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Conserved aromatic residues of the hepatitis B virus Precore propeptide are involved in a switch between distinct dimeric conformations and essential in the formation of heterocapsids

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ABSTRACT

The Hepatitis B virus Precore protein, present in the secretory pathway as the HBeAg precursor, can associate in the cytoplasm with the Core protein to form heterocapsids, likely to favor viral persistence. Core and Precore proteins share their primary sequence except for ten additional aminoacids at the N-terminus of Precore. To address the role of this propeptide sequence in the formation of Precore heterocapsids, we designed a Precore mutant in which the two propeptide tryptophans are replaced by glycines. This mutant retains the properties of the wild-type Precore, notably cell trafficking and ability to interact with Core. However, it is not incorporated into heterocapsids and forms stable dimers distinct from the labile HBe dimers and the presumably Core-like dimers assembled into heterocapsids. Our data highlights the essential role of Precore's propeptide in switching between different conformations for different functions and pinpoint the propeptide Tryptophan residues as central in these properties.

Introduction

The hepatitis B virus is an enveloped DNA virus with an icosahedral capsid composed of a single protein, the Core protein (Summers et al., 1975). The capsid encloses the viral DNA, covalently linked to the viral polymerase (Bartenschlager and Schaller, 1988). The Core protein (183 residues, Fig. 1A) is encoded by the same PreC/C open reading frame as the Precore protein precursor. They are translated from different in-frame AUG codons and consequently share the Core primary sequence, with an extended amino-terminal region of 29 amino acids (aa) for the Precore protein precursor. By convention the first residues of Core and Precore precursor are numbered (+1) and (−29), respectively (Fig. 1A). Translation from the Precore AUG leads to the Precore protein precursor (P25), which is co-translationally directed to the secretory pathway by a 19-amino acid signal peptide. The latter is cleaved during translocation of the nascent chain into the lumen of the endoplasmic reticulum (ER), leading to the Precore mature protein (22 kDa) (Ou et al., 1986). Thus, Precore differs from Core by two features: (i) an extended 10 aminoacids N-terminal sequence and (ii) a different intracellular localization since it is present in the secretory pathway while Core is a cytoplasmic protein. However, about 15% of Precore is released back to the cytosol by hijacking the ER-associated degradation pathway (Duriez et al., 2008; Guidotti et al., 1996; Messageot et al., 2003; Ou et al., 1989), while the majority of the Precore molecules are processed by removal of the C-terminal 29 residues to give rise to the secreted HBe (Messageot et al., 2003).

One important feature of HBV is its capacity to maintain a viral persistence. To achieve this goal, HBV has developed different strategies including immune escape, a process in which HBe appears as a key viral protein (Dandri and Locarnini, 2012; Milich et al., 1998). However, another precore gene product could be involved in a different mechanism favouring persistent infection. Indeed, several data indicate that the Precore protein present

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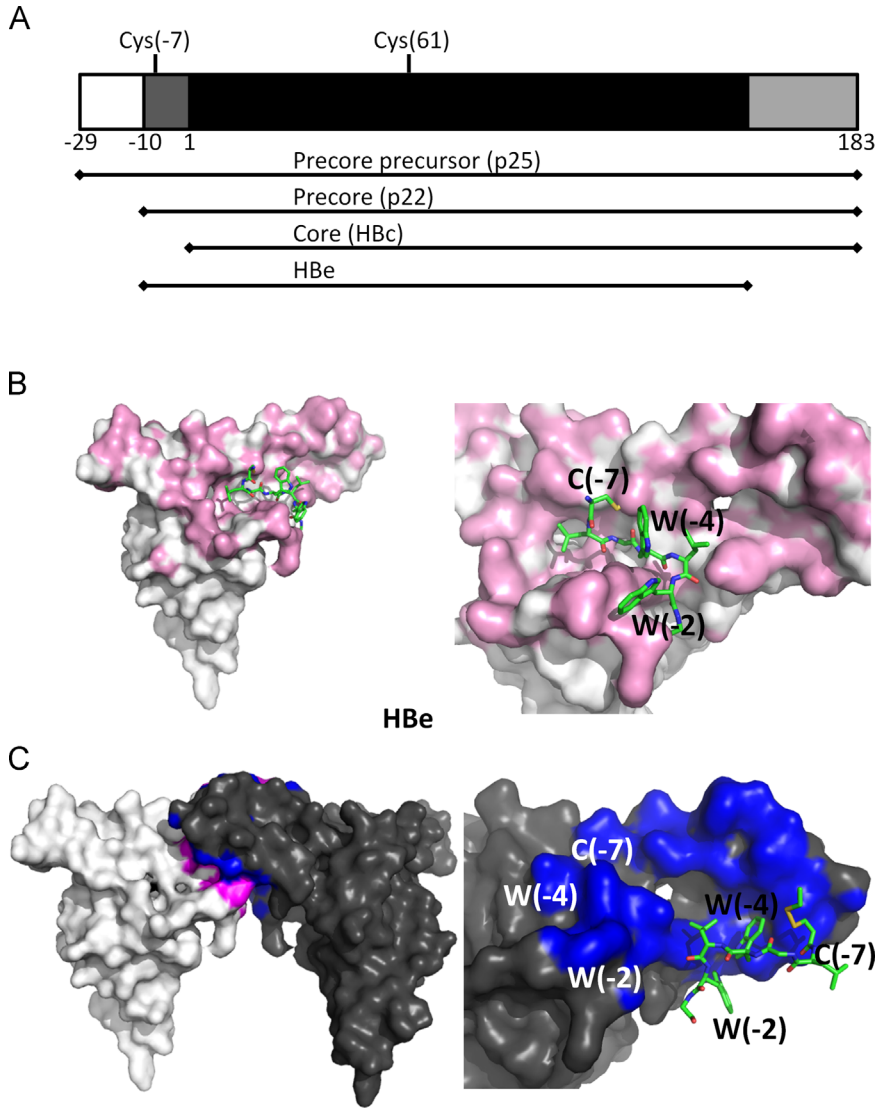


Fig. 1. Proteins encoded by the PreC/C open reading frame and propeptide location in the HBe structure. (A) Schematics of PreC/C gene products. Cysteines −7 and 61 that form an intramolecular disulfide bond in HBe are indicated. (B) Surface representation of a single HBe molecule with its propeptide in stick representation. Atoms that would be buried in a Core dimer interface are colored pink. Note that tethering of Cys(−7) through its disulfide bond leads to the propeptide covering part of this pink surface. (C) The HBe dimer. Left, surface representation of the dimer with the interacting surfaces colored magenta and blue. Note that the interface is much smaller than in the Core dimer, in accordance with its lower stability. Right, the tripeptide (−4)WLW(−2) provides a key hydrophobic bulge at the periphery of the HBe dimer interface. For clarity, the white molecule is hidden except for the propeptide that is in stick representation as in B/. The dimer has been rotated to view the propeptide from the opposite side with respect to (B).

in the cytoplasm regulates viral multiplication negatively. In this regard, HBV replication was reduced strongly in HBV transgenic mice crossed with animals overexpressing the Precore protein (Guidotti et al., 1996). Moreover, mutations leading to an abolition of Precore gene expression resulted in a significant increase of HBV replication in transfected cells (Lamberts et al., 1993; Scaglioni et al., 1997a). Finally, when Precore deleted of its signal peptide was over-expressed in cells, Core–Precore immature capsids were observed suggesting that the formation of unstable nucleocapsids containing cytosolic Precore would be a means to reduce viral secretion (Scaglioni et al., 1997b).

