphorylated CTD (Fig. 1) (13), tracked the virion DNA profile generally (Fig. 2B, C, and D versus A [lanes 1 to 7] and H), suggesting that capsids in complete HBV virions were nonphosphorylated, as we reported previously for DHBV (15). This was especially true for the A701 signal, which tracked the virion DNA signal almost perfectly (Fig. 2H). In contrast, HBc in empty virions from either HepG2 or Huh7 cells was not detected by A701 or C170 and was only weakly detectable by 25-7 (Fig. 2B, C, and D [lanes 8 and 9] and 3C [lanes 8 to 10]), indicating that few to no unphosphorylated capsids were contained in empty virions. In contrast, the B701 MAb, which was selective for the phosphorylated CTD (Fig. 1), reacted strongly with HBc from empty virions secreted by the Pol[−] mutant (Fig. 2E [lanes 8 and 9] and 3B [lanes 8 to 10]). For WT virions, B701-reactive HBc peaked in fraction 13 (Fig. 2E [lane 3] and H), the same as the bulk HBc peak detected by the NTD MAb, which contained very little viral DNA (i.e., the empty virion peak fraction), and was also readily detectable in the lighter (i.e., empty virion) fractions (Fig. 2E, lanes 1 and 2) like the NTD MAb-reactive HBc, suggesting that empty virions secreted by WT HBV, like those secreted by the Pol[−] mutant, also contained phosphorylated HBc, which was in agreement with their lack of nonphosphorylated HBc (i.e., negative or very weak signals with the A701, C170, or 25-7 antibody) (Fig. 2B, C, and D, lanes 1 and 2). In contrast, on the denser side of the gradient where the complete virions represented a larger proportion of total virions (as evidenced by the much higher levels of HBV DNA in fractions 15 to 17 than in fractions 11 and 12 [Fig. 2A]), the HBc signal detected by B701 (Fig. 2E, lanes 5 to 7) was similar

FIG 2 State of HBc CTD phosphorylation in cell culture virions as a function of genome content. (A to G) WT HBV virions harvested from induced HepAD38 cell culture supernatant were fractionated on an isopycnic CsCl density gradient. Fractions 11 to 17 were resolved using native agarose gel electrophoresis (lanes 1 to 7). Following transfer to a nitrocellulose membrane, virion-associated DNA, HBc, or HBs was detected by the HBV specific DNA probe (A), the indicated HBc specific antibodies (B to F), or the HBs-specific antibody (G). The peak virion fraction from the CsCl gradient fractionation of virions (all genome free) harvested from culture supernatant of HepG2 (lane 8) or Huh7 (lane 9) cells transiently transfected with the polymerase gene knockout (Pol⁻) HBV construct was also analyzed in parallel. The direction of ultracentrifugation is indicated by the arrow. pCTD, phosphorylated CTD. (H) The signals of viral DNA, HBcAg as detected by the different antibodies, and HBsAg in each individual fraction were quantified and expressed as percentages of the total signals from all the fractions for each parameter.

to or even lower than those detected by A701, C170, or 25-7 (all signals relative to their respective peak fraction) (Fig. 2B, C, and D [lanes 5 to 7] and H), all selective for nonphosphorylated CTD. This was again consistent with the notion that complete virions contained mostly, if not exclusively, HBc that was dephosphorylated at the A701, C170, and 25-7 epitopes, whereas empty virions contained phosphorylated CTD.

Although the empty virions peaked in fraction 13 and the complete virions peaked in fraction 14 (Fig. 2, lane 3 versus 4), empty virions are usually ca. 100-fold more abundant than the complete virions in the culture supernatant of cells replicating WT HBV (25). Therefore, there was no complete separation of the two WT virion populations on the density gradients shown in Fig. 2 or Fig. 3, and empty virions were also present in the later (heavier) fractions of the gradient, the HBc protein in which was readily detected by the phospho-CTD-selective antibody B701 (Fig. 2E [lanes 4 to 7] and

FIG 3 State of HBc CTD phosphorylation in HBV virions and naked capsids. Viral particles released to the culture supernatant of HepG2 cells transfected with the WT (lanes 1 to 6) or Pol[−] (lanes 8 to 13) HBV were fractionated by CsCl density gradient centrifugation. Selected virion fractions (WT, lanes 1 to 6; Pol[−], lanes 8 to 10) or naked capsid fractions (Pol[−], lanes 11 to 13) were resolved by agarose gel electrophoresis and detected by Western blotting using the indicated NTD or CTD MAbs. Cytoplasmic lysate from WT (lane 7) or Pol[−] (lane 14) HBV-transfected cells was analyzed in parallel. The direction of ultracentrifugation is indicated by the arrow. pCTD, phosphorylated CTD. V, virion; Ca, capsid. *, cross-reactive background signal detected by the A701 MAb.

