Minireview

Hepatitis B core particles as a universal display model: a structure-function basis for development

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Abstract Because it exhibits a remarkable capability to accept mutational intervention and undergo correct folding and self-assembly in all viable prokaryotic and eukaryotic expression systems, hepatitis B core (HBc) protein has been favored over other proposed particulate carriers. Structurally, the unusual α-helical organization of HBc dimeric units allows introduction of foreign peptide sequences into several areas of HBc shells, including their most protruding spikes. Progress toward full resolution of the spatial structure as well as accumulation of chimeric HBc-based structures has brought closer the knowledge-based design of future vaccines, gene therapy tools and other artificial particulate objects.

Key words: Hepatitis B core protein; Self-assembly; Protein structure; Electron cryomicroscopy; Molecular display; Antigenicity

1. Introduction

Supermolecular structures built in a symmetric manner from hundreds of proteins of one or more types, often called VLPs (virus-like particles), could serve as molecular carriers, providing a regular arrangement of wanted polypeptide chains at the desired positions on the outer surface of the carrier (see latest review articles [1–4]). Such structures ensure a specific three-dimensional conformation and a high density of introduced peptides per particle, which are the deciding factors for their functional activity in inducing immunological response, binding of receptors, or recognizing low molecular weight substrates.

The first models for VLP protein engineering came from rod-shaped representatives of the Inoviridae (filamentous bacteriophage f1) and Tobamoviridae (tobacco mosaic virus) families, as well as from the outer envelopes and the inner capsids of a representative of the double-stranded DNA virus family Hepadnaviridae, namely, human hepatitis B virus (HBV). Whereas phage f1 derivatives initiated a series of replication-competent viruses employed as particulate carriers, such as phage-display vectors, and some animal (polio, rhino) and plant (tobacco mosaic and cowpea mosaic) viruses (see recent review [4]), HBV nucleocapsid or core protein (HBc) remains as before the favorite example of non-infectious carriers.

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As a general advantage, the unusual flexibility and insensitivity to foreign invasions of the HBc molecule are manifested by its ability to participate in particles of both T = 3 and T = 4 symmetry and therefore to take not only the three A, B, and C, but also a fourth D conformation in a T = 4 arrangement [5]. HBc particles possess not only an outstanding ability to induce B cell, T helper (Th) and cytotoxic T cell (CTL) response, but also permit the inserted polypeptides to exhibit the appropriate immunological properties. HBc molecules retain their ability to form particles and high-level yields in practically all popular homologous and heterologous expression systems. Chimeric HBc derivatives are easy to purify because of their particulate nature, and they can be subjected to dissociation with subsequent re-association, thereby allowing removal of internal impurities. HBc chimeras are able to pack nucleic acids, for possible use in gene therapy experiments, or, alternatively, they can be deprived of this ability, to yield nucleic acid-free preparations.

Here we outline the relationship of functional features of the HBc shell to its structural peculiarities.

2. Multifunctionality as a major attribute of HBV C gene-encoded proteins

A portrait of gene C, as it is currently understood, is given in Fig. 1. Although gene C possess two in-frame initiation ATG codons, it is accountable for at least four different polypeptides, p25, p22, p21, and p17 [6]. The p21 polypeptide, or the HBc protein proper and the major constituent of the HBV nucleocapsid, is synthesized from the second initiation codon. It participates, besides its structural function, in the full cycle of viral replication and its regulation, including RNA packaging, DNA synthesis, viral maturation, recognition of viral envelope proteins and budding from the cell (see [7]). The p25 precore protein is started at the first ATG codon and becomes targeted by a signal peptide to a cell secretory pathway where N-terminal processing will create a p22 species. The latter undergoes further modification at the C-terminal region, after position 149, to generate p17, or e-protein, which is secreted from the cell as HBe antigen.

