Mosaic particles formed by wild-type hepatitis B virus core protein and its deletion variants consist of both homo- and heterodimers

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Abstract Co-expression in *Escherichia coli* of wild-type (wt) hepatitis B virus core protein (HBc) and its naturally occurring variants with deletions at amino acid positions 77–93 or 86–93 leads to formation of mosaic particles, which consist of three dimer subunit compositions. These compositions are wt/variant HBc heterodimers and two types of homodimers, formed by wt HBc or the variant HBc themselves. Mosaic particles were found also when both HBc deletion variants 77–93 and 86–93 were co-expressed in *E. coli*. These findings are discussed in terms of their significance for hepatitis B virus pathogenesis and prospective use of mosaic particles in vaccine development.

Key words: Hepatitis B virus; Hepatitis B virus core protein; Expression; Dimer formation; Mosaic particles

1. Introduction

Chronic infection with hepatitis B virus (HBV) may lead to liver cirrhosis and hepatocellular carcinoma. HBV genomes with internal in-frame C gene (HBV core protein (HBc) gene) deletions have been found frequently in chronic HBV carriers, including long-term immunosuppressed renal transplant recipients with development of end-stage liver disease [1,2]. Although their role in the pathogenesis of HBV infection remains unclear, their replication and enrichment appears to depend on the presence and amount of the wild-type (wt) HBc protein ([3–5], Meisel et al., unpublished data).

HBV nucleocapsids, or HBc particles, are formed by 90 or 120 HBc protein dimers [6]. The HBc protein contains four Cys residues at positions 48, 61, 107, and 183. Upon oxidation, Cys61 in one monomer can easily form a disulfide bridge to Cys61 in the second subunit of the dimer, whereas Cys183–Cys183 disulfides occur between dimers [7]. The tips of the spikes (in the vicinity of amino acids (aa) 74–83) carry the major immunodominant region (MIR) of the HBc and are frequently deleted in naturally occurring HBV variants from immunosuppressed renal transplant recipients [2].

Escherichia coli expression of naturally occurring HBc deletion variants in the presence of wt HBc as an 'assembly' helper resulted in the appearance of mosaic HBc particles consisting of both wt and HBc variants [8]. Here, mosaic particles formed by co-expression of wt HBc and natural HBc deletion variants are analyzed for their dimer composition. We provide evidence for existence of heterodimers, along with variant and wt homodimers, within mosaic particles for both investigated HBc deletion variants lacking aa 77–93 (#77/93) and 86–93 (#86/93).

2. Materials and methods

2.1. Expression vectors and purification of HBc particles

HBV genomes carrying internal C-gene deletions of 8 and 17 codons (designated, according to the codons deleted, as #86/93 and #77/93) were polymerase chain reaction (PCR)-amplified and cloned from sera of immunosuppressed renal transplant recipients with liver cirrhosis and end-stage liver disease [2]. Expression of wt HBc in a C-terminally truncated ('short') form (aa 1–144) was performed either in the vector pT31, a pBR322 derivative (ColE1 replicon) providing an ampicillin resistance [9], or in the vector pREPpT, a pREP4 derivative (p15A replicon) providing a kanamycin resistance [8]. For expression of the #77/93 variant in the short form, the respective pT31 and pREPpT derivatives p77/93pT and pREP77/93pT were used [8].

For expression of the wt HBc gene in full-length ('long') form, a site-directed PCR mutagenesis was performed within the plasmid pHBc3, a pBR322 derivative [10], to replace the C-terminal Cys183 codon TGT by the Ser-encoding TCT codon. This resulted in the generation of the HBc3* plasmid, where the asterisk denotes the Cys183Ser exchange in the corresponding HBc preventing its dimer oligomerization. To express the C wt-long* gene from a pREP4-derived plasmid, a *Pvu*II fragment from pHBc3* harboring the entire expression cassette was inserted into *Sal*I-restricted and Klenow-treated pREP4 vector (Qiagen), resulting in the pREPc3* plasmid. For expression of the variant #86/93-long, the *Bgl*II fragment from the pHBc3* vector was substituted by the corresponding fragment of the p86/93 plasmid, resulting in the p86/93* plasmid.

HBc derivatives were expressed in *E. coli* K12 strain K802. The cells were grown overnight in M9 medium supplemented with Casamino acids (Difco), and purification of HBc particles was performed as previously described [11]. Electron microscopy of particles was performed after negative staining with uranyl acetate or phosphotungstic acid in Zeiss EM 10A or JEM 100C electron microscope, respectively.

2.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Electrophoresis was performed in 15% polyacrylamide gel (PAAG).

