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Transient expression of modified Hepatitis B capsid protein in *Nicotiana benthamiana* plants for viral nanoparticles production

Gergana G. Zahmanova
Samir I. Naimov
Milena Mazalovska
Rumiana P. Valkova
Ivan N. Minkov

Authors' address:
Department of Plant Physiology and Molecular Biolohy, Faculty of Biology, Plovdiv University,
4000 Plovdiv, Bulgaria.

Correspondence:
Gergana Zahmanova
Department of Plant Physiology and Molecular Biolohy, Faculty of Biology, Plovdiv University,
24 Tsar Assen Str.,
4000 Plovdiv, Bulgaria.
Tel.: +359 32 261529
e-mail: gerganaz@uni-plovdiv.bg

ABSTRACT

Viral nanoparticles (VNPs) are a fascinating class of natural nanoparticles which provide excellent architectural templates that possess the ideal properties of monodisperse size, shape and composition and therefore offer great promise for developing innovative bioinspired nanomaterials. The use of VNPs as templates, carriers, containers and scaffolds for nanotechnology is a new field that has received increasing interest in the last decade. Hepatitis B virus core protein can form VNPs during self-assembly in vivo (in planta) and in vitro. HBV capsid protein forms highly stable icosahedral VNPs with diameter ≈ 30-33 nm. In this study we investigate the potential of modified HB core protein to self-assemble into VNPs during the expression in *Nicotiana benthamiana*. Interestingly, the stability of HBV VNPs can be controlled by alterations in the length of the capsid protein. In order to optimize chemical modification of HBV VNPs additional Lys residues have been added by site directed mutagenesis to surface exposed c/1 loop. A part of c/1 epitope has been replaced by peptide Gly-Lys-Gly. The introduced additional Lys residues that have reactive ε amino group side chains will facilitate interaction with chemical reagents such as activated NHS esters. This opens up a new opportunity to produce chemically conjugated HBV VNP for biomedical purpose.

Key words: Transient expression, Hepatitis B core protein, virus-like particle (VLPs), viral nanoparticles (VNPs)

Introduction

Viral nanotechnology has become one of the most rapidly developing fields of science and technology. Viruses have traditionally been studied as human, animal and plant pathogens, with the goal of understanding molecular and cell biology of viral infection and disease. During viral replication cycle, viruses demonstrate a remarkable plasticity in their structure, including self-assembly and disassembly and site-specific delivery of their cargo molecules. This knowledge has been translated into the idea of using viruses in nanotechnology. Although public perception of a "virus" is associated with harm, viruses can be easily re-organised into environmentally friendly and biosafe structures. Although the major focus of viral nanotechnology is to use plant viruses as a platform for producing nanomaterials, we also propose to use Hepatitis B virus like particles. This virus has been rigorously studied by us during a FP7–funded project PLAPROVA as a source for plant produced vaccines. It has been shown that core proteins of HBV can form VLPs to high yield, when produced in plants using the highly efficient pEAQ expression system (Meshcheriakova et al., 2006; Thuenemann et al., 2013). From a human health perspective, HBV VLPs are absolutely bio-safe due to the lack of viral nucleic acids. During self-assembly in vivo and in vitro the HBV capsid protein forms highly stable icosahedral capsids of two sizes, with triangulation numbers T = 3 (90 dimers, diameter ≈30 nm) and T = 4 (120 dimers, diameter ≈33nm) (Crowther et al., 1994). The HBV capsid protein consists of an N-terminal assembly "core" domain encompassing the

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first 140 amino acids and an arginine-rich "protamine" domain (residues 150-183 aa) connected by a linker peptide (Birnbaum & Nassal, 1990). The core domain dimerizes to form building blocks capable of self-assembly into VLPs, while the protamine domain ~50% arginine, binds nucleic acid (Watts *et al.*, 2002).

HBV VLPs have attracted our attention as a protein cage of imaging molecules or synthetic small RNAs for target delivery of their cargo. For the purpose of obtaining nucleic acids empty VNPs we constructed synthetic codon optimize HBc C-terminal modified genes in which the arginine-rich C-terminal portion of the protein, which is responsible for the incorporation of nucleic acid into core particles, was modified. To investigate the impact of C-terminal arginine rich domain on VLPs assembly we designed four different genes with modifications resulting into C-terminal truncation of HBc protein. The HBc C-terminal mutants were tested for their ability to form VLPs. Non modified HBc183 gene was used to generate HBc -Lys mutant by adding Lys residues into HBc surface exposed c/1 loop. This HBc -Lys mutant will be used for viral nanoparticles production. The additional Lys residue will facilitate the process of bioconjugation.