The exact role of the precore polypeptides p25 and p22 and of the HBe antigen in the biology of hepadnaviruses remains unknown. In the present review, we will restrict the discussion to the observation that the expression of p22 leads to the formation of nucleocapsids similar to those made with p21 core protein, and that p21 and p22 may form hybrid nucleocapsids [6]. The HBe protein p17 does not form particles, but enters the secretory pathway and is actively secreted by cells (see [8]).

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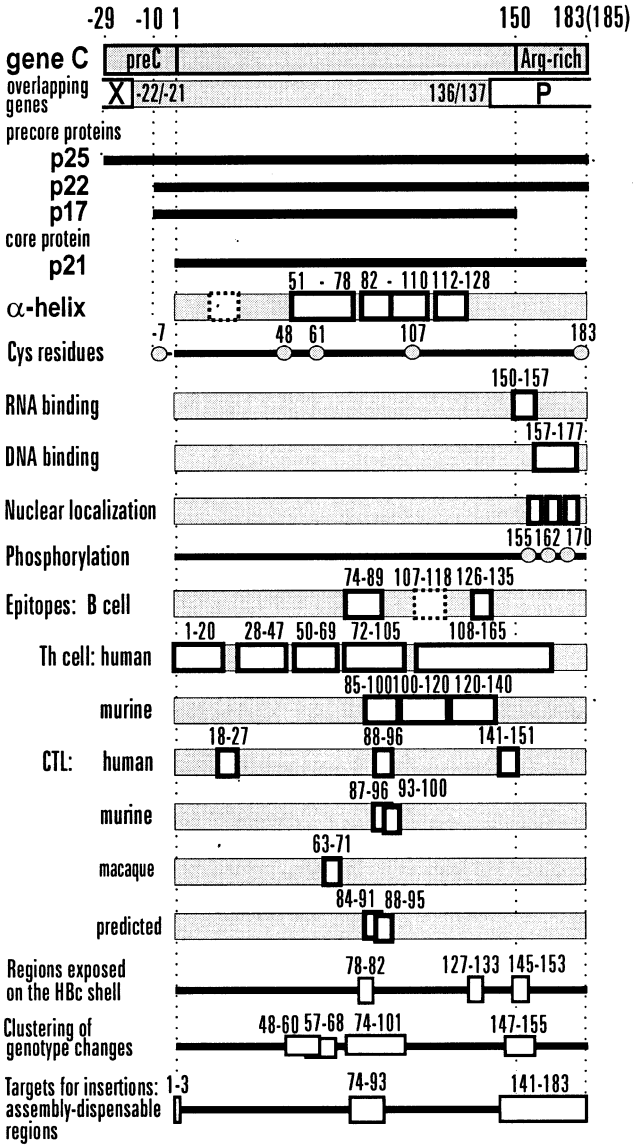

Fig. 1. Portrait of HBV gene C. Structural and functional peculiarities of the HBc molecule are compiled from the publications cited in the text. The 185-aa length of some HBc representatives belonging to the subtype *adw* is caused by a 2-aa insertion after position 152. For gene C overlap with genes X and P, the corresponding amino acid residues of the HBc molecule are indicated. The predicted N-terminal α-helix of 10 amino acid residues [28] is marked by a dashed line.

3. Evolutionary history of gene C

Gene C represents the only non-overlapping sequence in the HBV genome (Fig. 1), although it remains the mostly evolutionarily conserved. The HBV core sequence demonstrates high similarity (65–67% at the amino acid level) to cores of rodent: woodchuck (WHV), ground squirrel (GSHV), and arctic ground squirrel (ASHV) hepadnaviruses, but only traces of homology with cores of bird: duck (DHBV), and heron (HHBV) hepadnaviruses (for references see [9]). Recently, the woolly monkey hepatitis B virus (WMHBV), a probable progenitor of the human viruses, was found [10]. The WMHBV core is practically identical to HBV cores (85–87% similarity at the amino acid level). HBV, WHV, and GSHV, but not DHBV core proteins, are able to cross-oligomerize into mixed particles [11].