3B [lanes 5 and 6]). As expected, both complete and empty virion fractions had abundant HBsAg (Fig. 2G), which represented both envelope proteins on the outer surface of virions (both complete and empty) and HBsAg spheres and filaments that are devoid of the viral genome or capsid (25).

CTD-phosphorylated HBc, as detected by MAb B701 (and the NTD MAb), was also associated with naked (nonenveloped) capsids, both intracellular (lysate) (Fig. 3A and B, lanes 7 and 14) and extracellular (the naked capsid fractions in the CsCl gradient) (Fig. 3A and B, lanes 11 to 13), in both WT HBV and the Pol[−] mutant (Fig. 3A and B and data not shown). In contrast, whereas at least some WT naked capsids contained nonphosphorylated HBc CTD as detected by MAb A701 (Fig. 3C, lane 7), little to no A701 signal could be detected with the Pol[−] mutant (Fig. 3C, lanes 11 to 14). This result was thus consistent with the notion that HBc dephosphorylation (at least within the A701 epitope) was associated with, and was likely required for, capsid maturation in WT HBV, as we have shown before in DHBV (14, 15).

HBc CTD phosphorylation mutants were competent for empty virion secretion.

The above results on the phosphorylation state of HBV virions, as well as our previous studies using DHBV (14, 15), indicated that NC dephosphorylation, for both HBV and DHBV, is correlated with maturation and secretion of complete (DNA-containing)

FIG 4 Virion secretion by HBc CTD phosphorylation mutants. Viral particles released to the culture supernatant of HepG2 cells cotransfected with an expression plasmid (pCI-HBc) for the WT HBc (lanes 1 to 4) or the CTD mutant HBc 3A (lanes 6 to 9) or 3E (lanes 11 to 14) plus the core defective genomic HBV construct (pSVHBV1.5C⁻) were fractionated by CsCl density gradient centrifugation. Selected fractions (12 to 15) were resolved by agarose gel electrophoresis and transferred to a nitrocellulose membrane. Viral DNA and HBc protein were detected by a radiolabeled HBV DNA probe (A) and the HBc NTD-specific MAb (B), respectively. Cytoplasmic lysate from the WT (lane 5)-, 3A mutant (lane 10)-, or 3E mutant (lane 15)-transfected cells was analyzed in parallel. The direction of ultracentrifugation is indicated by the arrows. V, virion; Ca, capsid.

virions. Therefore, we were interested in determining if core protein dephosphorylation might indeed represent the long-sought-after maturation signal that is required to trigger mature NC envelopment and virion secretion. However, as introduced above, the dynamic state of CTD phosphorylation plays multiple roles in pgRNA packaging and DNA synthesis, steps preceding and prerequisite for complete virion secretion. As a result, it has remained difficult to decipher the role of the CTD phosphorylation state in the secretion of complete virions. On the other hand, the secretion of empty virions, which depends on neither pgRNA packaging nor viral DNA synthesis, afforded us the opportunity to test the role of the HBc CTD phosphorylation state in the secretion of empty HBV virions.

We thus transfected constructs to express the WT HBc or mutants with all three major sites of phosphorylation changed to either A (3A) or E (3E) (Fig. 1A). An HBV genomic construct defective in HBc expression but otherwise competent to initiate HBV replication and secretion was cotransfected into hepatoma cells together with the WT or mutant HBc expression construct to engender viral replication and assembly through *trans*-complementation. Virions in the culture supernatant of the transfected cells were fractionated over a CsCl gradient as described above (Fig. 2 and 3). As reported earlier (17, 32), the 3A or 3E mutant, in contrast to the WT HBc, was unable to support viral DNA synthesis so that little to no virion DNA was detectable from either 3A- or 3E-transfected cells in the fractions that contained HBV virion DNA in the case of WT HBc (Fig. 4A). However, abundant virion HBc was detected by the NTD MAb in the case of 3A or 3E, similar to the case for WT HBc (Fig. 4B). Furthermore, when all seven potential phosphorylation sites (three major plus four minor) in the CTD (Fig. 1A) were replaced with either A (7A) or E (7E), empty virion secretion still occurred in the absence of detectable virion DNA (data not shown), again supporting the notion that the CTD state of phosphorylation did not play a significant role in empty virion secretion.

