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Hepatitis B virus regulatory HBx protein binding to DDB1 is required but is not sufficient for maximal HBV replication

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ABSTRACT

Robust hepatitis B virus (HBV) replication is stimulated by the regulatory HBx protein. HBx binds the cellular protein DDB1; however, the importance of this interaction for HBV replication remains unknown. We tested whether HBx binding to DDB1 was required for HBV replication using a plasmid based replication assay in HepG2 cells. Three DDB1 binding-deficient HBx point mutants (HBx^{69}, $HBx^{90/91}$, HBx^{R96E}) failed to restore wildtype levels of replication from an HBx-deficient plasmid, which established the importance of the HBx-DDB1 interaction for maximal HBV replication. Analysis of overlapping HBx truncation mutants revealed that both the HBx-DDB1 binding domain and the carboxyl region are required for maximal HBV replication both *in vitro* and *in vivo*, suggesting the HBx-DDB1 interaction recruits regulatory functions critical for replication. Finally we demonstrate that HBx localizes to the Cul4A-DDB1 complex, and discuss the possible implications for models of HBV replication.

Introduction

Hepatitis B virus (HBV) infection is a serious health problem worldwide, with greater than 350 million people chronically infected and at risk for developing serious liver disease, including cirrhosis, fibrosis, and hepatocellular carcinoma (Beasley, 1988; Seeger et al., 2007). HBV is a 3.2-kb, partially double-stranded DNA virus that has four overlapping reading frames, which encode seven viral proteins: three surface antigens, a polymerase, two core proteins, and HBx. The HBx protein is the sole HBV regulatory protein and it has multiple functions both *in vitro* and *in vivo*, including transactivation of cellular and viral promoters (Spandau and Lee, 1988; Twu and Schloemer, 1987), activation of signaling pathways (Benn and Schneider, 1994; Cross et al., 1993), alteration of cell cycle progression (Benn and Schneider, 1995; Gearhart and Bouchard, 2010; Hodgson et al., 2008; Koike et al., 1994; Madden and Slagle, 2001), induction or prevention of apoptosis [reviewed in Bouchard and Schneider, 2004], and inhibition of cellular DNA repair (Becker et al., 1998; Groisman et al., 1999; Madden et al., 2000; Prost et al., 1998). HBx also acts as a tumor promoter in transgenic mice (Madden et al., 2001; Terradillos et al., 1997). HBx localizes to both the cytoplasm and the nucleus, where it presumably has different functions [reviewed in Bouchard and Schneider, 2004]. With the establishment of a plasmid-based HBV replication assay, it is now known that HBx is required for maximal virus replication (Bouchard et al., 2002; Keasler et al., 2007; Leupin et al., 2005; Tang et al., 2005) although the mechanism by which HBx facilitates HBV replication remain unclear.

HBx interacts with several cellular proteins and may mediate its role in virus replication through these interactions. The most well-characterized HBx binding partner is the damage-specific DNA binding protein 1 (DDB1) (Lee et al., 1995; Sitterlin et al., 1997; Lin-Marq et al., 2001) [reviewed in Keasler and Slagle, 2008]. DDB1 is a highly conserved, functional protein expressed in both the nucleus and cytoplasm (Liu et al., 2000). The interaction between HBx and DDB1 is conserved among the HBx proteins from all mammalian *hepadnaviruses* (Sitterlin et al., 1997), suggesting an important role for this interaction in virus replication. Further, interaction of the woodchuck hepatitis virus (WHV) *X* protein (WHx) with DDB1 is critical for WHV replication in woodchucks (Sitterlin et al., 2000). The minimal DDB1 binding domain on HBx has been identified by several laboratories to be amino acids 88–100 (HBx^{88-100}) (Fig. 1A).