Materials and Methods

Gene synthesis and cloning

All HBc gene variants have been based on NCBI protein id AAK58872.1, HBV, genotype B. The genes for HB core protein were codon optimized according to the nucleotide sequence of the plant host *N. benthamiana*. Parameters such as codon usage, GC content, cryptic splice sites, premature poly(A) sites, AT rich killer sequences, RNA secondary structures have been optimized. The codon optimized HBV core gene sequences were designed by synthesis (GeneArt AG, Regensburg, Germany). In addition the synthetic HBc genes contained unique restriction sites within N terminus and C terminus. The synthetic HBc genes were excited from the pMA-vector using Age I and XhoI restriction sites, and cloned into the pEAQ-*HT* (kindly provided by G. Lomonossoff, JIC, Norwich, UK) which is vector for transit expression in plants with modified 3'- and 5'-UTR of CPMV. Recombinant plasmids were transformed into *E. coli* XL1-Blue competent cells. The sequence of the resulting plasmids was verified by automatic sequencing at GATC, Norwich Research Park.

Site directed mutagenesis for Lys incorporation into surface exposed c/1 loop of HBc 183

We have used QuikChange Agilent mutagenesis kit following manufacturer's instructions and QuikChang Primer Design Program for design of overlapping primers.

The sequence of the resulting Lys mutant was verified by automatic sequencing, multiple sequence alignment of nucleotide sequences before and after site directed mutagenesis was done using AlignX (Vector NTI).

Oligonucleotide information:

Primer Name	Primer-Template Duplex
t221g_c225g_c226g_t227g	5'-cttgctacttgggtgggagggaagggtgagcaattgggaggttct-3' tgagaacgatgaacccaccctcacttggaactcgttaaccctccaagagac
t221g_c225g_c226g_t227g _antisense	actcttgctacttgggtgggagtgaaccttgagcaattgggaggttctctg 3'-gaacgatgaacccaccctcccttcccactcgttaaccctccaaga-5'

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Agroinfiltration of HBc mutant

Agrobacterium tumefaciens LBA4404 was transformed by electroporation with the plasmid pEAQ-*HT* containing all HBs constructs. *A. tumefaciens* cultures were grown for 2 days at 28°C in LB medium containing kanamycin (50 mg/L) and rifampicin (50 mg/L.). The cells were then harvested by centrifugation at 3000 rpm for 5 min and further resuspended in 10mM MES pH 5.6, 10 mM $MgCl_2$, 100 µM acetosyringone).The suspension was diluted to OD 600 = 0.3 and 3 hour incubation at room temperature. *N. bethamiana* leaves were infiltrated with the recombinant *A. tumefaciens*. Control infiltration included the empty pEAQ-*HT*. The leaf tissue was collected six days after infiltration.

Protein extraction and analyses - SDS PAGE, Western blot, Dot blot

Six days after infiltration, leave tissue was collected, homogenized in 3 volumes extraction buffer (10 mM Tris-HCl, pH 8.4; 120 mM NaCl; 1 mM EDTA; 0.75% (w/v) Sodium deoxycholate; Complete protease inhibitor) and centrifuged 5 min, 9000 x g. Supernatant was mixed with 2:1 w/3x reducing sample buffer and boiled 5 min. Extracts were separated on Invitrogen NuPage 12% gel, and either stained with Coomassie brilliantin blue or electrobloted to PVDF membrane using transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) Membrane was incubated with blocking buffer (5% NFDM, 1% BSA in PBS pH 7.2 with 0.05% Tween 20) for 1 h at RT. The membrane was inoculated overnight at 4°C with primary antibody (10E11, Abcam). After washing three times for 30 min with PBS with 0.05% Tween 20, the membrane was incubated for 1 h with anti-mouse HRP conjugated secondary antibody at 1:10000 dilution (Promega). The reaction was detected with Supersignal West Dura Chemiluminescent Substrate (Thermo Scientific). For Dot blot analysis the native protein extract was loaded on nitrocellulose membrane, the membrane was probed with rabbit polyclonal anti- HBc Ab (1:4.000) and anti-rabbit secondary Ab HRP conjugated. TSP were measured by a Bradford assay (Bio Rad) following manufacturer's instructions. The HRP signal was detected with an enhanced chemiluminescence (ECL) on the X-ray film.