HBV virions in patient blood contained HBc with a state of CTD phosphorylation that was dependent on their DNA content. As we reported recently, empty HBV virions are present in large excess (100-fold or more) over complete virions in the blood of HBV-infected patients, just as in cell culture supernatant or in the blood of experimentally infected chimpanzees (25, 26). Furthermore, upon antiviral treatment with RT

FIG 5 Loss of DNA-containing HBV virions in patient sera is associated with loss of nonphosphorylated HbC but not phosphorylated HbC. Serum samples at baseline (BL) and at the indicated weeks (w) posttreatment from two chronically HBV infected patients (001 and 003) were resolved by agarose gel electrophoresis. (A to H) HBV virion DNA (A) and virion HbC detected by the indicated antibodies (B to H) were detected as for Fig. 1. *, cross-reactive background signal detected by the A701 MAb. (I) The HbC signals as detected by the different antibodies at each time point for the two different patients were quantified and expressed as percentage of the baseline (BL) signals before treatment (relative amount).

inhibitors, HBV virion DNA levels in the blood dropped dramatically (by a millionfold or more), while virion HbC did not change or only decreased slightly (by ca. 2-fold) in most patients (26). Thus, we were interested in determining the CTD phosphorylation state in HBV virions in patient blood both at baseline and following antiviral treatment. We selected two different patients (001 and 003) (26) who were treated with the RT inhibitor tenofovir and experienced a dramatic loss of serum HBV DNA but almost no change in total (i.e., both phosphorylated and nonphosphorylated) virion HbC for analysis here. For both patients, total HbC in virions as detected by the polyclonal anti-HbC or the NTD MAb was present at high levels and changed little during therapy (Fig. 5B, C, and I), despite a dramatic loss (by over a millionfold) in viremia (i.e., blood virion DNA) (Fig. 5A) (26). Since most virions (ca. 99%) were DNA free even at baseline (26) and virtually all were empty after treatment, the serum total HbC levels essentially represented empty virions at all time points. When virion HbC was detected with the phospho-CTD-specific MAb B701 (Fig. 5D and I), its levels closely matched the total HbC signals (as detected by the Dako polyclonal antibody and the NTD MAb), indicating that most, if not all, empty virions in patient blood, as in cell culture supernatant as described above, contained phosphorylated CTD (at least within the B701 epitope).

In contrast, when blood virion HbC was detected with the non-phospho-CTD-selective MAb A701, it was decreased substantially following treatment, by ca. 10-fold in one patient (001) and by 10- to 30-fold in the other patient (003) (Fig. 5E and I), thus mimicking the loss of serum HBV DNA. However, the decrease in A701-reactive HbC signal following antiviral treatment, though much more significant than the decline of the HbC signal detected by the other HbC antibodies used here, was still not nearly as dramatic as the loss of complete virions (again, virion DNA levels decreased by over a

FIG 6 Density gradient analysis of HBV virions in a patient's serum. Serum from a chronically HBV-infected patient (003) was fractionated on a CsCl density gradient. Fractions around the virion peak were resolved by agarose gel electrophoresis and transferred to a nitrocellulose membrane. Virion DNA, Hbc protein, and HBs proteins were detected by a radiolabeled HBV DNA probe (A), the indicated Hbc-specific antibodies (B to E), and the S-specific antibody (F), respectively. The direction of ultracentrifugation is indicated by the arrows.