DDB1 functions as an adaptor protein for the Cul4A E3 ubiquitin ligase complex (Angers et al., 2006; Higa et al., 2006; Shiyanov et al., 1999). DDB1 recruits DDB1 Cullin Associated Factors (DCAFs), which in turn recruit substrates to the DDB1-Cul4A complex for subsequent ubiquitination and degradation by the proteosome (Angers et al., 2006; He et al., 2006; Higa et al., 2006). In this manner, DDB1 plays important roles in diverse cellular processes, such as DNA synthesis, gene expression, cell division, and apoptosis. The DCAFs have in common a 16-aa DDB1-binding WD40 (DWD) motif that is characterized not by amino acid sequence, but rather by the biophysical/biochemical characteristics of those amino acids (He et al., 2006). The minimal DDB1-

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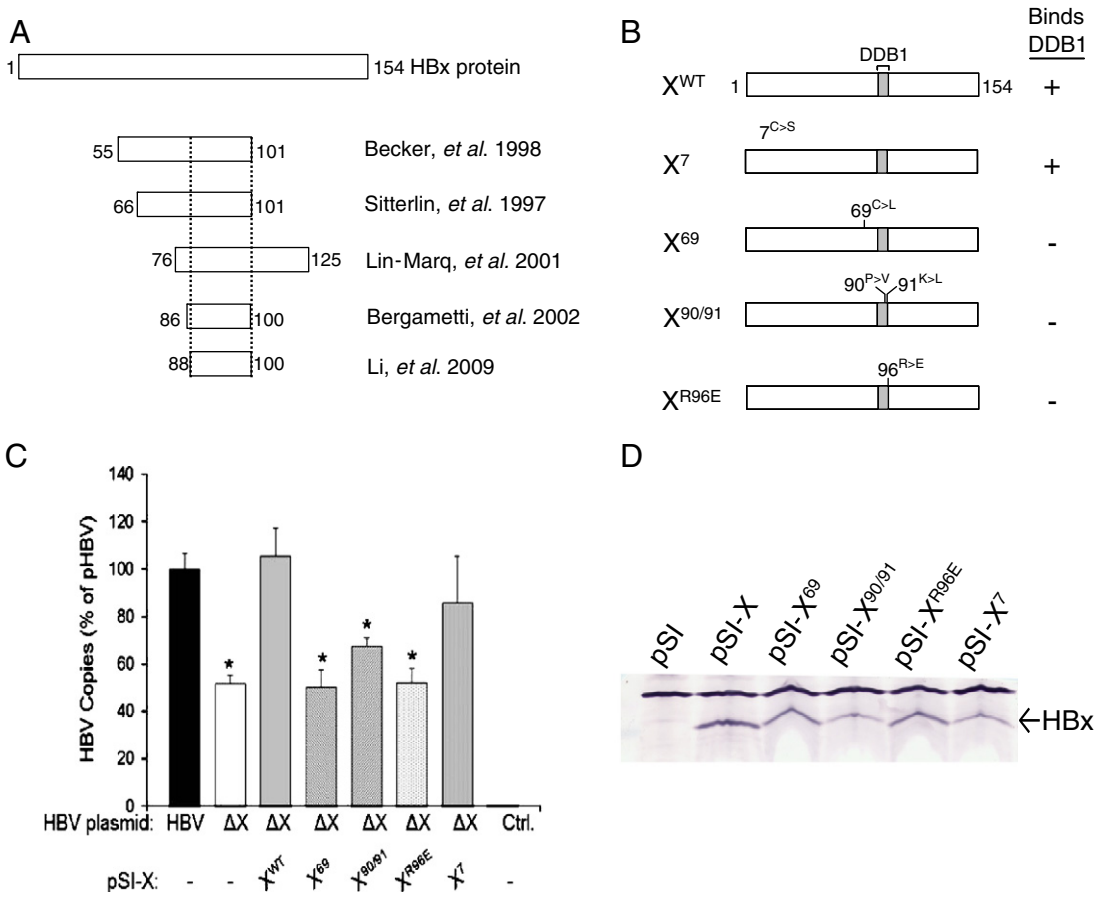