VLPs purification

VLPs were purified from agroinfiltrated leaves. Approximately 10 g of leave tissue was collected and blended with 3x volume extraction buffer and centrifuged 30 min, 9000 x g. The supernatant was collected and loaded onto 10 – 60% sucrose gradient in 10 mM Tris HCl pH 8.5, 120 mM NaCl) and then subjected to ultracentofugation for 2 h 30 min at 36000 rpm (SW41 Beckman). Gradient was fractioned and analyzed by SDS-PAGE, Western blot, Dot blot and EM.

Electron microscopy

For negative staining, one drop of a purified HBc solution containing the assembled core particles was applied to a carbon-coated grid and incubated for 1 min. The grid was washed with water and stained with 2% uranyl acetate for 1 min. Transmission electron micrographs were taken at JIC, Norwich, UK.

Results

Gene synthesis and cloning

The synthetic HBc C-terminal mutant genes were assembled from synthetic oligonucleotides and PCR products by GeneArt AG, Regensburg, Germany. Restriction sites at the 5' and 3' – terminus were introduced for appropriate cloning into pEAQ-*HT* vector.

The arginine-rich C-terminal domain of core protein is responsible for the incorporation of nucleic acid into HBV VLPs. In order to improved formation of empty HBV virus-like particles, a library of plant codon-optimized HBc genes was constructed in which the arginine-rich C-terminal portion of the protein, was modified or replaced (Figure 1). Arginine (R) clusters were replaced with alanine (A) or with lysine (K) residues. In the most extreme case the entire C-terminal region was deleted to give HBc149. A single cysteine residue was added to the C terminus with idea to stabilize the structure and to improve virus like particle formation.

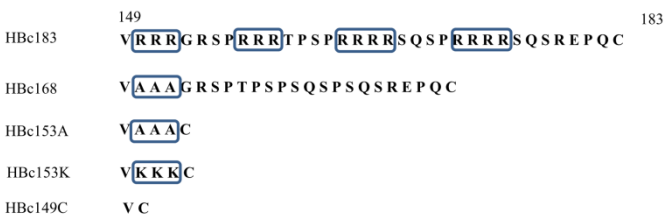

Figure 1. *List of amino acid sequences of the full-length version of HBc and the C- terminal mutants of HBc.*

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Expression of HBc C-terminal mutants and Characterization by SDS PAGE and Immunoblot

All C-terminal HBc mutants and HBc183 full version were expressed in *N. benthamiana* using the non-replicating CPMV-*HT* system. Western blot analysis confirmed that all HBc variants have been successfully expressed in inoculated leaves (Figure 2).

To investigate the influence of the C-terminal modification and truncations on particles formation, the proteins were partially purified by sedimentation on sucrose gradient. Of the C-terminal mutants HBc149 and HBc153K, sucrose gradient analysis indicated the formation of VLPs but these appeared to be less stable than those from HBcA183 (Figure 3, Figure 4). Only HBc183 gave sufficient levels of particles for further modification which showed that HBc183 could act as a source of VNPs (Figure 5).

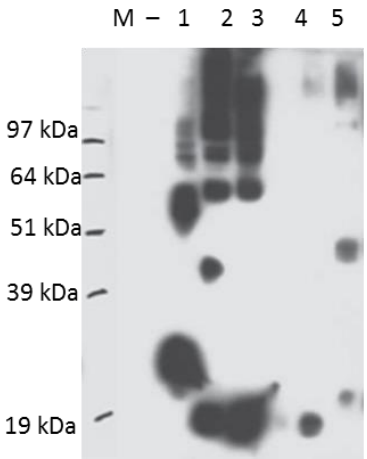

Figure 2. *Western blot probed with monoclonal anti-HBcAg (10E11). M – protein marker, – negative control, 1 – HBc183, 2 – HBc153A, 3 – HBc153K, 4 – HBc149, 5 - HBc168C. Protein samples of approx. 25 µg of total soluble protein per lane extracted from infiltrated leaf tissue.*