millionfold) in these patients (26), suggesting that empty virions in patient blood might contain some capsids with the A701 epitope also nonphosphorylated; i.e., nonphosphorylated CTD might be present not only in complete virions but also in some empty virions in patient blood. Interestingly, virion Hbc detected by C170 decreased only slightly (by ca. 3-fold) during treatment and mostly tracked the total Hbc and phospho-CTD (B701) signals (Fig. 5F and I). This suggested that the Hbc CTD in empty virions in patient blood was unphosphorylated to a greater degree at the C170 epitope (at the end of the Hbc with one major site of phosphorylation S170 and two minor sites S176 and S178 [Fig. 1A]), in contrast to the N-terminal part of CTD (i.e., within the A701 and B701 epitopes), which was mostly phosphorylated (reacted much more with B701 than with A701). Virion Hbc detected by MAb 25-7 was also decreased (by ca. 10-fold) following antiviral treatment (Fig. 5G and I), almost to the same extent as that detected by A701 (Fig. 5E and I), suggesting that the CTD site within the 25-7 epitope (Fig. 1A) was also dephosphorylated in complete virions although it was partially dephosphorylated in empty virions from patient blood.

It was reported before that Hbc contained in DNA-free virions in patient blood have an abnormal Hbc-related antigen (the so-called HBcrAg) that was claimed to have an N-terminal extension and CTD truncation relative to Hbc (33). Our results above using several different CTD antibodies clearly indicated that the CTD was intact in Hbc in empty virions. To determine if Hbc in empty virions has any CTD truncation at all, we took advantage of our recently developed MAb, 6-1, whose epitope includes the very C-terminal residue (183) (Fig. 1A) (13). The serum virion Hbc was clearly detected by MAb 6-1 (Fig. 5H), indicating that the majority, if not all, Hbc from empty virions in patient blood had an intact C terminus. Since MAb 6-1 also detects nonphosphorylated CTD (13), the modest drop in the 6-1 signal during antiviral therapy suggested that the 6-1 epitope, containing one or two minor phosphorylation sites at the very end of CTD (S175/S178 [Fig. 1A]), was also less phosphorylated in complete virions than in empty virions, although it was also dephosphorylated in empty virions to some extent (Fig. 5I).

To further characterize the Hbc CTD phosphorylation state in virions from patient blood, virions in the baseline serum sample from patient 003 (Fig. 5) were analyzed by CsCl gradient fractionation. As shown for cell culture-derived virions (Fig. 2 and 3), dephosphorylated Hbc in serum virions, as detected by A701 (Fig. 6D), tracked the virion DNA (Fig. 6A) on the gradient, peaking in the denser fraction 17 and being undetectable in the lighter fraction 15. This further supported the conclusion above that complete virions in patient serum, like those in cell culture supernatant, contained

nonphosphorylated CTD at the A701 epitope. In contrast, phosphorylated CTD, as detected by B701 (Fig. 6C), followed the total virion HBc signal (as detected by the anti-NTD MAb) (Fig. 6B), peaking in fraction 16 and being more abundant in the lighter fraction 15 than in the heavier fraction 17. This again supported the notion that empty virions in patient serum, like those in cell culture supernatant, contained phosphorylated CTD. Also as described above for cell culture empty virions, serum empty virion HBc signal detected by the MAb 25-7 showed an intermediate distribution profile over the density gradient between those of A701 and B701, being strong in both the virion DNA peak and empty virion peak (Fig. 6E, fraction 16 and 17), suggesting that empty virions in patient blood might contain some CTDs that were not phosphorylated at the 25-7 epitope (similar to DNA-containing virions), as suggested above from the analysis of unfractionated patient sera (Fig. 5G). As expected, the HBs signals, mostly representing HBsAg spheres and filaments (i.e., no HBc or genome), peaked in the lighter fraction 15 (Fig. 6F, lane 2), which was even lighter than the empty virion peak (fraction 16), which contained HBs (Fig. 6F, lane 3), high levels of HBc (Fig. 6B and C, lanes 3), and little HBV DNA (Fig. 6A, lane 3).