In this paper, we focused our attention on the role of the N-terminal 10 aa sequence of Precore, here referred to as the propeptide sequence, that is almost identical in all orthohepadnaviruses (Revill et al., 2010). Recent data relative to HBe, the processed and secreted form of Precore, have attributed two functions to this unique region. First, as it contains a Toll/IL-1 receptor motif it could allow HBe to interfere with the innate immune system (Lang et al., 2011). Second, crystallographic studies of HBe expressed in *Escherichia coli* have revealed that the propeptide induces a spectacular quaternary structure switch in HBe relative to Core (DiMattia et al., 2013). Indeed, whereas the tertiary structures of the two proteins are very similar and they both form dimers (Watts et al., 2011), the second subunit is rotated by 140° in the HBe dimer relative to the Core dimer in the capsid structure. This radical change is directly caused by the propeptide that (i) shields atoms that would be engaged in the Core dimer interface, thus preventing Core-like dimerization (Fig. 1B) (ii) is itself largely buried in the HBe dimer interface (Fig. 1C). Central to these features are (i) an intramolecular disulfide bond between Cys(−7) and Cys(61) (Nassal and Rieger, 1993) that tethers the propeptide to this part of the Core fold (Fig. 1B), and (ii) the (−4)-Trp-Leu-Trp(−2) tripeptide that provides a hydrophobic bulge bridging the HBe dimer interface (Fig. 1B and C). In contrast, in Core dimers the two Cys(61) lie opposite each other across the dimer interface and can form an intermolecular disulfide bond connecting the two monomers. This led us to study the role of the (−4)-Trp-Leu-Trp(−2) tripeptide in Precore in a relevant

experimental system. To this end, we developed a new replicating HBV cell line mimicking natural infection conditions and we designed a mutated form of Precore in which the two Trp residues of the hydrophobic tripeptide were replaced by two Gly residues. In addition, plasmids encoding an HA-tagged Precore were constructed to allow us to distinguish Core and Precore proteins in the absence of specific antibodies. We demonstrated that the Precore mutant is biosynthesized, secreted and retrotransported to the cytosol as the wild-type protein, excluding a role of the 2 Trp residues in these mechanisms. Interestingly, the Precore mutant still forms dimers, that are surprisingly more stable than the wild type, and is still able to interact in vitro with the Core protein but cannot form heterocapsids in our HBV replicating cell lines. Taken together, our results show that the replacement of the two Trp residues of the propeptide sequence by glycine residues disrupts the formation of cytosolic Core–Precore heterocapsids even though the mutant Precore forms stable dimers whether in a reducing or an oxidizing environment. Thus the two Trp side chains that contribute to presenting the propeptide in such a way that formation of the Cys(−7)-Cys(61) and hence an HBe-like dimer is facilitated in an oxydizing environment (DiMattia et al., 2013) are also necessary for Precore to form a Core-like dimer in the reducing environment of the cytosol. These observations hint at a further, previously unsuspected dimerization mode of Precore besides the Core-like and HBe dimers.

Results

The Trp residues of the propeptide sequence are not required for the retrotransport of Precore from the ER to the cytoplasm

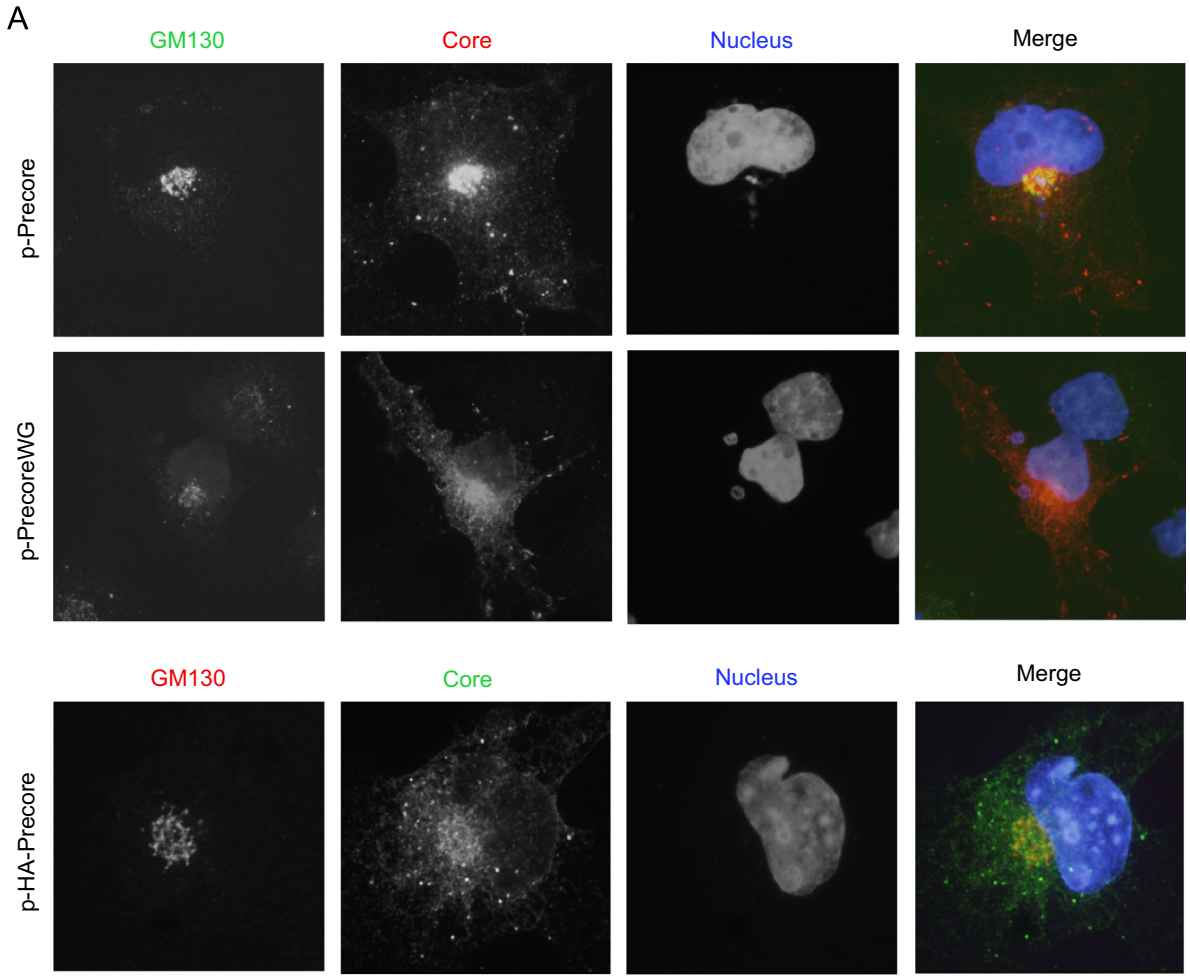
To obtain new insights into the role of the propeptide sequence of the cytoplasmic form of Precore, we focused our studies on the hydrophobic residues present in this sequence. To achieve this goal, we replaced the two Trp residues at (−4) and (−2) positions by Gly ones. In a first step we examined whether the mutations modified the intracellular localization of Precore. A cell system highly transfectable is required to detect the cytosolic part of Precore, as it represents around 10% of the total expressed protein. To this purpose, HEK-293 T cells were transfected with plasmids encoding the wild-type (Precore) or the mutated protein (PrecoreWG). Cells were processed for immunofluorescence analyses with antibodies directed against either the Golgi marker GM130 or the Precore protein (the anti-Core antibody detected both Precore and Core proteins). As shown in Fig. 2A, both Precore and PrecoreWG proteins were readily detected by the polyclonal anti-Core antibody and were present mainly in the secretory pathway as demonstrated by their colocalization with the Golgi compartment. Interestingly the localization of the HA-tagged Precore protein was identical (Fig. 2A), confirming that it could be used to distinguish Precore from Core in the following experiments. To go further, it is of importance to examine whether the Trp mutations affect the retrotransport of Precore from the ER to the cytoplasm. To this end, a cytosolic fractionation was performed on HEK-293 T cells transfected with plasmids encoding Precore, HA-Precore or HA-PrecoreWG. The cytosolic and non-cytosolic fractions were analyzed by Western blot (Fig. 2B). As a control, the blot was probed with an antibody directed against the luminal ER protein PDI. The absence of this protein in the cytosolic extracts allowed us to exclude that proteins from the ER had contaminated the cytosolic fractions. As expected, Precore was detected in the cytosolic and non cytosolic fractions with the polyclonal antibody anti-Core. Similarly, HA-Precore and HA-PrecoreWG proteins were detected in both fractions, demonstrating that neither the HA-tag nor the Trp replacements impair the retrotransport of Precore (Fig. 2B, lanes 6–8). The presence of BiP protein in cytosolic fractions (Fig. 2B, lanes 6–8) was expected as Precore retrotransport targets a subset of BiP protein to the cytoplasm (Duriez et al., 2008). We next investigated whether the mutation of the Trp residues could modify Precore secretion. To this purpose, the amounts of HBe secreted in the media of HEK-293 T cells expressing the mutated or the wild-type protein were quantified. As shown in Fig. 2C, no significant difference in the levels of secreted HBe was observed. In conclusion, these results demonstrate that the presence of an N-terminal HA-tag or the replacement of Trp residues by Gly do not affect Precore localization, HBe secretion or Precore retrotransport from the ER to the cytoplasm.

