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An N-terminal extension to the hepatitis B virus core protein forms a poorly ordered trimeric spike in assembled virus-like particles

Richard McGonigle [a], Wei Boon Yap [b,1], Swee Tin Ong [b], Derek Gatherer [a,2], Saskia E. Bakker [a], Wen Siang Tan [b,c], David Bhella [a,*]

[a] MRC-University of Glasgow Centre for Virus Research, Sir Michael Stoker Building, Garscube Campus, 464 Bearsden Road, Glasgow G61 1QH, Scotland, UK
[b] Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[c] Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

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ABSTRACT

Virus-like particles composed of the core antigen of hepatitis B virus (HBcAg) have been shown to be an effective platform for the display of foreign epitopes in vaccine development. Heterologous sequences have been successfully inserted at both amino and carboxy termini as well as internally at the major immunodominant epitope. We used cryogenic electron microscopy (CryoEM) and three-dimensional image reconstruction to investigate the structure of VLPs assembled from an N-terminal extended HBcAg that contained a polyhistidine tag. The insert was seen to form a trimeric spike on the capsid surface that was poorly resolved, most likely owing to it being flexible. We hypothesise that the capacity of N-terminal inserts to form trimers may have application in the development of multivalent vaccines to trimeric antigens. Our analysis also highlights the value of tools for local resolution assessment in studies of partially disordered macromolecular assemblies by cryoEM.

1. Introduction

Vaccines play a crucial role in combating infectious diseases and have led to the eradication of one notable human pathogen: variola virus that caused smallpox. Despite many successful vaccination campaigns, major threats to human health remain for which we have no adequate vaccines. Development of new vaccines is a difficult and expensive process. Traditionally, vaccines for the prevention of viral disease consist of killed or live-attenuated virus. More recently subunit vaccines, containing the antigenic components of the target pathogen have been successfully introduced, these may comprise adjuvant-peptide complexes or particulate assemblies of antigen (Rueckert and Guzmán, 2012).

Viral structural proteins can frequently be made to assemble into structures that closely resemble authentic virus particles. These assemblies are known as virus-like particles (VLPs). As a vaccine candidate VLPs have a number of advantages over peptide based subunit vaccines: ordered, multivalent antigens are more readily recognised by the immune system, and present viral antigens in an authentic conformation, stimulating a strong B-cell response (Noad and Roy, 2003). VLP based vaccines are also safer than live attenuated ones. There is no risk of reversion to virulence such as that which has hindered poliovirus eradication (Modlin, 2010). VLP based vaccines are easier than killed vaccines to produce safely since their production does not require large scale propagation of live pathogenic viruses which may pose a hazard to production workers or a risk of accidental release. VLP based subunit vaccines are currently used to vaccinate against hepatitis B virus (HBV) and human papillomavirus (HPV).

HBV is an enveloped virus that contains a partially double stranded DNA genome synthesized via a reverse transcription step and enclosed within an icosahedral capsid. HBV vaccines consist of the hepatitis B surface antigens (HBsAg), which are the major components of the viral envelope. VLPs of HBsAg are produced in HBV chronic carriers and the first-generation vaccine was prepared by purifying these non-infectious VLPs from the blood of infected individuals (Shepard et al., 2006). The modern form of HBV vaccine contains HBsAg VLPs produced in yeast via recombinant DNA technology (Valenzuela et al., 1982).

VLPs are also capable of being assembled from the dimer of core protein (HBcAg), which is 183 amino acids (aa) in length (or 185 aa

* Corresponding author.
E-mail address: david.bhella@glasgow.ac.uk (D. Bhella).
[1] Current address: Program of Biomedical Sciences, School of Diagnostic and Applied Health Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, 50300 Jalan Raja Muda Abdul Aziz, Kuala Lumpur, Malaysia.
[2] Current address: Division of Biomedical and Life Sciences, Lancaster University, Lancaster LA1 4YT, United Kingdom.

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in some variants of the virus). HBcAg VLPs were the first to be proposed as epitope display particles for the development of multivalent vaccines (Pumpens and Grens, 1999). HBcAg particles are dimorphic; they may be formed from 240 subunits to produce a $T = 4$ icosahedrally symmetric particle that is 34 nm in diameter or 180 subunits to make a 30 nm diameter particle with $T = 3$ symmetry (Crowther et al., 1994; Karpenko et al., 2000). Both forms of particle have prominent spikes on their surfaces that are four-helix bundles consisting of two anti-parallel helices donated by each monomer. The loop connecting the two helices in each monomer is the major immunodominant epitope (MIE) of the core protein. The C-terminal 43 residues of HBcAg form a basic domain that interacts with the viral genome. For heterologous VLP production this region can be deleted without hindering assembly (Gallina et al., 1989).

HBcAg can activate a B-cell response independent of T-cell activation, and confers this property to normally T-cell dependant antigens displayed on the VLP (Whitacre et al., 2009). HBcAg can also spontaneously self-assemble, in the absence of other viral proteins (Karpenko et al., 2000). Assembly has been observed in a wide range of expression systems including: mammalian (Hirschman et al., 1980), insect (Hilditch et al., 1990; Seifer et al., 1998), plant (Tsuda et al., 1998) and bacterial cells (Pasek et al., 1979; Schödel et al., 1990).