Fig. 1. HBx point mutants that do not bind DDB1 fail to restore HBx-deficient replication. (A) Schematic representation of HBx protein showing minimum domain required for binding to DDB1 [adapted from Keasler and Slagle, 2008]. (B) Schematic of wildtype and point mutant HBx proteins. DDB1 binding determined previously (Becker et al., 1998; Lin-Marq et al., 2001). (C) Quantitation of capsid-associated viral DNA as described in Materials and methods section. Mean copy number from cells transfected with pHBV was set to 100% and compared to others. Error bars (SE) from three independent experiments. Statistical significance compared to pHBV is noted by an asterisk ($p<0.05$). (D) Western blot detection of wildtype and point mutant HBx proteins with rabbit anti-HBx.

binding domain of HBx (aa 88–100) contains a sequence similar to that of DWD motifs in cellular DCAFs (Keasler and Slagle, 2008; Li et al., 2010). A recent high resolution crystal structure demonstrated the direct interaction between DDB1 and an HBx^{88-100} peptide (Li et al., 2010). The specific region on DDB1 to which HBx binds is shared with at least 79 other DCAFs (He et al., 2006). This raises the possibility that HBx binding to DDB1 may benefit virus replication by displacing DCAFs and thereby altering the spectrum of DCAFs and their substrates recruited to the Cul4A complex.

In the present study, we used the plasmid-based HBV replication assay in cultured HepG2 cells and in hydrodynamically injected mice to investigate the contribution of the HBx-DDB1 interaction to HBV replication. Three HBx point mutant proteins that no longer bind DDB1 were unable to restore HBx-deficient replication, demonstrating that the HBx-DDB1 interaction is required for maximal HBV replication. However, further analysis of HBx truncation mutants revealed that HBx-DDB1 binding is not sufficient to restore HBx-deficient replication, and that an additional function(s) residing in the carboxyl half of the HBx protein is essential for maximal replication.

Results

HBx interaction with DDB1 is required for virus replication

The importance of the HBx-DDB1 interaction has been suggested by the observation that an HBx point mutant that no longer binds to DDB1 (HBx^{R96E}) was unable to restore HBx-deficient pHBVΔX replication (Leupin et al., 2005). In the present study, the same plasmid-based HBV replication assay that requires HBx expression for maximal virus replication was used to investigate the HBx-DDB1-mediated requirements for HBV replication. Human liver HepG2 cells were transfected with a plasmid encoding a greater-than-genome length (129%) HBV genome (pHBV) (Melegari et al., 1998) or an identical plasmid encoding HBV with a point mutation in the *X* open reading frame that prevents HBx expression (pHBVΔX) (Scaglioni et al., 1997). Cells receiving pHBVΔX were additionally co-transfected with plasmids encoding HBx proteins that either bind DDB1 (*e.g.*, HBx, HBx^{7}) or do not (HBx^{69}, $HBx^{90/91}$, HBx^{R96E}) (Fig. 1B). Quantitation of capsid-associated DNA revealed that HBx-deficient replication from pHBVΔX was reduced by approximately 60% compared to wildtype pHBV (Fig. 1C). Replication from pHBVΔX was restored to wildtype pHBV levels by co-transfection of a second plasmid encoding HBx^{WT}, as reported previously (Bouchard et al., 2002; Keasler et al., 2007, 2009; Kumar et al., 2011; Leupin et al., 2005; Tang et al., 2005) and also by the HBx^{7} point mutant that retained DDB1-binding (Fig. 1C). Importantly, the three HBx point mutants that do not bind DDB1 failed to restore pHBVΔX replication. We have previously shown that the amount of HBx required for maximal replication in the HepG2 assay is well below the limit of detection by our sensitive western blot assay (Keasler et al., 2009). Therefore, expression of these HBx mutants at detectable levels (Fig. 1D) indicates that sufficient protein was present to restore HBVΔX replication. No conclusions can be drawn from this experiment regarding the relative levels of the HBx and mutant proteins, as we are not certain the polyclonal anti-HBx serum reacts equally with all HBx proteins. Together, these results confirm the previous finding that HBx^{R96E} is unable to