The tryptophan residues in the propeptide sequence are not critical for Precore interaction with Core

We then asked whether the Trp residues were essential for the Core–Precore interaction described previously (Scaglioni et al., 1997b). HEK-293 T cells were co-transfected transiently with Core and HA-Precore, HA-PrecoreWG or the irrelevant SEAP-HA protein expression vectors. Crude extracts were prepared and immunoprecipitations directed against the HA epitope were performed. Crude extracts and IP products were analyzed by Western blot with a polyclonal antibody directed against the Core protein. The Core protein (arrow) as well as the HA-Precore wild-type and mutant proteins are detected in crude extracts by the polyclonal antibody anti-Core (Fig. 3, lanes 2–5). As shown in Fig. 3, lanes 7 and 8, the Core protein (arrow) was co-immunoprecipitated with HA-Precore or HA-PrecoreWG but not with SEAP-HA (lane 9), demonstrating a specific interaction between Core and HA-Precore which is not dependent of the presence of the 2 Trp residues. We also investigated if the mutations of Trp residues on the propeptide sequence disrupted the interaction of Precore with its cellular partner gC1qR (Laine et al., 2003). This was not the case as gC1qR was co-immunoprecipitated with the wild-type and mutated Precore proteins (Fig. 3, lanes 7 and 8). These data suggest that mutations of the two hydrophobic Trp residues in the propeptide sequence do not modify drastically the tertiary structure of the Precore protein and indicate that the two Trp are not critical for its interactions with Core or gC1qR.

Replacement of the 2 Trp residues in the propeptide sequence stabilizes Precore dimers

The fact that HA-Precore as HA-PrecoreWG interacts with the Core protein strongly suggests that both proteins can form heterocapsids with Core in the reducing environment of the cytosol. However, different data obtained with a recombinant HBe indicate that the propeptide sequence plays a role in the stability of the HBe dimers, allowing incorporation into capsids in reducing conditions (presumably as Core-like dimers) but inducing the alternate HBe dimer in oxidizing conditions (DiMattia et al., 2013; Watts et al., 2011). Thus, we next investigated whether the wild-type and mutated Precore could dimerize and if these dimers had an identical stability. HA-Precore and HA-PrecoreWG were extracted from HEK-293 T in the presence or absence of dithiothreitol (DTT) and subjected to SDS-PAGE. Core-like disulfide-linked dimers will migrate as dimers if kept in oxidizing conditions but dissociate into apparent monomers in reducing conditions, while non-disulfide-crosslinked dimers will dissociate in both conditions. Interestingly we observed that the two proteins did not have the same behavior in oxidizing or reducing conditions. Oxidized HA-Precore protein migrated under the two forms, monomeric and dimeric and mostly as a monomer when treated with the reducing agent DTT prior to electrophoresis (Fig. 4, lanes 1 and 2). This behavior is very similar to that observed for Core



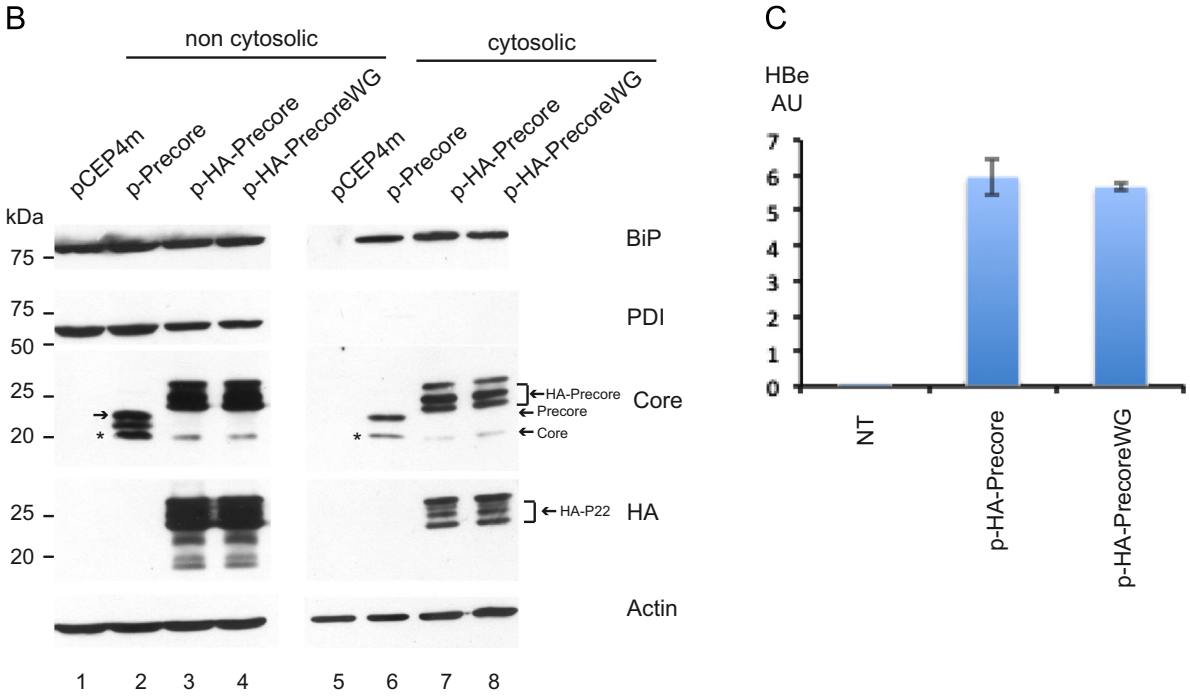


Fig. 2. PrecoreWG mutant is biosynthesized and secreted as the wild-type protein. HEK-293 T cells were transfected with p-Precore, p-PrecoreWG or p-HA-Precore. (A) Immunostaining experiments were performed using an anti-Core antibody to detect Precore (red for Precore and PrecoreWG, green for HA-Precore) and GM130 (green for Precore and PrecoreWG, red for HA-Precore). Nuclei were stained with DAPI (blue). A merge of the three stainings was realized. (B) After cytosolic fractionation, cytosolic and non-cytosolic extracts were subjected to a SDS-PAGE. Blots were probed with the antibodies indicated on the right. The band corresponding to the Precore form is marked by an arrow (lane 2), whereas Core, which is slightly expressed by this vector, is indicated with an asterisk (previously characterized in (Duriez et al., 2008)). The two bands corresponding to HA-Precore are indicated (lane 8). The upper band is not always present in our experiments and could be the result of post-translational modification (Duriez et al. 2008). (C) Supernatants of non transfected cells (NT) or transfected cells (p-HA-Precore and p-HA-PrecoreWG) were analyzed by ELISA to quantify HBe. Experiments were repeated at least three times.