Serologically, HBV genomes were classified into four main subtypes *adw, adr, ayw* and *ayr*, in accordance with the HB seroreactivity. Structurally, six HBV genotypes A–F were determined [12], and two core genotypes representing the core sequences of HBV genotype C and most genotype B strains (core genotype 1) and of HBV genotypes A and D (core genotype 2) have been described [13]. The mutation clustering regions for these core genotypes 1 and 2 were located at residues 84–99 and 48–60, respectively [13]. For HBV genotype F, a mutation clustering region was localized within the amino acid stretch 57–68 [14]. In total, the sequence 74–101 was found to be the most 'hypervariable' where frequent variations occur in amino acid residues [15]. Recent investigations have shown that clustering changes may be directly responsible for the outcome of the disease, in particular, HBc deletions and missense mutations are related to the development of liver injury (for example, [16]).

4. Extraordinariness of the spatial structure of HBc molecules

4.1. Direct structural investigations

Early structural investigation of HBV core particles was undertaken in the mid-1970s when the first data on their shape, size, and polypeptide composition appeared [17]. The existence of two size classes of HBV cores, with a predominant larger and a less frequently occurring smaller species, has been detected with particles from HBV-infected human liver using either electron microscopy of a negatively stained specimen [18] or gel filtration [19]. Nevertheless, the first precise electron microscopic ultrastructure of native HBV cores fixed for more than 10 years a notion of their composition according to $T = 3$ icosahedral symmetry, built up by 180 subunits [20]. Since HBV failed to be reproduced in cell cultures, further developments in the understanding of HBc structure required heterologous sources of authentic HBc particles.

Expression of the HBV C gene, not only in various eukaryotic systems: yeast *Saccharomyces cerevisiae*, insect *Spodoptera*, frog *Xenopus* oocytes, mammalian cell cultures, vaccinia virus (for references see [2,4]), and, recently, in plant *Nicotiana tabacum* [21], but also in bacteria such as *Escherichia coli*, *Bacillus subtilis*, *Salmonella* and *Acetobacter* [2,4], demonstrated high-level production of the HBc protein. Over-expressed HBc protein showed correct self-assembly into naturally shaped particles, in the absence of any other viral components. Direct electron microscopy comparison of HBc particles isolated from virions and infected hepatocytes and the particles from *E. coli* [18] and from yeast [22] confirmed their ultrastructural superimposability. The size heterogeneity of bacterial [18,19] and yeast [22] HBc particles suggested the ability of these expression systems to employ homologous or similar machinery for synthesis, translocation and folding of HBc protein as the human cell.

Of particular value in understanding the structure of HBc particles was the clear demonstration of the dispensability of the C-terminal protamine-like arginine-rich domain 150–183 of HBc polypeptide both for their ability to form particles, and, more or less, for the size and morphology of capsids [23,24]. These HBcΔ particles are practically indistinguishable from full-length HBc particles using high resolution electron cryomicroscopy [5]. In contrast to the full-length HBc par-