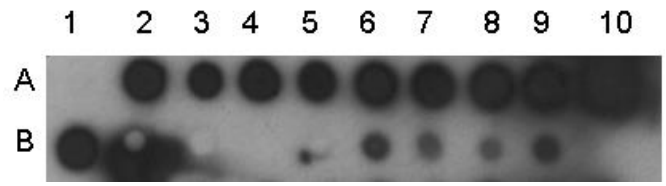

Figure 3. *Dot blot analyses with rabbit anti core polyclonal Ab. HBc153K fraction A.1 – negative control; A.2 positive control; A.3 – B.10 - fraction 1 to fraction 17 from top to bottom. Partially purified on 10-60% sucrose gradients, TH641 rotor, (40 000 rpm, 2h 30 min. 4 C).*

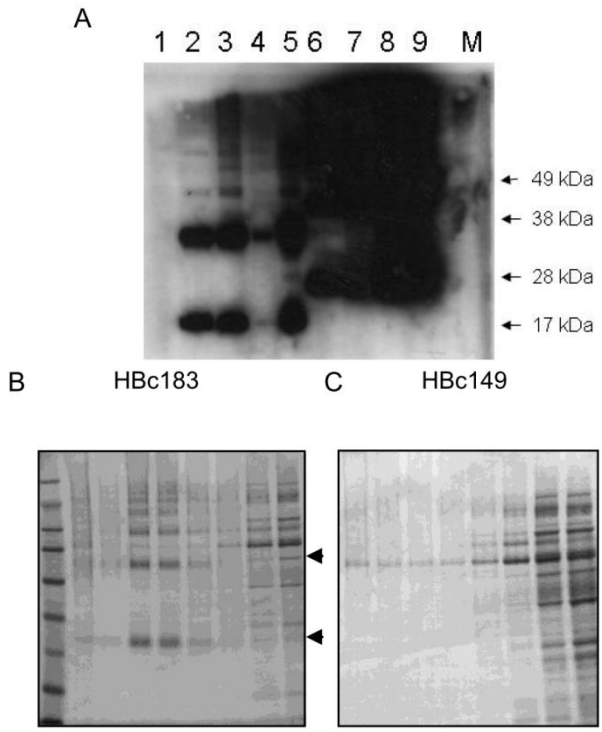

Figure 4. ***A*** *- Western blot probed with monoclonal anti-HBcAg (10E11) Ab.* ***1*** *- negative control;* ***2*** *- HBc149 fraction 7 and 8 mix;* ***3****- HBc149 fraction 5 and 6 mix;* ***4*** *- HBc149 fraction 3 and 4 mix;* ***5*** *- HBc149 fraction 1 and 2 mix;* ***6*** *- HBc183 fraction 7 and 8;* ***7*** *- HBc183 fraction 5 and 6 mix;* ***8*** *- HBc183 fraction 4;* ***9*** *- HBc183 fraction 3;* ***M*** *- SeeBlue NuPage protein standard.* ***B*** *and* ***C*** *– SDS PAGE Coomassie-stained gel (Invitrogen NuPage 12% gel).*

Electron microscopy of purified HBc C-terminal mutants

To investigate the ability of HB C-terminal mutants to form VLPs, we prepared sucrose density gradient and analysed the positive fractions by electron microscopy. HBcAg183, as expected, was found to be the most efficient though the particles formation (Figure 5). Of the C-terminal mutants, EM analysis indicated the formation of VLPs, but we can clearly see particles as well as other aggregates which are partially assembled or disassembled material these appeared to be less stable than those from HBcAg183.

Only HBc183 gave sufficient levels of particles for further analysis which showed that C-terminal mutants could not be used as a source of viral nanoparticles. HBc183 was investigated as a source for site directed mutagenesis and incorporation of Lysine into VLPs.