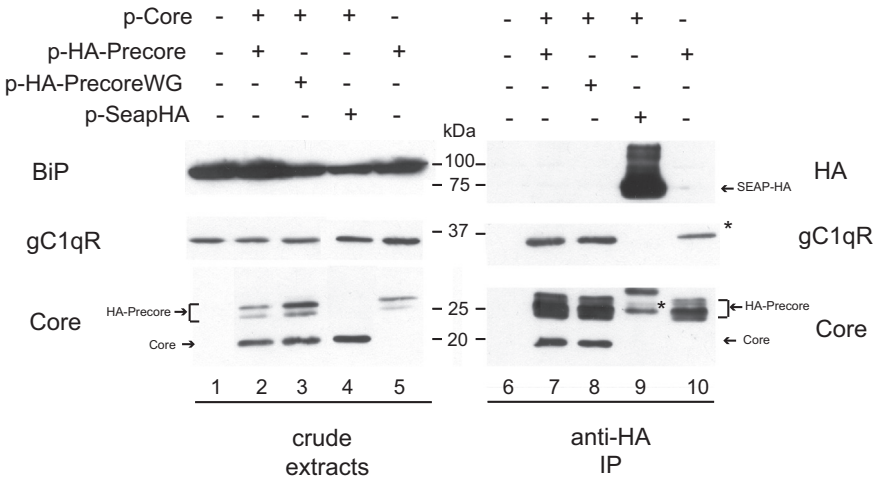


Fig. 3. The Core protein co-immunoprecipitates with HA-Precore and HA-PrecoreWG proteins. HEK-293 T cells were transfected with vectors indicated on the top of the Figure. Crude extracts and anti-HA immunoprecipitates (IP) were analyzed by a 12.5% SDS PAGE. A western blot was performed with antibodies indicated on both sides. An asterisk indicates light IgG chains. Presence of HA-Precore, Core and SEAP-HA proteins are indicated. Experiments were repeated three times with similar results.

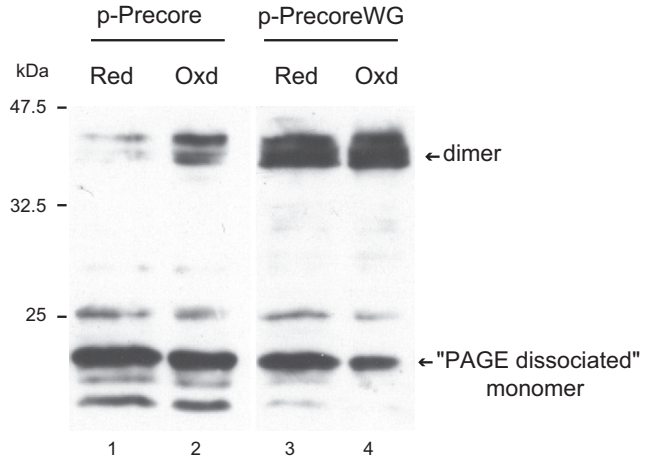


Fig. 4. Removal of Trp side chains in the propeptide sequence of Precore stabilizes a dimeric form of Precore. HEK-293 T cells were transfected with p-Precore or p-PrecoreWG. A total protein extract was performed in a Laemmli buffer with DTT (Red) or without DTT (Oxd) and boiled for 5 min at 100 °C. Extracts were subjected to SDS-PAGE and blots were probed with an anti-Core antibody. The bands corresponding to Precore PAGE dissociated monomers and Precore dimers are indicated. Experiments were repeated three times with identical results.

(not shown) and indicates that a portion of Precore molecules forms a dimer with the formation of an intermolecular disulfide bond, presumably a Core-like dimer. Another portion of Precore may form HBe-like dimers with an intramolecular bond between the Cys(−7) and the Cys(61) as shown for recombinant HBe molecules (Watts et al., 2011). However, the present experiment does not allows detection of this intramolecular bond since HBe dimers are less stable than Core dimers (Watts et al., 2011), and HBe-like Precore dimers will dissociate and migrate as monomers in SDS-PAGE, regardless of oxidation state. At any rate, any HBe-like dimers would be expected to be destabilized in the mutant Precore since the two removed tryptophan side chains are prominently involved in the HBe dimer interface (Fig. 1B and C). Surprisingly, HA-PrecoreWG migrated almost entirely as a dimer in all cases (Fig.4, lanes 3 and 4). Thus PrecoreWG is more stable as a dimer than Core-like or HBe-like dimers. Since we observe no detectable difference between oxidizing and reducing conditions, we cannot assess whether this PrecoreWG dimer involves a disulfide crosslink. Taken together, our results show that PrecoreWG predominantly assumes a dimeric conformation distinct from both the previously reported Core-like and HBe-like dimers (DiMattia et al., 2013), suggesting that the Glycine–rich peptide in the mutant favours a new conformational state.

New cell lines allowing infection-like expression of Core and Precore show that the replacement of the two propeptide Trp residues by glycines disrupts the formation of Core–Precore heterocapsids

We then decided to analyze the role of the two Trp residues of the propeptide sequence in HBV cell cycle. To this end, we established hepatoma HuH-7 cell lines that replicate an HBV genome defective in Precore synthesis, p-HBV(precore−) in which HA-tagged wild-type or mutated Precore proteins were provided in trans. HuH-7 cells carrying HBV(p25−) genome and doxycycline inducible HA-Precore or HA-PrecoreWG vectors were selected as described in material and methods. The HBV(p25−) genome delivered in HuH-7 is expressed under the control of endogenous viral regulatory sequences and carries a stop mutation in place of the −2 Trp codon of Precore (this precore gene mutation naturally occurs in HBV patients).

In order to assess the expression of viral proteins expression in these new cell lines, a total protein extract was performed and proteins analyzed by western blot (Fig. 5A). Detection of PDI served as a control of total protein extraction (Fig. 5A, panel PDI). As expected the HBV replicating cell lines expressed Core (Fig. 5A, panel Core) as well as HA-Precore or HA-PrecoreWG proteins (Fig. 5A, panels Core and HA). Interestingly, as observed in natural infection, Core expression was much higher than Precore expression in the HBV replicating cell lines when both proteins were detected with the polyclonal anti-Core antibodies (Fig. 5A, panel Core). As previous reports indicated that Precore reduces the viral secretion, we next asked whether the WG Precore mutant influenced viral secretion. To this end, HBV particles from cell supernatants were purified using a 30% sucrose cushion and the presence of Core and Middle envelope (M) proteins probed by Western blot. The presence of the M envelope protein should reflect nucleocapsid containing particles (infectious virions) and subviral empty particles, a hallmark of hepadnavirus infection, whereas Core detection is only due to the presence of nucleocapsid containing particles. As a control, a coomassie staining was performed to ensure that the same amounts of proteins were loaded (data not shown). As shown on Fig. 5B, HBV M envelope proteins were detected in similar amounts in HuH-7 cell lines expressing the HBV(p25−) genome alone or in combination with

