Effects of point mutations in the cytidine deaminase domains of APOBEC3B on replication and hypermutation of hepatitis B virus in vitro

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APOBEC3 cytidine deaminases hypermutate hepatitis B virus (HBV) and inhibit its replication in vitro. Whether this inhibition is due to the generation of hypermutations or to an alternative mechanism is controversial. A series of APOBEC3B (A3B) point mutants was analysed in vitro for hypermutational activity on HBV DNA and for inhibitory effects on HBV replication. Point mutations inactivating the carboxy-terminal deaminase domain abolished the hypermutational activity and reduced the inhibitory activity on HBV replication to approximately 40%. In contrast, the point mutation H66R, inactivating the amino-terminal deaminase domain, did not affect hypermutations, but reduced the inhibition activity to 63%, whilst the mutant C97S had no effect in either assay. Thus, only the carboxy-terminal deaminase domain of A3B catalyses cytidine deaminations leading to HBV hypermutations, but induction of hypermutations is not sufficient for full inhibition of HBV replication, for which both domains of A3B must be intact.

Whether APOBEC3 proteins can inhibit the propagation of retroelements by a second mechanism independent of hypermutations is a matter of debate (Hakata & Landau, 2006). Hypermutation-independent restriction of retroelements, including human T-cell leukemia virus type 1, HIV-1 and the IAP, Alu and LINE-1 retrotransposons, has been proposed for several APOBEC3 enzymes (Bogerd et al., 2006a, b; Chiu et al., 2005, 2006; Hulme et al., 2007; Muckenfuss et al., 2006; Sasa et al., 2005; Stenglein & Harris, 2006). Newman et al. (2005) demonstrated that catalytically inactive A3G mutants (E259Q, C288S and C291S) inhibit HIV replication and postulated that hypermutation might not be more than a side effect of this main inhibitory effect. In contrast to these findings, Hakata & Landau (2006) reported that catalytically inactive A3G mutants, notably E259Q, were inactive with respect to HIV restriction, suggesting that mutational inactivation of the viral genome is the physiologically relevant mechanism for the inhibition of retroviral replication by APOBEC3 editing enzymes.

Turelli et al. (2004) were the first to demonstrate that A3G inhibits HBV in vitro and to suggest a hypermutation-independent mode of inhibition, as they found inhibition of HBV replication by two catalytically inactive A3G mutants and failed to detect G-to-A hypermutations in replicating HBV DNA. In line with this assumption, A3G has been shown to increase the susceptibility of encapsidated prereplicative HBV RNA for degradation, indicating that A3G might interfere with RNA packaging by binding to viral RNA or proteins or by inducing structural changes in the nucleocapsids (Rosler et al., 2005). The establishment of three-dimensional PCR (3D-PCR) enabled a reproducible detection of G-to-A hypermutations as a result of cytidine deaminase activity of APOBEC3 editing enzymes in retroviruses.
replicating HBV DNA both in vitro and in vivo (Bonvin et al., 2006; Suspene et al., 2005a). However, the proportion of hypermutated viral genomes in patients with chronic HBV infection was as low as $10^{-4}$ of the total HBV genomes escaping APOBEC3 restriction (Suspene et al., 2005a).

Here, we studied APOBEC3B (A3B) as a model enzyme to investigate in vitro whether the inhibition of HBV replication is dependent on the catalytic cytidine deaminase activity and the generation of G-to-A hypermutations. Similarly to A3G and A3F, A3B has two cytidine deaminase domains (Jarmuz et al., 2002; Wedekind et al., 2003), edits HBV DNA during viral replication (Suspene et al., 2005a) and inhibits the accumulation of HBV replicative intermediates in vitro (Bonvin et al., 2006). The extent of inhibition of HBV replication achieved by A3B is similar to that achieved by A3G or A3F (Bonvin et al., 2006). Although the in vivo role of A3B in HBV infection has not been established, it is conceivable that the double-domain editing enzymes A3B, A3F and A3G, all of which are upregulated by alpha interferon in primary human hepatocytes, act cooperatively in vitro to restrict HBV replication in the liver as part of the innate immune response (Bonvin et al., 2006; Suspene et al., 2005a). In human hepatoma HuH-7 cells, A3B mRNA levels are about 10 times lower than in primary human hepatocytes (Bonvin et al., 2006). Most notably, the A3B protein, which is localized in the nucleus in the absence of HBV, redistributes into the cytoplasm of HuH-7 cells upon HBV expression, suggesting that the A3B protein is either exported from the nucleus together with HBV pre-genomic RNA or trapped within the cytoplasm by HBV-encoded proteins, such as HBcAg (Bonvin et al., 2006).