Virion secretion by DHBC CTD phosphorylation mutants. To test the role of CTD phosphorylation state in the secretion of complete (DNA-containing) virions of hepadnaviruses, we turned to DHBV. As we reported earlier, DHBC in complete WT virions is completely dephosphorylated (15), suggesting that DHBC dephosphorylation may be required for virion secretion. To test this idea, we analyzed a number of our previously described DHBC CTD phosphorylation mutants (14) for their potential effects on virion secretion, using a *trans*-complementation assay whereby an expression plasmid for either the WT or mutant DHBC protein was cotransfected with a DHBC-defective but otherwise functional genomic construct (14). As we reported before (14), the S/T-to-D substitutions in the DHBC CTD were severely defective in the production and stability of mature NCs containing RC DNA (Fig. 7). However, some mature NCs still were accumulated by these phosphomimetic DHBC mutants, thus providing the opportunity to test whether these mutants, which mimic constitutive phosphorylation but are unable to undergo dephosphorylation normally associated with NC maturation, were blocked in the secretion of DNA-containing DHBV virions.

As we reported before (14), the S/T-to-D substitutions mutants shown in Fig. 7 affected the integrity of the mature NCs such that exogenous nuclease treatment, routinely used during extraction of NC-associated viral DNA to remove DNA not protected by NCs (e.g., plasmid DNA) but not viral DNA inside NCs, led to a substantial underestimation of intracellular mature NCs formed by the mutants. This was especially true for the SSDDDD and DDDSDS mutants (Fig. 7A [lanes 4 versus 5 and 19 versus 20], B [lanes 4 versus 5 and 7 versus 8], and C) (14). Therefore, to measure the levels of total mature NCs (stable and unstable), we extracted intracellular NC DNA using two different methods, with (C1) or without (C2) (Fig. 7A and B) the nuclease digestion (14, 34). The amount of virion RC DNA secreted by each mutant was then compared to the intracellular RC DNA extracted by either method (Fig. 7C). To more precisely examine the maturity of the intracellular NC DNA and extracellular virion DNA, we also measured the levels of full-length (3 kb) plus strands (upon denaturation of the RC DNA and the minor double-stranded linear DNA [dsIDNA]) in intracellular NCs and purified virions by first denaturing the viral DNA and then detecting the plus strands selectively using a strand-specific riboprobe (Fig. 7B).

As shown in Fig. 7, all the S-to-D mutants tested allowed complete virion secretion. When the virion DNA signal was normalized to intracellular viral DNA extracted with nuclease digestion, the virion secretion efficiency, defined by the ratio of extracellular RC DNA (V) (complete virions) versus intracellular RC DNA (C1) (mature NCs, following nuclease digestion), by the core mutants was in fact up to 3- to 4-fold higher than that of the WT DHBC (Fig. 7B [lanes 6 versus 4 for SSDDDD and 9 versus 7 for DDDSDS, compared to lane 3 versus 1 for the WT HBc] and C). This was due to the underestimation of intracellular mature DNA extracted from the mutant mature NCs using this

FIG 7 Secretion of DHBV virions by DHBc CTD phosphorylation mutants. The chicken hepatoma LMH cells were cotransfected with the core-defective DHBV genome pCMV-DHBV/C⁻ together with the WT (lane 1 to 3) or indicated mutant (lane 4 to 21) core expression plasmid. (A) Viral DNA from the peak CsCl virion fraction (V) (lanes 3, 6, 9, 12, 15, 18, and 21) or from the intracellular NCs isolated with (C1) (lanes 1, 4, 7, 10, 13, 16, and 19) or without (C2) (lanes 2, 5, 8, 11, 14, 17, and 20) micrococcal nuclease (MNase) digestion was analyzed by Southern blotting hybridization using a DHBV DNA probe. RC, relaxed circular DNA; DSL, double-stranded linear DNA; SS, single-stranded DNA. Note that 5-fold more DNA, relative to that from the wild type (lanes 1 to 3), was loaded for all the core mutants (lanes 4 to 21) to compensate for their lower levels of DNA synthesis and accumulation. (B) Analysis of secreted plus-strand DHBV virion DNA. LMH cells were cotransfected with the core-defective DHBV genome pCMV-DHBV/C⁻ together with the indicated wild-type (WT) or mutant (SSDDDD and DDDSDS) core expression plasmid. Viral DNA isolated from the peak CsCl virion fraction (V) (lanes 3, 6, and 9) or from intracellular NCs with (C1) (lanes 1, 4, and 7) or without (C2) (lanes 2, 5, and 8) MNase digestion was heat denatured prior to Southern blot analysis using a riboprobe specific for the plus-strand DNA. Heat-denatured (single-stranded) DNA markers are indicated on the left. (C) Summary of results. RC¹ and RC², RC DNA from intracellular NCs isolated with or without MNase digestion, respectively. +++++, 100% (WT); +++, 10% to 100% of WT; ++, 1% to 10% of WT; +, 0.1% to 1% of WT; -, <0.1% of WT.