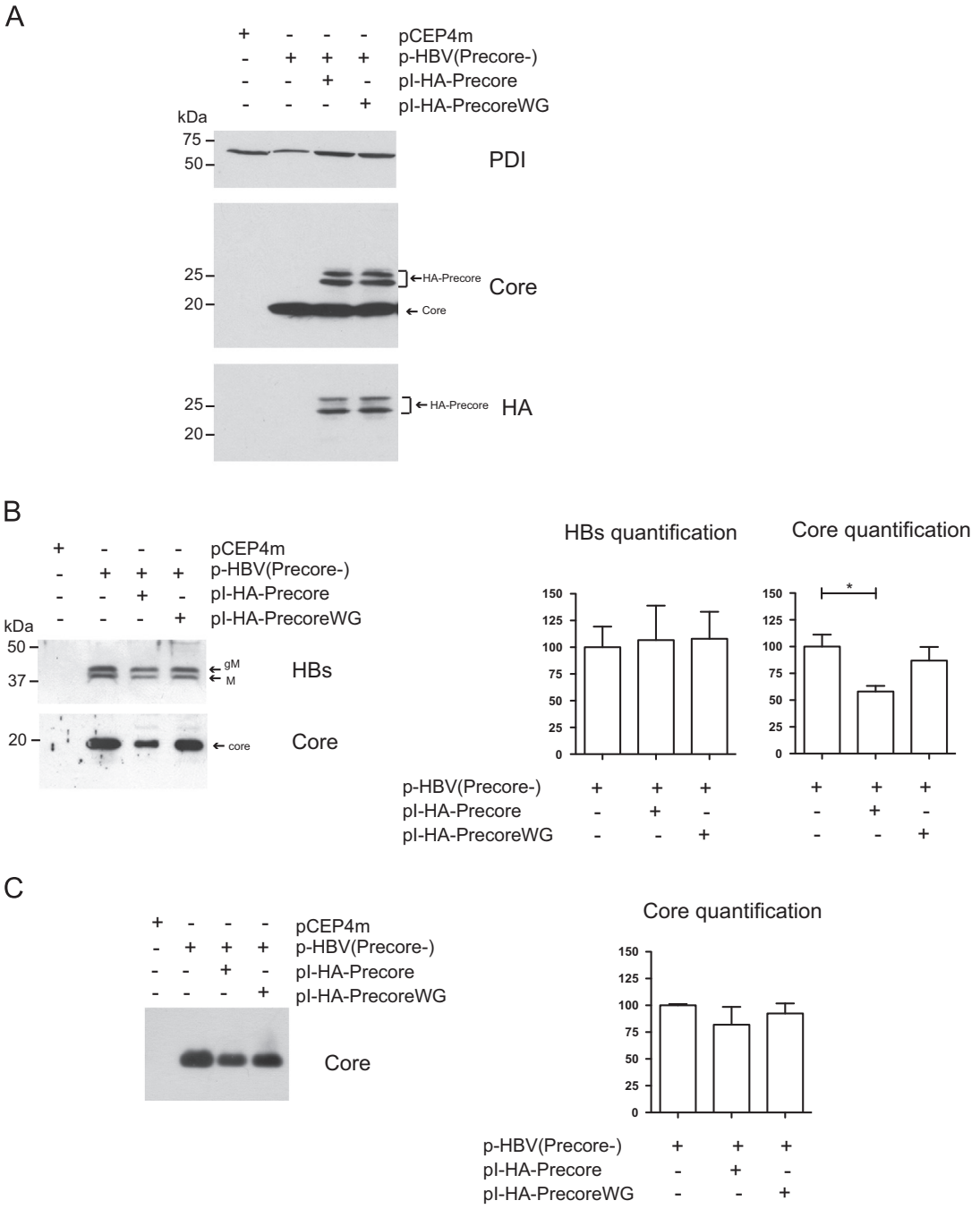


Fig. 5. Trp residues from Precore are not required for intracellular and extracellular nucleocapsid detection. HBV-expressing cell lines containing the hygromycin B resistant plasmid p-HBV(precore$^-$) and either pI-HA-Precore or pI-HA-PrecoreWG plasmids were selected as described in material and methods. (A) Cells were lysed in Laemmli buffer and extracts were subjected to electrophoresis on a 12.5% polyacrylamide-SDS gel. Blots were probed with antibodies indicated on the right. The bands corresponding to Core and HA-Precore proteins are indicated. Experiments were repeated three times with similar results. (B) Extracellular media from cells were concentrated then centrifuged in a 30% sucrose cushion. The resulting pellets were resuspended in Laemmli buffer and subjected to a 12% SDS PAGE. Viral proteins were detected by western blotting with antibodies indicated on the right. The rabbit anti-HBs antibody directed against the preS2 HBV envelop protein allowed mainly the detection of the M envelop protein (glycosylated or not). Quantifications were performed with three independent experiments. * Denotes significant difference ($p=0{,}0274$, unpaired t-test). (C) NP40 extraction was performed as described in material and methods. Extracts were loaded on a 1.2% agarose gel. After migration in non-denaturing conditions proteins and nucleocapsids were transferred onto a nylon membrane. Western blotting analysis was performed with an antibody directed against Core to reveal the presence of the intracellular capsid. Three independent experiments showed no significant variation between Precore and PrecoreWG.

HA-Precore or HA-PrecoreWG proteins. As expected we observed a significant reduction of Core detection in presence of the Precore protein (Fig. 5B, quantification of three independent experiments), confirming that Precore protein induces a reduction of infectious virions as already described by Scaglioni et al. (1997b). Interestingly this reduction was not observed in presence of PrecoreWG, meaning that the replacement of Trp residues abolished Precore effect on viral secretion. In a second step, intracellular nucleocapsids were extracted and analyzed by Western blot in non denaturating conditions (Fig. 5C). The amounts of intracellular nucleocapsids were appreciably similar in HuH-7 cell lines expressing the HBV(p25$^-$) genome alone or together with HA-Precore or HA-PrecoreWG proteins. This suggests that the expression of Precore (wild-type or mutated) does not quantitatively affect the

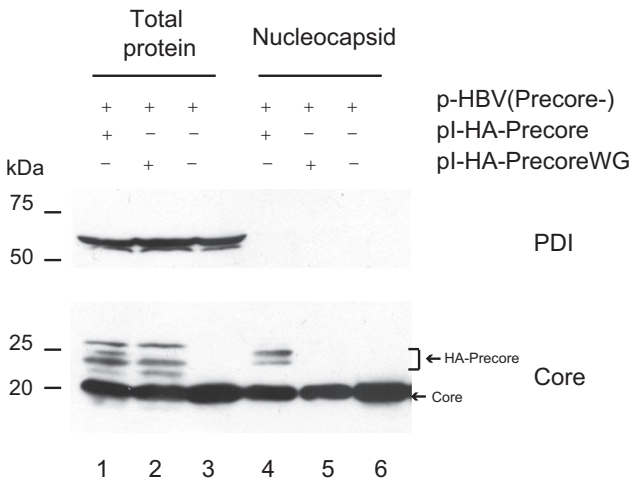


Fig. 6. Trp residues are essential for Precore association with nucleocapsids. HBV-expressing cell lines containing the p-HBV(precore⁻) plasmid and pI-HA-Precore or pI-HA-PrecoreWG were selected as described in Fig. 4. NP40 extraction was performed as described in material and methods. Extracts were centrifuged for 2 h at 50,000 rpm through a 30% sucrose cushion. Total protein extracts (Total protein) and resuspended pellets (Nucleocapsid) were subjected to a 12% SDS-PAGE. A western blotting assay was performed with antibodies indicated on the right. PDI was detected as a control. Presence of HA-Precore and Core proteins are indicated. Experiments were repeated three times with similar results.

global level of intracellular capsids. This result is consistent with the hypothesis of Scaglioni and co-workers, who proposed that P22 could interfere with the assembly of viral nucleocapsids and participate to the formation of hybrid nucleocapsids devoid of pregenomic viral RNA.