To successfully exploit the above properties requires that potential vaccine constructs be designed such that the antigen is exposed on the VLP's surface and does not interfere with particle formation. Only 3 sites, the MIE, the N- and C-termini, allow insertion of foreign antigen while fulfilling these criteria (Ulrich et al., 1998). The most immunogenic of the three insertion sites is the MIE loop, followed by the N-terminus (Schödel et al., 1996). While it is not possible to definitively predict whether a VLP will assemble with a given insert, certain characteristics have been deduced which impact on VLP formation (Nassal et al., 2008). Factors that can disrupt VLP formation include the presence of: cysteine residues (Janssens et al., 2010), β-pleated sheets and extensive regions of hydrophobicity (Karpenko et al., 2000). Formation can also be disrupted by homomeric interactions between inserts, for example propensity to form anti-parallel dimers might disrupt core dimerization (Vogel et al., 2005). The size of the insert is important; smaller inserts are more likely to permit VLP formation, owing to reduced steric hindrance. Exceptions exist however, such as the successful insertion of GFP into the MIE loop (Kratz et al., 1999). Finally incorporating flexible linker regions between insert and core increases the probability of success. For inserts into the MIE, close proximity of the N- and C-termini also improves the chances of VLP assembly as such inserts are less likely to disrupt formation of the four-helix bundle (Nassal et al., 2008).

Here we describe the structure of VLPs produced by heterologous expression of a HBcAg construct containing a polyhistidine tag within a 37 residue N-terminal extension (His-β-L HBcAg) (Yap et al., 2009). Cryo-electron microscopy and three-dimensional reconstruction at intermediate resolution revealed the presence of a second, apparently trimeric, spike on the VLP surface. Icosahedral reconstructions were calculated for both $T = 3$ and $T = 4$ VLPs. Attempts to achieve sub-nanometre resolution in these maps were frustrated however; Fourier shell correlation analysis indicating that the structures were limited to 10 and 12 Å respectively. Two local resolution assessment algorithms were used to investigate the resolution of the maps in more detail revealing that sub-nanometre resolution was achieved in the HBcAg component of the construct while the N-terminal insert was poorly ordered. Nonetheless we hypothesise that formation of a second trimeric spike on the VLP surface may have application in the design of vaccines to elicit immunity against proteins that normally exist in a trimeric state.

2. Materials and methods

2.1. Expression of N-terminal extended HBcAg

The plasmid pHis-β-L-HBcAg was previously described (Yap et al., 2009) and the amino acid sequence of its translated product was described in (Lee et al., 2012). Briefly the construct His-β-L HBcAg comprises a 37aa region (MRGSHHHHHHGMASMTGGQQM GRDLYDDDDKDPLEFH) fused to residues 3–145 of HBcAg. The N-terminal extension contains a 6-His tag, the T7 gene 10 leader, the Xpress™ epitope, amino acid sequence of pRSET vector, two residues of β-galactosidase and a 3 residues flexible linker.

Escherichia coli cells harbouring pHis-β-L HBcAg were cultured in 1 L Luria–Bertani (LB) medium at 30 °C at 200 rpm. When the cultures reached OD_{600} 0.6–0.8, recombinant protein expression was induced with 0.5 mM isopropyl β-d-thiogalactoside (IPTG) and incubated for 16–18 h. The cells were pelleted by centrifugation at 4000×*g* for 20 min; the pellet was then resuspended in HEPES buffer (25 mM; 2 ml; pH 7.6). The cells were disrupted by ultrasonication for 10 min (20 s between pulses) then centrifuged at 30,000×*g* for 20 min. The supernatant was incubated with DNA-ase (10 μg/ml) at 37 °C for 1 h and then precipitated with 35% ammonium sulphate saturation. This mixture was spun at 8000×*g*, dissolved in binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazol, pH 7.4) and dialysed at 4 °C overnight.

2.2. Purification of VLPs

VLPs were purified by immobilised metal affinity chromatography. A HisTrap HP column (Amersham Bioscience, Pittsburg, USA) was equilibrated with 5 column volumes (CV) of binding buffer, 10 mg of sample was loaded then washed with 10 CV of binding buffer, followed by 5 CV of washing buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4; 100 mM imidazole) and then eluted with elution buffer [(20 mM sodium phosphate; 500 mM NaCl), pH 7.4; 500 mM imidazole] (Yap et al., 2009).

2.3. Cryo-electron microscopy

Purified VLP suspension (5 μl) was loaded onto freshly glow-discharged quantifoil perforated carbon support films (R2/2; Quantifoil, Jena, Germany), blotted, and plunged into liquid ethane (Adrian et al., 1984). Vitrified specimens were imaged at low temperature and under low-electron dose conditions (between 12 and 15 e/Å^2) in a JEOL 2200 FS cryo-microscope operated at 200 kV. Specimens were held in a Gatan 626 cryo-stage cooled to 95 K. To reduce the contribution of noise to the image by inelastic scattering of electrons, energy filtered imaging was performed with a slit-width of 20 eV. Images were recorded at 150,000× magnification (corresponding to a pixel size 0.69 Å in the specimen) on a Gatan Ultrascan 4k × 4k charge-coupled device camera.

2.4. Icosahedral reconstruction

Micrographs were binned by a factor of two prior to analysis, giving a sample frequency of 1.39 Å/pixel. The *BSOFT* package was used to estimate defocus values for each micrograph (which ranged from 0.8 to 3.8 μm) and apply CTF correction (Heymann, 2001). Particle images were processed using *SPIDER* (Frank et al., 1996) to remove low-frequency variations in density and to exclude outlying grey levels caused by e.g. X-rays. Images were then classified into two sub-populations based on correlation with fuzzy ring models representing the $T = 3$ and $T = 4$ classes. Finally