method, resulting from their elimination (but not the WT NCs) during the nuclease digestion procedure as described above. When normalized to the intracellular viral DNA extracted without the nuclease digestion, virion DNA secretion efficiency by the mutants was similar to that by the WT DHBc (Fig. 7A to C), indicating that these phosphomimetic substitutions in the DHBc CTD, while destabilizing mature NCs, did not significantly impair their envelopment and virion secretion (Fig. 7C). Furthermore, no immature DNA (ssDNA) was secreted by any of these mutants (Fig. 7A, lanes 6, 9, 12, 15, 18, and 21), and the secreted virions contained predominantly full-length plus-strand DNA like the WT HbC (Fig. 7B and data not shown). Therefore, DHBc CTD dephosphorylation, while associated with and required for intracellular NC maturation (14, 15), was not essential for the selective interaction of mature NCs with the envelope proteins for virion secretion. The A substitution mutants showed little to no viral DNA synthesis, as we reported before (14); their effects, if any, on complete DHBV virion secretion could not be tested (Fig. 7C).

FIG 8 Differential phosphorylation state of the HBc CTD in complete versus empty HBV virions. The thick-lined hexagons symbolize the RC DNA (double-lined circles in the hexagons)-containing (i.e., mature) NC and the empty capsids, both sharing a structural property allowing their envelopment, which is absent from the immature NCs (hexagons with thin lines) containing either the pgRNA (wavy line) or ssDNA (single-lined circle inside the hexagons). The immature NCs instead may display a single-strand blocking signal, signified by the thin outlines, that inhibits their envelopment. The large circles surrounding the capsid signify the viral envelope (Env). P, phosphorylated CTD.

DISCUSSION

Through the analysis of HBV virions harvested from cell culture as well as from patient blood, we have demonstrated here that the highly dynamic and functionally critical HBc CTD is phosphorylated in empty (genome-free) virions but dephosphorylated in DNA-containing virions. Secretion of empty HBV virions, without the need for pgRNA packaging or DNA synthesis, also allowed us to determine the role of the CTD phosphorylation state in empty virion secretion, without the complication of CTD effects on RNA packaging and DNA synthesis, which are prerequisites for secretion of complete virions. We have shown that substitutions in the CTD to mimic constitutive phosphorylation or dephosphorylation in the three major or all seven potential S/T sites had little effect on secretion of empty HBV virions, suggesting that the state of HBc CTD phosphorylation does not play a major role in determining empty virion secretion (Fig. 8). Moreover, we have also demonstrated that phosphomimetic substitutions at all known phosphorylation sites in the DHbC CTD also did not block secretion of DHBV virions containing viral DNA. With the caveat that E or D substitutions may not completely mimic phosphorylated S/T residues, these results indicate that CTD dephosphorylation, though associated with NC maturation in both DHBV and HBV, is not essential for subsequent envelopment of mature NCs during complete virion secretion or envelopment of empty capsids during empty (genome-free) virion secretion.

Instead, our results are consistent with the notion that some aspect of capsid conformation may be involved in regulating capsid-envelope interactions for virion formation. Assuming that formation of empty (genome-free) and complete (dsDNA-containing) virions share a similar mechanism, empty and dsDNA-containing capsids should share some structural property, which is absent from the RNA or ssDNA capsids (Fig. 8). As complete virions contain dephosphorylated capsids while empty virions contain phosphorylated capsids, the state of capsid phosphorylation would unlikely to be a candidate for such a common property involved in triggering capsid envelopment. On the other hand, we have reported recently that both mature HBV NCs and empty HBV capsids are less stable than immature NCs (containing pgRNA or ssDNA) (35), hinting at some common conformational (destabilizing) feature of these virion formation-competent capsids, which is absent from those that are unable to form virions. Interestingly, the hyperdestabilization of mature NCs induced by multiple S-to-D substitutions in the DHbC CTD mutants apparently did not preclude the generation of the secretion (maturation) signal in the context of the maturation signal hypothesis (4, 27) or the sequestration of the single-strand blocking signal on immature NCs in the context of the single-strand blocking model of virion secretion (25).