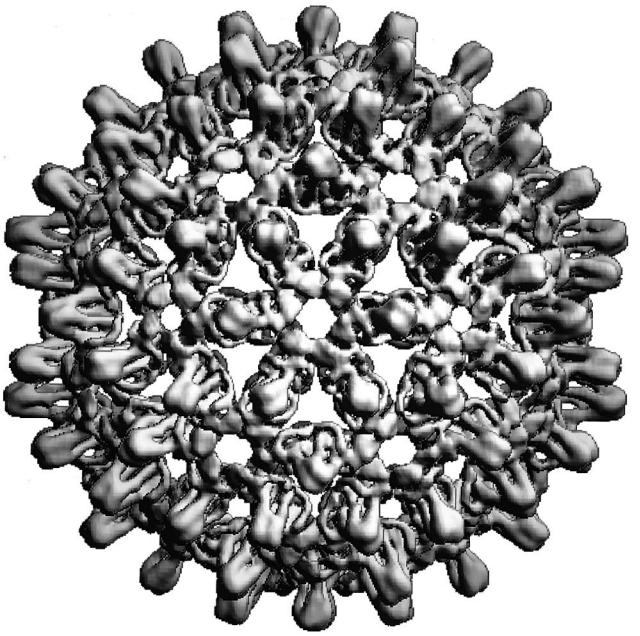

Fig. 2. Surface-shaded three-dimensional map of the hepatitis B core shell. The map was computed at 7.4 Å resolution from electron micrographs of frozen hydrated specimens and shows a 2-fold view of the 240-subunit shell. The fold of the core protein, which is largely α-helical, was deduced from the map (see [28]). The map was a generous gift of Dr. R.A. Crowther.

ticles, they are less stable, and usually appear as empty shells, [23–26]. Moreover, the first attempts to apply NMR for analysis of HBc particles revealed an unusual mobility of the C-terminal protamine-like domain [27]. Such HBcΔ particles play an important role in structural modeling and seem to be good prospects for further applications such as vaccine or gene therapy carriers.

The fine 3D organization of HBc particles (Fig. 2) was revealed at last by electron cryomicroscopy and image processing [5], and was found to be largely α-helical and quite unlike previously solved viral capsid proteins [28]. It turned out that HBc molecules are able to assemble into two particle sizes, large and small, which corresponded to triangulation number T = 4 and T = 3 dimer clustered packings, containing 240 and 180 HBc molecules, respectively [5]. Particles are approximately 34 nm and 30 nm in diameter and show 120 or 90 protruding spikes. The local packing of subunits was very similar in the two sizes of particle and showed characteristic spikes and holes on the shell surface. The arrangement of these spikes and holes led to the proposal that the putative basic subunit of HBc particle is a dimer having a hammer head shape, with the protruding spike forming the 'handle' and the 'head' formed by wedges of the shell domain extending towards the centers of the rings of 6 or 5 dimers. Full-length HBc particles contained additional material, probably RNA, which appeared as a partially icosahedrally ordered inner shell in the 3D map [5].

At a resolution accuracy of 7.4 Å, the 3D map revealed the complete fold of the HBc polypeptide dimer, which consisted of radial bundles of four long α-helices with the central positions 78–82 of the major immunodominant region, or MIR (Fig. 1), of the HBc protein at the tip of the spike [28]. Each HBc monomer participates in this bundle by an antiparallel α-helical hairpin about 42 Å in length, with the straight α-helix 51–78 bounded by conserved proline residues P50 and P79, and with the kinked α-helix 82–110 finished at glycine G111 (Fig. 1). This α-helix would contain a conserved glycine at position 94, which might correspond to the position of the kink, whereas the next, C-terminal α-helix would then correspond to residues 112–128, being terminated by conserved proline residue P129. A fourth α-helix of the HBc monomer would lie at the N-terminal part. Most of the monomer-monomer interface within the dimer consists of interactions between the two homologous straight α-helices in the two opposed hairpins [28]. The total length of α-helices corresponds to about 81 aa, which is in good agreement with circular dichroism measurements [29]. After the tip of the spike, the next most exposed and accessible feature on the shell surface is region 127–133, a further B cell epitope beyond the MIR (Fig. 1) lying at the end of the C-terminal α-helix and making a small protrusion on the surface close to the 5-fold and 6-fold positions. The accessibility of this region to specific monoclonal antibodies was confirmed by immune competition [30] and electron microscopy [31] experiments.

An independent cryo-electron microscopy map of the HBc particle at a resolution of 9 Å revealed the same protruding spikes consisting of a 4-helix bundle of the dimeric subunit, but placed the residues 76–82 on the sides of the spikes [32]. Recently, the location of these residues on the tips of the spikes was confirmed by electron cryomicroscopy of complexes of HBc shells with anti-HBc monoclonal antibodies recognizing amino acid residues 78–83 [33]. Electron cryomicroscopy and 3D image reconstruction were also applied to compare DHBV and HBV cores of natural and bacterial origin [31]. In spite of strong differences between duck and human proteins in both length (262 versus 183 residues) and amino acid composition, the only significant structural differences observed are the lobes of density on the lateral edges of the projecting domain of the HBc dimer. Since the authors used native HBc isolated from human liver, this study re-confirmed on an advanced level the faithful reproduction of the native core structure in bacteria, even in the absence of the complete viral genome, as well as the idea of evolutionary variability of the projecting domain.