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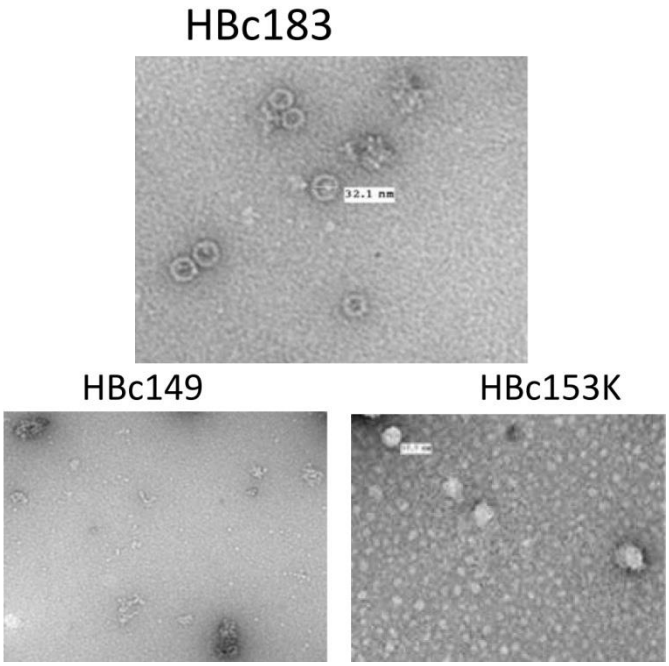

Figure 5. *Electron microscopy of virus-like particles (VLPs) in HBc183; HBc149; HBc153K plant extract.*

Site directed mutagenesis of HBc183

Non modified HBc183 gene was used to generate HBc - Lys mutant by adding Lys residues into HBc surface exposed c/1 loop (Figure 6). We used Glycine as a linker between Lysine and HBc amino acids. The amino acids 78-D, 79-Q and 80-L were replaced with 78-G, 79-K and 80-G by site directed mutagenesis. The sequence of the resulting Lys mutant was verified by automatic sequencing and multiple sequence alignment of nucleotide sequences before and after site directed mutagenesis. Lys - HBc mutant and was expressed in *N. benthamiana* using the non-replicating CPMV-HT system. Western blot analysis confirmed that Lys - HBc mutant has been successfully expressed in inoculated leaves (Figure 7).

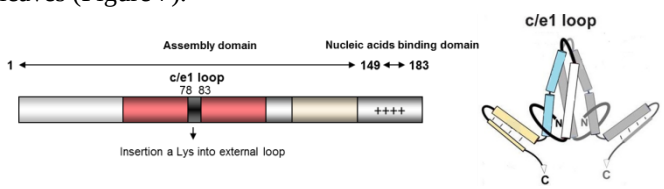

Figure 6. *A - Scheme of the HBc assembly domain and nucleic acids binding domain (Wynne et al., 1999). B - The dimer has the overall shape of a capital T whose stem extends outwards as a spike. In the dimer the c/e1 epitope locates to the loop.*

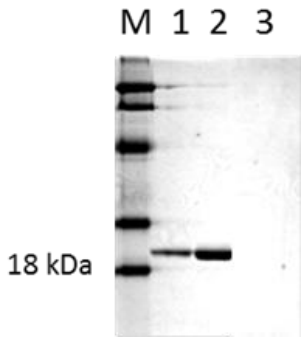

Figure 7. *Western blot probed with monoclonal anti-HBcAg (10E11) antibody. 1 – positive contro l50 ng HBcAg; 2 – Lys-HBc183 mutant; 3 – 5 µl plant extract from inoculated leaves with Lys – HBc mutant construct.*

The sucrose gradient analysis indicated the formation of VLPs from Lys-HBc mutant (Figure 8).

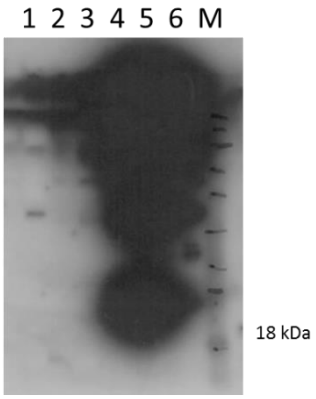

Figure 8. *Western blot probed with monoclonal anti-HBcAg (10E11) Ab. From 1 to 6 –different Lys - HBc sucrose gradient fractions from top to bottom. M - SeeBlue NuPage protein standard.*

Discussion

Although natural HBV is a DNA-containing virus, the HBV VNPs have the further advantage that they can incorporate various foreign RNAs during assembly in vitro with the same efficiency as homologous viral RNA (Porterfield et al., 2010). This opens up a new opportunity to produce HBV VNP-carriers for delivery of various RNAs [including small regulatory RNAs and mRNAs] for various purposes which will be investigated. It is also important that HBV VLPs are tolerant to genetic manipulation. An important next step will be to evaluate the ability of the plant-expressed Lys-HBc VLPs to be chemically conjugated.

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