The CTD state of phosphorylation during the different stages of viral replication and assembly is highly dynamic and remains to be more fully characterized, including the exact stoichiometry of phosphorylated versus nonphosphorylated CTD in empty capsids or different populations of intracellular NCs at different stages of maturation or in

extracellular empty versus complete virions. Our previous analysis of DHBc indicated that mature NCs, both intracellular and in extracellular virions, are completely dephosphorylated (15). Whether this is also the case for HBV remains to be determined, but our results here indicate that the general trend of maturation-associated NC dephosphorylation also holds true for HBV (Fig. 8). Given the large excess of empty HBV virions that always accompanies complete virions, it will be challenging to isolate the minority of complete virions, which contain the mature NCs, away from the excess empty virions for detailed biochemical analysis. Our results here further suggest that the individual phosphorylation sites in the HBc CTD may also have states of phosphorylation that are different from each other. Thus, the N-terminal part of CTD, containing the epitopes for A701 and B701, including two of the three major CTD phosphorylation sites, S155 and S162 (Fig. 1A), was heavily phosphorylated in empty capsids (and empty virions) and likely immature NCs, since they barely reacted with A701 but strongly reacted with B701. As a loss of complete virions in the patient serum samples (Fig. 5), which accounted for only ca. 1% of total virions (26), could lead to a decrease of the A701 signal by 10- to 30-fold (Fig. 5I), the fraction of A701-reactive HBc in complete virions from the human sera, albeit detectable, was very small (ca. 0.1% or less of the total virion HBc). On the other hand, two antibodies, 25-7 and C170, both selective for nonphosphorylated CTD (toward its C-terminal part [Fig. 1A]), could clearly detect capsids in empty virions from patient sera (Fig. 5). C170 reacted with empty virions in patient sera more strongly than 25-7, suggesting that significantly more HBc in empty virions was unphosphorylated at the C170 epitope than at the 25-7 epitope. Although we have not specifically used antibodies selective, individually, for the phosphorylated S170, a major CTD phosphorylation site, and each of the three minor sites (S168, S176, and S178), which are potentially within the 25-7 and C170 epitopes (Fig. 1A), it was reported decades ago that an MAbs targeted to this part of the CTD (positions 165 to 175) and selective for the phosphorylated state (i.e., phosphorylation at S168 and/or S170) readily detected serum HBV virions (36), which, although it was unknown at the time, are predominantly empty. These results together indicate that capsids in empty HBV virions were predominantly phosphorylated at the CTD, although some were unphosphorylated, especially toward the C-terminal end of the CTD. The levels of nonphosphorylated CTD, as detected by all three CTD antibodies selective for the nonphosphorylated CTD (A701, C170, and 25-7) (Fig. 1A), also appeared to be higher in serum empty virions than empty virions from HepG2 cells, since the empty virion HBc from cell culture supernatant was either undetected (by A701 and C170) or detected at only low levels (by 25-7) (Fig. 2 and 3), whereas the empty virion HBc from the human sera was more strongly detected (C170 > 25-7 > A701) (Fig. 5 and 6). Thus, the exact state of CTD phosphorylation in intracellular capsids and extracellular virions may also be influenced by the host cell environment, which of course is not unexpected given that host kinases/phosphatases are responsible for CTD (de)phosphorylation. Further studies using additional phosphorylation state-selective antibodies targeting each CTD site and detailed mass spectrometry mapping (15) will help to more completely define the dynamic state of CTD phosphorylation during viral replication and assembly, in a CTD phosphorylation site-specific manner.

In contrast to HBV virions, which contained both phosphorylated HBc CTD (in empty virions) and dephosphorylated CTD (in complete virions), DHBc contained in virions is all dephosphorylated (15). These results suggest that either DHBV does not secrete empty virions (i.e., all NCs inside DHBV virions contain dsDNA and thus with dephosphorylated DHBc CTD) or if DHBV secretes empty virions, they contain only dephosphorylated DHBc, in contrast to the case for HBV. Although future studies will be required to resolve these alternatives, we have recently obtained preliminary evidence in support of the former possibility; i.e., DHBV does not secrete empty virions (X.N. and J.H., unpublished results). Studies to elucidate this interesting difference in virion secretion between these two related hepadnaviruses are ongoing.