4.2. Functional complementations to the 3D structure. Role of specific elements in its maintenance

4.2.1. Dimers and Cys residues. The described HBc fold [28], although quite different from the standard β-sheet jelly-roll packings found in many viral capsids, is consistent with a large body of functional information about HBV cores. First of all, it explains the proposed composition of HBc particles from dimers [34] and the linkage within these dimers by homologous intermolecular disulfide bridges, upon aging or oxidation, between cysteines C61 [35–37], at the base of hairpin, and about half-way up the straight α-helix, respectively. Of four HBc cysteine residues at positions 48, 61, 107 and 183, the latter constituting the C-terminus, each was replaced individually, or in all combination, by alanine or serine, and all were found non-essential for HBc particle formation. However, cysteine residues stabilize HBc dimers and capsids [37]. Normally, C107 exists as a free thiol buried within the particle structure, C48 is present partly as a free thiol which is exposed at the surface of the particle [35]. C61 is always and C48 is partly involved in interchain disulfide bonds with the identical

residues of another monomer, leading to dimerization, whereas C183 is always involved in a disulfide bond between dimers generating polymers of S-S-linked dimeric HBc molecules [35–37]. No intra-chain disulfide bonds occurred. Interestingly, WHV core shells have the same pattern of bonding, whereas the DHBV core lacks any disulfide bonds, and the single free thiol, C153, which is equivalent to C107 of HBc, is buried [35]. Direct HBV replication studies showed, however, that none of the steps in the viral life cycle from reverse transcription to envelopment was principally impaired by replacement of all cysteines by serine residues, although the stability of the mutant enveloped particles was lowered in comparison to the wild type [38].

4.2.2. Cys residues and formation of HBe antigen. During maturation of the HBe protein (for references see [8]), 19 of the 29 preC amino acids, as well as about 34 amino acids of the C-terminal domain are cleaved from the precore p25 protein (Fig. 1). A 10-aa signal sequence and specifically cysteine C(−7) within it are responsible for the properties which distinguish the HBe from HBc antigens. The HBc map [28] explains the block of HBc formation by a possible disulfide bond between preC cysteine C(−7) and either cysteine C48 or C61, preventing dimerization and therefore shell assembly.

4.2.3. Assembly. C-terminally truncated cores. The interactions between dimers to form the icosahedral shell occur around the 5-fold and 6-fold axes and are mediated by the C-terminal helices and tails [28]. This explains why truncation of the HBc protein before residue 140 inhibits shell assembly [26], as these contacts could not then be formed, and also why the C-terminal truncation may influence the distribution between T = 3 and T = 4 shells in bacterially expressed HBc derivatives ([39,40], Borisova and Ose, unpublished observations).

According to the map [28], HBc protein consists roughly of an assembly domain 1–149 and a protamine-like (RNA binding) domain 150–183. Detailed assembly studies in *Xenopus* oocytes led to the understanding that nascent HBc polypeptides rapidly dimerize, but accumulation of free dimers to a signature concentration (approximately 0.8 μM) triggers a highly cooperative capsid assembly reaction, whereas deletion of a protamine-like domain markedly increases the concentration of dimers needed to drive capsid assembly [41]. According to recent data [40], the last assembly-competent HBc derivative terminates at position 140, and shows a proportion of T = 4 isomorphs of approximately 18%. The proportion of T = 4 capsids increases systematically with shorter deletions from the C-terminus, and the 142-, 147-, and 149-aa variants form about 52, 79 and 94% of T = 4 capsids, respectively. The density map of a HBc mutant with a single gold-labeled cysteine at its C-terminal residue 150 supported expecting a bulky C-terminus to favor formation of the T = 4 capsids [42].