To go further, we determined if the wild-type and mutated Precore proteins were present in the intracellular nucleocapsids. After cell lysis, intracellular nucleocapsids were purified through an ultracentrifugation on a 30% sucrose cushion, loaded onto a SDS-PAGE and analyzed by western blot. In addition, total protein extracts were performed to ensure that HA-Precore and HA-PrecoreWG were expressed at similar levels (Fig. 6, lanes 1 and 2). Interestingly, analysis of purified intracellular nucleocapsids from HA-Precore and HA-PrecoreWG HBV cell lines revealed a drastic difference. Indeed, HA-Precore was observed in purified intracellular nucleocapsids altogether with Core protein (Fig. 6, lane 4), in agreement with the data reported previously (Scaglioni et al., 1997b). On the opposite, HA-PrecoreWG was absent from these structures (Fig. 6, lane 5), although it was expressed in the same amount as the wild-type Precore. Overall, these results demonstrate that the replacement of the two Trp residues of the propeptide sequence by glycine inhibits the formation of Core–Precore heterocapsids.

Discussion

In this report, we provide a novel molecular insight for the formation of Core–Precore heterocapsids. We show that two hydrophobic Trp residues in the propeptide sequence of Precore are essential for the formation of these heterocapsids. Importantly, it appears that the replacement of the two Trp residues by Glycines does not impair Core–Precore interaction but prevents formation of a Core-like dimeric structure.

The role of the cytosolic form of Precore in the viral life cycle is still not totally understood. Years ago, several reports using surrogate HBV expression systems have showed that Precore down regulates the level of HBV replication (Buckwold et al., 1996; Guidotti et al., 1996; Lamberts et al., 1993), most likely by the association of Precore and Core proteins in capsids devoid of pregenomic RNA (Scaglioni et al., 1997b). To better define the role of Precore in this mechanism, we developed a new HBV expression model using a vector, which replicates as an extrachromosome episome in human cells, mimicking the viral cccDNA form. Moreover the model allowed us to detect specifically Precore with a ratio of Precore to Core expression similar to the natural infection (less Precore produced than Core). Thus, this experimental system is very relevant for our studies on the ability of Precore and PrecoreWG to form heterocapsids. Using this relevant experimental system, we confirmed that Precore and Core proteins can form heterocapsids, as previously shown (Scaglioni et al., 1997b). Moreover we observe a significant reduction of viral secretion when wild-type Precore assembles with Core in nucleocapsids. In previous studies Precore was mostly over expressed without its signal peptide sequence and consequently only present in the cytoplasm. In our experimental system Precore is expressed at a relatively low level and is present both in the secretory pathway and the cytoplasm, thus our data show that the presence of heterocapsids is not dependent on the overabundance of Precore in the cytoplasm. According to Scaglioni et al. such an interaction would lead to the formation of immature capsids, which are not competent for replication and consequently cannot be enveloped and secreted.

We sought to shed light on this and other functions of Precore by mutating the 10-residue Precore propeptide sequence. It was suggested, years ago, that the Cys(−7) and the two Trp residues (−4) and (−2) of the propeptide sequence are required to prevent HBeAg assembly into core-like structures (Wasenauer et al., 1992). Moreover, recent crystallographic studies of HBe expressed in *E. coli* have shown that the formation of the intramolecular disulfide bond between Cys(−7) and Cys(61) locks the propeptide into place, stabilizing Gly(−5)-Trp(−4)-Leu(−3)-Trp(−2)-Gly (−1) into a hydrophobic bulge that comes into close contact with the central part of the protein (Fig. 1B). Consequently, Core-like dimers cannot be formed but a radically altered mode of dimerization of HBe, relative to Core, occurs which cannot assemble in capsids (DiMattia et al., 2013). However, in a reducing environment, the intramolecular disulfide bridge is disrupted, and HBe dimers are able to form capsids, indicating a Core-like mode of association. If we assume that Precore tertiary structure is close to that of HBe, we believe that in the cytosol, a reducing environment, Precore could homodimerize in a Core-like dimer conformation, allowing the formation of heterocapsids. The two Trp residues (−4) and (−2) are central in the oxidized intramolecular disulfide state of HBe both in preventing Core-like dimer formation (Fig. 1B) and stabilizing the alternate HBe dimer (Fig. 1C) that is presumably the secreted form. What could be the specific role of these Trp residues in the cytosolic fraction of Precore? Surprisingly, the rather drastic change of mutating both Trp residues to glycines produced a rather mild phenotype in our experimental system. The PrecoreWG mutant was still expressed, processed and located as the wild type. The secreted fraction and the retro-transported fraction were detected in the same quantities as the wild type and the latter cytosolic PrecoreWG fraction still interacted with Core. It is all the more striking that PrecoreWG has specifically lost the ability to be associated with the nucleocapsids. Furthermore, PrecoreWG formed a very stable dimer independently of oxidation conditions. This suggests that the PrecoreWG dimer is distinct from both the disulfide-linked Core-like dimer and the weaker HBe dimer (DiMattia et al., 2013; Watts et al., 2011). Since PrecoreWG behaved otherwise identically with Precore, it is tempting to speculate that this mutant has trapped a previously unsuspected minor Precore conformation. At any rate, our results suggest a particular role of the two aromatic residues in the formation of heterocapsids. Thus, the same residues that prevent a Core-like dimer formation under oxidizing conditions for the secreted HBe promote it under reducing conditions for the retro-transported Precore.

Viruses with a compact genome usually optimize its use by having the same sequences serve several functions. One strategy for this is coding alternate polypeptide lengths, as in the HIV reverse transcriptase P66/P51 heterodimer where P51 displays a very different conformation despite being only a shorter form of P66 (Kohlstaedt et al., 1992). Another common strategy is to have the same polypeptide chain that serves different functions in different contexts, as in the HBV Core, which is known or suspected to have gene expression tuning functions in its dimeric form in addition to its primary role in virion formation. Central to this switch of Core is the role of allostery in HBV capsid assembly (Alexander et al., 2013; Packianathan et al., 2010). It is noteworthy that the crystal structure of the pre-assembly conformation of the Core dimer was obtained with the Y132A mutant (Packianathan et al., 2010), that shows two important similarities with the PrecoreWG mutant. First, Y132 is an aromatic residue at the periphery of an important (interdimer) interface in the capsid, in a similar way to W(−4) and W(−2) for the HBe dimer interface (Fig. 1C). Second, mutating the Y132 side chain not only weakens the interdimer interface to which it contributes but also leads to long-distance remodeling of the Core dimer interface. In such a plastic protein as the Core fold, a similar effect may explain in part the alternate dimer of PrecoreWG.

HBV is an extreme example with respect to genome compaction. We show here that the single PreC/C open reading frame may code for even more functions than previously suspected. Particularly the Precore protein's cytosolic fraction may have different dimeric conformations that may serve different functions in HBV replication.