MATERIALS AND METHODS

Plasmids. Plasmids pCI-HBc, -3A, -3E, -7A, and -7E, for expression of WT or mutant HBc (13), and pSV-HBV1.5/C⁻ expressing an HBc-defective HBV genome (29) were described before. pCMV-DHBV/C⁻, a genomic DHBV construct defective in DHBc expression, and pCMV-DHBVΔXM, expressing WT DHBc, were also described previously (14). The DHBc CTD phosphosite mutations were introduced into pCMV-DHBVΔXM by replacing the phosphorylation site residue S or T by D as described previously (14).

Antibodies. The HBc NTD-specific MAb targeted to the very N-terminal end of HBc (25) and the two HBc CTD antibodies, 25-7 and C170, both preferentially reacting with nonphosphorylated CTD, have been described previously (13). Another CTD-specific MAb, 6-1, targeted to the extreme C-terminal end of HBc, has also been described (13). The rabbit polyclonal antibody against HBc was from Dako. Additional HBc CTD-specific antibodies (A-701 and B701) were made for this study (see Results for details).

Transient transfection and analyses of core protein expression and viral DNA synthesis. HBc expression constructs and/or HBV genomic constructs were transfected into the human hepatoma cell line HepG2 or Huh7 as previously described (37, 38). For analysis using DHBV, the chicken hepatoma cell line LMH was transfected by the calcium phosphate method using pCMV-DHBVΔXM plus pCMV-DHBV/C⁻, as previously described (14). Transfected cells were harvested on day 5 posttransfection. Core proteins in the cell lysates were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described previously (14, 25). Core DNA from NCs was isolated from cytoplasmic lysate either with nuclease digestion or without nuclease digestion and analyzed by Southern blotting as described previously (14, 39). Core DNA from virions was isolated as described previously (40). A genome-length, ³²P-labeled HBV or DHBV DNA probe was used to detect the viral DNA replicative intermediates by Southern blotting. Where indicated, ³²P-labeled strand-specific riboprobes were used to detect either the minus-strand or plus-strand DNA.

Isolation of viral particles released into cell culture supernatant. To isolate and analyze the enveloped DHBV virion particles, we applied the two-step stringent procedure described previously (40). Cell-free culture medium was first precipitated with polyethylene glycol (PEG) and subjected to pronase and nuclease digestion to remove plasmid DNAs and naked NCs. The pronase- and nuclease-treated precipitate was then subjected to CsCl isopycnic gradient ultracentrifugation to fractionate virion particles. For HBV, pronase digestion proved to be ineffective to remove naked NCs and so was omitted; only DNase digestion was used (to remove plasmid DNAs) before the CsCl gradient fractionation (25). In selected experiments as indicated, the nuclease-treated PEG precipitate was directly analyzed without the second step of CsCl centrifugation, as described before (25). HBV virions in human serum samples was fractionated on the CsCl gradient as previously described (25, 26).

Native agarose gel electrophoresis for analyzing NC assembly and virion secretion. Native agarose gel electrophoresis of intact NCs from cell lysate, virion particles obtained after pronase and/or nuclease digestion of PEG-precipitated culture medium or following CsCl gradient fractionation, or unfractionated or fractionated human serum samples from HBV-infected patients (26) was carried out by using previously reported procedure (14, 25). Viral DNA was detected using a DHBV or HBV DNA probe, and the same membranes were subsequently probed with the indicated core- or surface-specific antibody to detect the core or surface proteins, as previously described (25, 26).

ELISA. ELISA (96-well) plates were coated with peptides (100 ng/well) overnight at 4°C. After washing unbound peptides, the wells were blocked with 2% (wt/vol) bovine serum albumin (BSA) (200 μl/well) at 37°C for 2 h. Primary antibodies were then added and incubated at 37°C for 1 h. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (secondary antibody) was then incubated at 37°C for 1 h. The HRP substrate, 3,3',5,5'-tetramethylbenzidine (TMB), was then added for color development, and the optical densities at 450 nm (OD₄₅₀) were read. For competition assays, competing peptides were added before the primary antibodies.

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