In the study of protease resistance of HBc particles, the only protease-sensitive region 145–153 was suspected to constitute a hinge between the assembly and protamine-like domains [43]. This region was suggested to form an arm-like structure, where a single mutation of the proline at position 138, responsible for dimer interactions, to glycine prevented self-assembly into particles of the full-length HBc protein dimers, whereas replacement of conserved prolines at positions 134, 135, and 144 did not affect HBc particle formation [44].

Two-hybrid and pepscan techniques recently identified two strongly interacting regions: 78–117 (region I), which probably forms part of the dimer interface, and 113 and 143 (region II) located at the basal tips of the dimer and contributing to dimer multimerization [45].

4.2.4. N-terminal interactions. There may also be contacts around the 3-fold axes between N-terminal residues of neighboring dimers [28], which might explain why N-terminal deletions appear to inhibit shell assembly [46]. Although monoclonal antibody mapping suggested rather interior location of the N-terminus of the HBc molecule [47], direct structural analysis revealed the superficial location of 9-aa β-galactosidase amino acid residues added to the N-terminus [48].

5. Mapping of specific functional sites on the HBc molecule

5.1. RNA encapsidation

The location of positions around 140–149 in the map [28] is consistent with the putative continuation of the protamine-like tail into the interior of the shell to interact with nucleic acid. The HBc sequence 1–164 was sufficient not only for RNA binding in a heterologous system [26], but also for specific HBV pregenome encapsidation and formation of enveloped virions [49]. However, cores from the 1–164 variant failed to support production of relaxed circular HBV DNA, and further C-terminal residues at least to position 173 were necessary to restore this ability. Careful analysis attributed major RNA-recognizing activity to the sequence 150–157, and DNA-recognizing activity to three repeated SPRRR motifs within the sequence 157–177 (Fig. 1), although four arginines following the first 149 amino acid residues also provided RNA binding [50]. Capsids formed in *E. coli* by full-length HBc protein packaged bacterial RNAs in amounts equivalent to the viral pregenome [42].

5.2. Phosphorylation and nuclear localization

HBc protein appears as a phosphoprotein with the phosphorylation of the C-terminal stretch of the molecule [51], on serine residues within three repeated SPRRR motifs, which could serve as acceptors of phosphate with equal efficiency [52] (Fig. 1). Non-phosphorylated HBc protein showed a higher affinity for nuclear localization than did phosphorylated protein [52]. Moreover, phosphorylation of the nucleic acid binding sites in the HBc protein that occurs within the particles after encapsidation of viral polymerase and pregenomic RNA may serve as a signal for viral genome maturation [51].

5.3. Traces of proteolytic domains

One of the most intriguing and unclear features of the HBc protein remains its relatedness to proteolytic enzymes [53]. However, the direct mutagenic analysis of the highly conserved HBc sequence 30-LLDTAS-35, similar to the active site motifs of both aspartic acid and retroviral proteases, suggested strongly that HBc processing to HBe does not depend on the presence of an intact protease-like sequence [54].

5.4. Immunological epitopes

From the immunological point of view, HBc particles induce the strongest B cell, Th cell and CTL responses, among other HBV polypeptides, and function as both T-cell-dependent and T-cell-independent antigen (see [1]). Recently, the enhanced immunogenicity of HBcAg was explained by its ability

to be presented by B cells as the primary antigen-presenting cell in mice [55].