Materials and methods

DNA constructs

Plasmid pCEP4m was previously described (Sitterlin et al., 2000). Plasmid p-Seap-HA (formerly pCEP4mSeap-HA) was previously described (Duriez et al., 2008). Plasmids p-Core and p-Precore derive from pCEP4m and allow the expression of Core and Precore genes under control of the cytomegalovirus promoter, respectively (Duriez et al., 2008). Plasmid p-PrecoreWG was generated by site-directed mutagenesis on p-Precore (Trp codons at position −4 and −2 were replaced by two Gly codons). Plasmid p-HA-Precore allows the expression of a Precore protein fused to a HA-tag (ASYPYDVPDYAPG) at its N-terminus end (HA-Precore). This construct was obtained by inserting a HA-tag coding sequence downstream of the sequence coding for the signal peptide of the Precore P25 precursor. Thus, after cleavage of the 19-residues signal peptide, the HA-tag will be present at the N-terminus of the mature Precore. Plasmid p-HA-PrecoreWG was generated by site-directed mutagenesis on p-HA-Precore (Trp codons at position −4 and −2 were replaced by two Gly codons). It allows the expression of a mutated Precore (HA-PrecoreWG). Plasmid pI-HA-Precore allows expression of HA-Precore under the control of an inducible promoter. The CMV inducible promoter containing tetracycline operator sequences from the pcDNA™4/TO (Life technologies) was amplified and inserted at the SalI-HindIII site of p-HA-Precore, in place of the CMV promoter. The pI-HA-PrecoreWG, in which the −4 and −2 Trp codons were replaced by two Gly codons, was generated by site-directed mutagenesis on pI-HA-Precore. Plasmid p-HBV(precore⁻) carries a hygromycin B resistance gene and contains a 1.2-HBV genome unit-length from nucleotides 1402 to 3182 and 1 to 1986 (genotype D, subtype ayw, Genbank#v01460 (Galibert et al., 1979)) with a mutation in the precore gene at nucleotide 1896 leading to a stop codon in the propeptide sequence (TGG to TAG). As the CMV promoter has been deleted from the pCEP4m vector, HBV RNA transcripts are expressed under the control of endogenous viral regulatory sequences.

HEK-293T cells transfections

HEK-293 T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% of heat-inactivated fetal calf serum and 100 units/ml of penicillin and streptomycin. HEK-293 T cells were transfected with 10 μg of total DNA for 5×10^6 cells using the calcium phosphate procedure as previously described (Laine et al., 2003).

HBV cell line selection

A HuH-7 stable cell line that constitutively expressed the Tet repressor was established (HuH-7 Tet Rep). HuH-7 cells were transfected with the pcDNA6/TR (Life technologies) and selected with blasticidin (5 μg/ml) to isolate a single stable cell line expressing the Tet repressor protein. The resulting HuH-7 Tet Rep cells were grown in William's E Medium supplemented with blasticidin 5 μg/ml, 10% of heat-inactivated fetal calf serum and 100 units/ml of penicillin and streptomycin. The expression was assessed by Western blot with a Tet repressor antibody (data not shown). HBV replicating HuH-7 cell lines were obtained as follows: 2×10^6 of HuH-7 Tet Rep cells were seeded in 100-mm cell dish and transfected using the calcium phosphate procedure with 20 μg of an equal mixture of p-HBV(p25⁻) and pI-HA-Precore or pI-HA-PrecoreWG. Two days post-transfection, cells were grown in the presence of hygromycin B (0.25 μg/ml, Life technologies). Medium was changed every two days during one week and cells were replated to a low confluence. At the end of the selection, 0.5 μg/ml of doxycycline (Clontech) were added during 24 h to induce HA-Precore or HA-PrecoreWG expression. The CMV inducible promoter containing tetracycline operator sequences allowed lower expression than a basic CMV promoter, thus preventing cell toxicity due to a strong expression of Precore. However, we found out that this "inducible" promoter was extremely leaky, as the control cells expressed the same level of HA-Precore as doxycycline treated cells. Therefore, we further used this plasmid without doxycycline induction.

Cytosolic fractionations and total protein extractions

Cytosolic fractionations were performed using a procedure described previously (Duriez et al., 2008). Forty percent of the corresponding soluble extract (cytoplasmic) and 10% of the insoluble fraction (non-cytoplasmic) were subjected to SDS-PAGE. Total protein extractions were performed on HEK-293 T cells 48 h after transfection and 24 h after doxycycline addition on HBV replicating HuH-7 cells. Cells were washed twice with PBS and suspended in 300 μl of Laemmli buffer (for 10^7 cells) containing 100 mM DTT, except when protein dimer formation was evaluated. Proteins were boiled for 5 min and 10% of the total protein extract was analyzed by SDS-PAGE.

Immunoprecipitations

Cells were washed twice with PBS and lysed in 1 ml PBS-1% NP40 buffer containing protease inhibitors purchased from Sigma (0.5 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin). Lysates were cleared of debris by centrifugation at 16,000 *g* for 10 min at 4 °C. For each reaction, 2.5 μl of anti-HA antibody (Covance Research Products, MMS101R) were coupled to 50 μl of protein A Sepharose (Sepharose CL-4B, GE Healthcare 17-0780-01) and incubated with cell lysate overnight at 4 °C on a rotating wheel. Beads were washed with PBS-NP40 1% buffer five

times and boiled in Laemmli's sample buffer. Half of the immunoprecipitate and 20% of the crude lysate were subjected to SDS-PAGE.

Western blot and antibodies

Protein extracts were submitted to electrophoresis on 12.5% SDS-Polyacrylamide gels and then blotted onto a nitrocellulose membrane using a semi dry transfer Bio-Rad apparatus following the manufacturer's instructions. HBV Precore and Core proteins were both detected using a polyclonal rabbit anti-Core antibody (Dako, B0586). Mouse antibodies against BiP (BD Biosciences, G73320), PDI (Stressgen, SPA891), HA (HA-11, Covance, MMS101R), Actin (Sigma, A-2066) and gC1qR (Covance Research Products, MMS-604 R) were used as primary antibodies. A rabbit anti-HBs antibody directed against the preS2 HBV envelope protein was kindly provided by Dr Sureau (INTS, Paris). Goat HRP-conjugated antibodies anti-rabbit (W4011) and anti-mouse (W4021) were purchased from Promega. Membranes were revealed using the ECL SuperSignal West Pico kit (Pierce) and exposed to Hyperfilm (GE Healthcare Life Sciences)).

Immunostaining

Cells were seeded on cover slips (Knittelgläser) and transfected. Forty eight hours after, cells were fixed in a 4% paraformaldehyde-PBS buffer for 10 min and permeabilized 15 min with 0.5% X100 Triton-PBS buffer. Incubations with antibodies were done during 45 min in 1% BSA-PBS. Primary antibodies rabbit anti-Core (Dako, B0586), mouse anti-GM130 (BD Biosciences, G65120) and relevant Alexa Fluor secondary antibodies (Life technologies) were used. For nuclei detection, cells were stained for 10 min with 2 μg/ml DAPI (Euromedex). Immunostainings were analyzed with a fluorescence Leica DMRHC research microscope with a 63×1.32 oil immersion objective.

Purification and content analysis of intracellular nucleocapsids.

HBV replicating HuH-7 cells (1×10^7) were lysed in 1 ml of buffer containing 50 mM Tris–HCl pH 8, 100 mM NaCl, 1 mM EDTA, 10 mM Mg acetate and 0.7% NP40 during 15 min at 37 °C. Nuclei and cellular debris were removed by centrifugation (5 min at $5000 \times g$), and then the supernatant was incubated for 15 min at 37 °C after addition of 200 μg DNase (Sigma, DN-25), 100 μg RNase (Sigma R48-75) and $MgCl_2$ to a final concentration of 6 mM. After addition of EDTA to a final concentration of 15 mM, the lysate was centrifuged at 18,000 g for 10 min at 4 °C to clarify the extract. Quantification of protein was done using the BCA protein assay reagent (Pierce). Protein extracts containing intracellular nucleocapsids were either analyzed by electrophoresis on native agarose gel or by SDS-PAGE. For native agarose gel, protein extracts (20 μg) were loaded on a 1.2% agarose gel. After a 2h30 migration at 50 V, proteins and nucleocapsids were blotted overnight onto Amersham Hybon™-N+ membrane (GE Healthcare) with a 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA buffer. The membrane was incubated in a blocking solution (1X TBS, 0.1% Tween, 10% milk) and subjected to classical Western blotting analysis. For SDS-PAGE analysis, nucleocapsids (100 μg of proteins extract) were concentrated by centrifugation through a 30% sucrose solution (w/v) in an SW60 rotor (Beckman Colter) for 2 h at 10 °C at 50,000 rpm. The pellet fraction was resuspended at 4 °C in 50 μl of PBS and then 50 μl of Laemmli buffer were added. One third of the resuspended pellet was loaded on SDS polyacrylamide gel and subjected to Western Blot analysis.