A human wild-type cDNA of A3B (GenBank accession no. NM_004900), kindly provided by Michael M. Malim, Guy’s, Kings and St Thomas’ Medical School, Kings College London, UK, was used to generate point mutations of both catalytic domains CD1 and CD2 by site-directed mutagenesis. The conserved histidine (H66R, H253R) and cysteine (C97S, C284S) residues of both cytidine deaminase domains, which have been shown to be of critical importance for catalytic activity, were replaced by arginine or serine residues, respectively (Fig. 1). Wild-type and mutant A3B, both tagged with a haemagglutinin (HA) epitope, were co-expressed in human hepatoma HuH-7 cells together with pCMV-HBV, which induces HBV replication in vitro, and with pCMV-LacZ, to correct for differences in transfection rates between individual experiments (Bonvin et al., 2006). In three independent cDNA pools from HuH-7 cells transfected with 0.7 μg A3B cDNA, the A3B mRNA levels measured by real-time RT-PCR were $24.4 \pm 7.2\text{-fold (±SD)}$ higher than those in mock-transfected HuH-7 cells with only endogenous A3B mRNA expression. These determinations were performed in duplicate assays and normalized to GAPDH mRNA levels as described previously (Bonvin et al., 2006). Thus, in HuH-7 cells transiently transfected with A3B cDNA, the A3B mRNA levels are approximately 2–3-fold higher than endogenous A3B mRNA levels in primary human hepatocytes (Bonvin et al., 2006).

HBV replicative intermediates were immunopurified and analysed by Southern blot (Fig. 2). HBV DNA was quantified by phosphorimaging and normalized to the amount of co-transfected β-galactosidase activity (Bonvin et al., 2006).

![Fig. 1. Schematic of A3B showing the conserved domains and the amino acid residues critical for cytidine deamination. Each domain coordinates a zinc atom via the His (H) and one of the Cys (O) residues, whilst Glu (E) is involved in proton transfer. The His and one of the Cys residues from both domains were mutated into non-functional Arg (R) and Ser (S) residues.](http://vir.sgmjournals.org)

**Fig. 2.** A3B inhibitory activity on HBV replication. (a) Human hepatoma HuH-7 cells were transiently cotransfected with pCMV-HBV, with pcDNA6 expression plasmids containing the cDNAs of wild-type (WT) or mutant A3B or with empty-vector pcDNA6 control, and with pCMV-LacZ to correct for transfection efficiency. HBV replicative intermediates were purified from the cell lysate and detected by Southern blotting. (b) Expression of the HA-tagged A3B WT or mutant proteins in the cell lysates was analysed by Western blotting. (c) Immunopurified HBV replicative intermediates from three individual transfection experiments were analysed by Southern blots performed on separate days. HBV DNA of these replicative intermediates was quantified by radiophosphorimaging and normalized for transfection efficiency by determination of cotransfected β-galactosidase activity. The reduction of HBV replication intermediates in the presence of the WT A3B protein, compared with the empty-vector control, was defined as 100% inhibition of HBV replication. Means ± SEM are given.
In all experiments, a constant ratio of A3B:β-galactosidase cDNA was used; therefore, the determination of β-galactosidase activity enabled us to correct for variations in transfection efficiency and expression levels between independent experiments. In HuH-7 cells transfected with wild-type A3B, the replication of HBV was reduced to 20% relative to mock-transfected cells. This extent of inhibition of HBV replication achieved by wild-type A3B was defined as 100% inhibition. The inhibitory activities of the different A3B mutants were expressed as a percentage of this wild-type inhibitory activity, and the mean ± SEM of three independent experiments was calculated. The CD1 mutant C97S inhibited HBV replication to the same extent as wild-type A3B protein (Fig. 2). The CD1 mutant H66R showed an impaired inhibition of HBV replication with a mean inhibitory activity of 63%, and the CD2 mutants H253R and C284S had an inhibitory activity of only approximately 40% (Fig. 2). Titration experiments demonstrated that an increase of transfected A3B cDNA to 1 and 1.5 µg, respectively, did not augment the inhibition of HBV replication, whilst a decrease of A3B cDNA (0.5, 0.25, 0.1 µg) led to reduced inhibition of HBV replication, demonstrating that the transfection conditions were optimized for the ratio of A3B mRNA and HBV RNA expression.

HBV DNA from immunopurified HBV replicative intermediates was amplified by differential DNA denaturation (3D-) PCR, using degenerate primers and a two-round procedure (Bonvin et al., 2006; Suspene et al., 2005a, b). For each HBV DNA sample, the conditions for 3D-PCR were optimized by performing gradients with decreasing denaturation temperature from 95 to 85 °C to determine the lowest denaturation temperature for PCR amplification (data not shown). HBV DNA from HuH-7 cells transfected with wild-type A3B or with mutants H66R, C97S or H66R/C97S could be amplified at a denaturation temperature as low as 87 °C, in contrast to HBV DNA from mock-transfected cells or from cells transfected with the mutants H253R, C284S, H66S/C284S, C97S/C284 or H253R/C284S, all of which required a denaturation temperature of at least 91 °C for amplification. A representative 3D-PCR experiment is shown in Fig. 3(a).