Fig. 1 summarizes existing data on the location of immunological epitopes on the HBc molecule. B cell epitopes are mapped, first of all, within the MIR on the tip of the spike, carrying so-called c and e1 epitopes [56,57]. Only one group [58] positioned the main B cell epitope to the right of aa 80, at the region 107–118. The other HBe epitope, e2, was assigned originally to the region 130–138 [56]. Later, human and murine anti-HBe antibodies were found to recognize a linear region around position 130 within the amino acid stretch 126–135 defined now as the e2 epitope, and the most essential sequences there are 129-PPA-131 129-PP-Y-132 [59]. Refinement of HBc regions exposed or internalized at the surface of the particle with monoclonal antibodies led to the final conclusion that sequence 127–133, along with region 78–83, occupies a superficial position on the native HBc shell, whereas other regions, for example, 133–145 and 9–20, were hidden [30,60].

In acutely infected hepatitis B patients, the immunodominant Th epitope 50–69 is recognized irrespective of HLA background, two further important epitopes are 1–20 and 117–131, and a series of other sequences covering practically the whole HBc polypeptide were described (see [61]). In mice, examination of the fine specificity of Th cell recognition revealed predominant epitopes specific for each murine strain, dependent on the H-2 haplotype: 120–140 (haplotype H-2s,b), 100–120 (haplotype H-2f,q), and 85–100 (H-2d mice) [62].

A single HLA-A2-restricted CTL epitope 18–27 has been identified in man (see [61]). Additionally, an HBc epitope 141–151 has been defined by CTL clones from patients with acute hepatitis B, which is restricted by both HLA-Aw68 and HLA-A31 molecules. Recent studies allowed the direct isolation of a naturally processed HBc peptide 88–96 recognized by HLA-A11-restricted CD8+ CTLs [63]. In mice, the HBc peptides 93–100 [64] and 87–96 [65] were found as CTL epitopes in the context of Kb binding (H-2b mice) and Kd binding (H-2d mice), respectively. In macaques, the long-lived CTL response was directed against a HBc peptide 63–71 [66]. Peptides 84–91 and 88–95 were predicted as good candidates for CTL epitopes [15].

6. Capacity of the HBc carrier. Structural and functional fate of inserted sequences

Long-term empirical search for appropriate targets of protein engineering manipulations by insertion of various foreign B cell and T cell epitope sequences (reviewed in [1–4]) pointed to the MIR region, or the tip of the spike, and to the N- and C-termini of the HBc molecule, which is in good agreement with the high-resolution structural data [28]. Summarizing briefly all existing data on the insertions of foreign epitopes, the present conclusions are as follows. Deletions of more than three amino acid residues are not allowed at the N-terminus of the HBc molecule. The maximal length of N-terminal insertions reaches 50 amino acids, but the superficial accessibility of insertions is not very high. For C-terminal insertions, where positions 144, 149, and 156 are used most frequently as targets, the capacity of vectors exceeds 100 amino acid residues, and inserted sequences may be exposed, at least partly. Foreign epitopes inserted at the N- and C-termini are more or less immunogenic, but chimeras maintain strong intrinsic HBc antigenicity/immunogenicity.

The situation is much more interesting in the case of foreign insertions into the MIR of the HBc molecule, which is now selected as a target of choice and where the region between two conserved glycines G73 and G94 seems to be dispensable for HBc self-assembly. It is extremely important that deletions of proper amino acid residues within this region abrogate the intrinsic HBc antigenicity/immunogenicity and simultaneously kake it possible to enhance the immunogenicity of inserted sequences [67]. Recent investigations also showed the unusually high capacity, up to 120 amino acids, of the MIR for foreign insertions [68]. Moreover, strong experimental evidence has been found for the self-assembly competence of some natural HBc deletion variants (e.g. 86–93 and 77–93) occurring in patients with progressive liver disease [69].

In general, the HBc carrier ensures a high level of B cell and T cell immunogenicity to foreign epitopes, especially when they are inserted into the tips of the spikes. Besides the ability of the HBc carrier moiety to provide T cell help to inserted sequences, the HBc shell guarantees the T cell-independent character of the humoral response against inserted epitopes, due to the high degree of repetitiveness of the latter and the proper spacing between them [70]. By using HBc carriers, not only neutralizing antibodies and specific CTL responses, but also protection of experimental animals against challenge with the pathogens were demonstrated (see [4]).

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