Analysis of extracellular virions

Extracellular medium from HBV HuH-7 cells was recovered and concentrated on a 10 kDa Amicon Ultracentrifugal filter. Half of the residual solution was then loaded onto a 30% sucrose cushion and processed as described for the intracellular nucleocapsids.

HBe quantification in cell media

Extracellular medium from HBV replicating HuH-7 cells was recovered and HBe quantification was done by ELISA assay (bioMérieux clinical diagnostics).

Structure analysis and display

Coordinates and structure factors were retrieved from the Protein Data Bank for the HBe dimer (chains E and F in PDB 3V6Z (DiMattia et al., 2013)), the Core dimer in the $T=4$ HBV capsid (chains A and B in PDB 1QGT (Wynne et al., 1999)), and an assembly-defective Core dimer (mutant Y132A, chains E and F in PDB 3KXS (Packianathan et al., 2010)). Dimer interfaces were computed with the software PISA (Krissinel and Henrick, 2007). Structures were visualized for analysis and rendered for Fig. 1 with PYMOL (http://www.pymol.org/).

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References

Alexander, C.G., Jurgens, M.C., Shepherd, D.A., Freund, S.M., Ashcroft, A.E., Ferguson, N., 2013. Thermodynamic origins of protein folding, allostery, and capsid formation in the human hepatitis B virus core protein. Proc. Natl. Acad. Sci. U. S. A. 110, E2782–E2791.

Bartenschlager, R., Schaller, H., 1988. The amino-terminal domain of the hepadnaviral P-gene encodes the terminal protein (genome-linked protein) believed to prime reverse transcription. EMBO J. 7, 4185–4192.

Buckwold, V.E., Xu, Z., Chen, M., Yen, T.S., Ou, J.H., 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J. Virol. 70, 5845–5851.

Dandri, M., Locarnini, S., 2012. New insight in the pathobiology of hepatitis B virus infection. Gut 61 (Suppl 1) (i6-17).

DiMattia, M.A., Watts, N.R., Stahl, S.J., Grimes, J.M., Steven, A.C., Stuart, D.I., Wingfield, P.T., 2013. Antigenic switching of hepatitis B virus by alternative dimerization of the capsid protein. Structure 21, 133–142.

Duriez, M., Rossignol, J.M., Sitterlin, D., 2008. The hepatitis B virus precore protein is retrotransported from endoplasmic reticulum (ER) to cytosol through the ER-associated degradation pathway. J. Biol. Chem. 283, 32352–32360.

Galibert, F., Mandart, E., Fitoussi, F., Tiollais, P., Charnay, P., 1979. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. Nature 281, 646–650.

Guidotti, L.G., Matzke, B., Pasquinelli, C., Shoenberger, J.M., Rogler, C.E., Chisari, F.V., 1996. The hepatitis B virus (HBV) precore protein inhibits HBV replication in transgenic mice. J. Virol. 70, 7056–7061.

Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A., Steitz, T.A., 1992. Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science 256, 1783–1790.

Krissinel, E., Henrick, K., 2007. Inference of macromolecular assemblies from crystalline state. J. Mol. Biol. 372, 774–797.

Laine, S., Thouard, A., Derancourt, J., Kress, M., Sitterlin, D., Rossignol, J.M., 2003. in vitro and in vivo interactions between the hepatitis B virus protein P22 and the cellular protein gC1qR. J. Virol. 77, 12875–12880.

Lamberts, C., Nassal, M., Velhagen, I., Zentgraf, H., Schroder, C.H., 1993. Precore-mediated inhibition of hepatitis B virus progeny DNA synthesis. J. Virol. 67, 3756–3762.

Lang, T., Lo, C., Skinner, N., Locarnini, S., Visvanathan, K., Mansell, A., 2011. The hepatitis B e antigen (HBeAg) targets and suppresses activation of the toll-like receptor signaling pathway. J. Hepatol. 55, 762–769.
Messageot, F., Salhi, S., Eon, P., Rossignol, J.M., 2003. Proteolytic processing of the hepatitis B virus e antigen precursor. Cleavage at two furin consensus sequences. J. Biol. Chem. 278, 891–895.
Milich, D.R., Chen, M.K., Hughes, J.L., Jones, J.E., 1998. The secreted hepatitis B precore antigen can modulate the immune response to the nucleocapsid: a mechanism for persistence. J. Immunol. 160, 2013–2021.
Nassal, M., Rieger, A., 1993. An intramolecular disulfide bridge between Cys-7 and Cys61 determines the structure of the secretory core gene product (e antigen) of hepatitis B virus. J. Virol. 67, 4307–4315.
Ou, J.H., Laub, O., Rutter, W.J., 1986. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proc. Natl. Acad.Sci. U. S. A. 83, 1578–1582.
Ou, J.H., Yeh, C.T., Yen, T.S., 1989. Transport of hepatitis B virus precore protein into the nucleus after cleavage of its signal peptide. J. Virol. 63, 5238–5243.
Packianathan, C., Katen, S.P., Dann 3rd, C.E., Zlotnick, A., 2010. Conformational changes in the hepatitis B virus core protein are consistent with a role for allostery in virus assembly. J. Virol. 84, 1607–1615.
Revill, P., Yuen, L., Walsh, R., Perrault, M., Locarnini, S., Kramvis, A., 2010. Bioinformatic analysis of the hepadnavirus e-antigen and its precursor identifies remarkable sequence conservation in all orthohepadnaviruses. J. Med. Virol. 82, 104–115.
Scaglioni, P.P., Melegari, M., Wands, J.R., 1997a. Biologic properties of hepatitis B viral genomes with mutations in the precore promoter and precore open reading frame. Virology 233, 374–381.
Scaglioni, P.P., Melegari, M., Wands, J.R., 1997b. Posttranscriptional regulation of hepatitis B virus replication by the precore protein. J. Virol. 71, 345–353.
Sitterlin, D., Bergametti, F., Transy, C., 2000. UVDDB p127-binding modulates activities and intracellular distribution of hepatitis B virus X protein. Oncogene 19, 4417–4426.
Summers, J., O'Connell, A., Millman, I., 1975. Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles. Proc. Natl. Acad. Sci. U. S. A. 72, 4597–4601.
Wasenauer, G., Kock, J., Schlicht, H.J., 1992. A cysteine and a hydrophobic sequence in the noncleaved portion of the pre-C leader peptide determine the biophysical properties of the secretory core protein (HBe protein) of human hepatitis B virus. J. Virol. 66, 5338–5346.
Watts, N.R., Conway, J.F., Cheng, N., Stahl, S.J., Steven, A.C., Wingfield, P.T., 2011. Role of the propeptide in controlling conformation and assembly state of hepatitis B virus e-antigen. J. Mol. Biol. 409, 202–213.
Wynne, S.A., Crowther, R.A., Leslie, A.G., 1999. The crystal structure of the human hepatitis B virus capsid. Mol. Cell 3, 771–780.