Cloning and sequencing demonstrated extensive hypermutations in all PCR products that were amplified at a denaturation temperature of 87 °C (see Supplementary Fig. S1, available in JGV Fig. 3. A3B-induced hypermutations in replicating HBV DNA. (a) Wild-type (WT) or mutant A3B was cotransfected into HuH-7 cells together with pCMV-HBV plasmid to induce HBV replication in vitro. HBV replicative intermediates were purified from the cell lysates and HBV DNA was amplified by 3D-PCR with denaturation at 87 °C (selective temperature) or 95 °C (permissive temperature). (b) Percentage of G-to-A transitions in 3D-PCR amplification products of replicating HBV DNA in the presence of WT A3B or mutants H66R, C97S or the double mutant H66R/C97S, relative to the total number of G residues in the locus. Four or five independent HBV sequences were cloned and analysed per A3B variant. A x² analysis revealed no significant difference in the number of G-to-A transitions induced by WT A3B or by A3B mutants H66R, C97S or H66R/C97S (P≤0.2). (c) Dinucleotide context associated with A3B editing. The y-axis represents the substitution frequency as a function of the 5’ nucleotide. The deaminated C residue is underlined in the dinucleotide sequence. The expected value is represented by a horizontal bar and corresponds to the base composition of the locus. A x² analysis showed that the observed frequencies for WT A3B, H66R, C97S and H66R/C97S deviated significantly from the expected values for the locus (P≤0.001), whilst not differing significantly from each other (P≤1).
Online). Notably, the H66R/C97S double mutant with an inactivated amino-terminal deaminase domain did not generate fewer G-to-A mutations than wild-type A3B \((P \leq 0.2)\) (Fig. 3b) and the mutation patterns revealed similar dinucleotide-context preferences \((P \leq 1)\), with a significant divergence from the base composition of the locus \((P \leq 0.001)\) (Fig. 3c). In contrast, the PCR products amplified at a denaturation temperature of 91 °C did not contain hypermutated sequences, as shown by cloning and extensive sequencing of recombinant clones (data not shown).

Thus, mutations in the amino-terminal deaminase domain (CD1) of A3B had no effect on the hypermutation of HBV DNA, whilst replacing only one of the critical amino acid residues in the carboxy-terminal deaminase domain (CD2) resulted in a complete loss of hypermutation activity. These results confirm recent experiments on the editing activity of A3B in a DNA mutator assay in bacteria (Bogerd et al., 2006b), demonstrating that the catalytic activity of A3B resides exclusively in the carboxy-terminal deaminase domain, as shown previously for A3G and A3F (Hache et al., 2005; Newman et al., 2005; Stenglein & Harris, 2006). In the HIV system, however, CD1 of A3B has been shown to mediate some residual mutational activity preferentially at GG dinucleotides, whereas CD2 favours GA sequences (Bogerd et al., 2007). In contrast, we did not observe any difference in the dinucleotide-context preferences between wild-type A3B and the H66R/C97S double mutant (Fig. 3c), suggesting further that the impact of A3B CD1 on HBV-editing activity is negligible.

In conclusion, here we show that (i) A3B-mediated cytidine deamination of HBV DNA is at least preferentially, but probably exclusively, mediated by the carboxy-terminal deaminase domain, (ii) catalytically inactive A3B mutants retain inhibitory activity on HBV replication, and (iii) the H66R mutation in CD1 reduces inhibitory activity without affecting cytidine deamination. These results demonstrate that the induction of G-to-A transitions in HBV DNA is not sufficient for full inhibition of HBV replication in vitro and suggest a second mode of inhibition in addition to hypermutation. In support for this assumption, Bogerd et al. (2006b) showed that a catalytically inactive A3B mutant (E255Q) retained full inhibitory activity against the LINE-1 retrotransposon, whereas the catalytically active A3B mutant E68Q exerted only about 30 % inhibitory activity in this system. Very recently, Nguyen et al. (2007) reported that HBV inhibition by A3G is independent of DNA editing, confirming previous results (Turelli et al., 2004).

The results of our study suggest that full inhibition of HBV replication in vitro by A3B requires an intact CD1, in addition to cytidine deamination with generation of G-to-A hypermutations by CD2. The mechanism of this editing-independent, CD1-mediated mode of inhibition for APOBEC3 proteins remains to be uncovered. Moreover, it remains to be investigated whether this presumptive editing-independent mode of HBV restriction by A3B can also be detected in vivo. Navarro et al. (2005) showed that the amino-terminal deaminase domain of A3G binds HIV RNA and proposed that A3G binding could impede RNA packaging or cDNA synthesis sterically when over-expressed. Inhibition of initiation or early elongation of the reverse transcription reaction or alterations of pre-genomic RNA conformation are possible explanations (Nguyen et al., 2007). It would be of interest to know whether the three-dimensional structure of the A3B amino-terminal deaminase domain is unaffected by the C97S mutation and whether full inhibition can take place even without zinc coordination. Notably, Opi et al. (2006) reported that the C97 residue of A3G is essential for RNA-dependent multimerization, but dispensable for catalytic deamination and HIV restriction. These and other questions concerning the presumptive inhibitory function of CD1 in the APOBEC3 enzymes and its possible role in vivo should be addressed in future studies.

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References


The retroviral hypermutation specificity of APOBEC3F and APOBEC3G is governed by the C-terminal DNA cytosine deaminase domain. 