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Abbreviations: aa, amino acid(s); β-ME, β-mercaptoethanol; C gene, HBV core protein gene; HBc, HBV core protein; HBV, hepatitis B virus; MIR, major immunodominant region; PAAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; wt, wild-type

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Variant and wt HBc proteins were detected by immunoblotting with the monoclonal anti-HBc antibody 13C9 recognizing the aa residues 136–144 [12] and anti-mouse IgG peroxidase conjugate (Dako Diagnostika).

2.3. Analysis of HBc dimers

To fix the intermolecular disulfide bridges between monomers within the dimers, purified HBc particles were treated with 100 μM $CuSO_4$ according to Nassal et al. [7]. Samples were boiled for 10 min. To avoid overoxidation, the treated samples were immediately mixed with the sample buffer and loaded onto the SDS–PAGE. Gels were stained with Coomassie brilliant blue. Dimer bands were cut from PAAG and eluted in SDS–PAGE buffer (25 mM Tris; pH 8.3; 190 mM glycine; 0.1% SDS) with a Model 422 Electro-Eluter device (Biorad). Eluted dimers were dissolved in Laemmli buffer containing 5% β-mercaptoethanol (β-ME) and analyzed by immunoblotting.

3. Results and discussion

3.1. Strategy for the analysis of the dimer subunit composition of mosaic HBc particles

Although HBc dimers are suggested according to the X-ray map [6] to contain intermolecular disulfide bridges connecting Cys61 residues, these disulfide bridges appear only during purification and storage but not during formation of HBc particles in *E. coli*. In order to fix the dimers of the mosaic particles covalently, we induced oxidation at the Cys61 [7]. To avoid polymerization of dimers by formation of intermolecular Cys183 disulfide bridges, the Cys183 codon was mutated to a Ser codon in long wt and variant #86/93 HBc.

We combined long and short forms of wt and variant HBc (#86/93 or #77/93) proteins in co-expression experiments with different expression vector combinations, and expression products were characterized for their solubility and assembly competence (Table 1). Combination of compatible pBR322-derived (ColE1 replicon) and pREP4-derived (p15A replicon) vectors allowed us to vary the relative amounts of wt and variant HBc proteins, since pREP-driven expression levels were always lower for both HBc variants as well as for the wt HBc. Use of short and long HBc forms ensured also a better separation of homo- and heterodimers in SDS–PAGE (Figs. 1–3).

3.2. The HBc variants #77/93 and #86/93 differ in their assembly competence when expressed alone

The variant #86/93-long showed a similar level of accumulation and particle formation as the wt HBc. No particles were found for the variant #77/93-long (Table 1) and it was hardly detectable even by immunoblotting, independently of the vector (pBR- or pREP-derived) used (data not shown). However, the variant #77/93-short was expressed very efficiently. Although it was mostly insoluble, some particles in the *E. coli* lysate demonstrated its principal self-assembly competence (Table 1). Comparable expression levels of #86/93-long and wt-long proteins suggest that the low expression level of the #77/93-long variant is caused by rapid protein degradation rather than by low transcription and/or translation level.

3.3. Co-expression with the wt HBc improves solubility of the #77/93-short variant

Presence of wt-short helper in both vector combinations led to markedly improved solubility of the #77/93 variant (Table 1, Nos. 1, 2). When the variant #77/93-short was expressed from the medium-copy number plasmid pREP4, but the wt HBc from the high-copy number plasmid pT31 (Table 1, No. 2), the variant #77/93 was more soluble and its content in purified particles was higher. This combination was selected for dimer analysis of mosaic particles formed by short forms of variant #77/93 and wt protein (Fig. 1).

A strong helper effect of wt HBc on the solubility of the #77/93-short variant was also found in one of the combinations, where full-length wt HBc was used. Thus, a sufficient amount of particles from co-expression of #77/93-short+wt-

Table 1
Particle formation and dimer composition after co-expression of wt and variant HBc by different vector combinations

No.	Co-expression: gene combination	Vector	Solubility[a]	Self-assembly[b]	Composition of HBc particles (%)		
					Variant[c]	Homodimers[d] (variant/wt)	Heterodimers[e]
	#77/93-short	p77/93pT; pREP77/93pT	+	+	100	–	–
1	#77/93-short+wt-short	p77/93pT+pREPpT	++	+++	5–15	ND	ND
2	#77/93-short+wt-short	pREP77/93pT+pT31	+++	+++	15–25	5–15/65–75	15–25
3	#77/93-short+wt-long*	p77/93pT+pREPc3*	–	–	ND	ND	ND
4	#77/93-short+wt-long*	pREP77/93pT+pHBc3*	+++	+++	45–55	30–40/30–40	30–40
	#77/93-long	p77/93; pREP77/93	–	–	–	–	–
5	#77/93-long+wt-short	pREP77/93+pT31	+++	+++	5–15	ND	ND
6	#77/93-long+wt-long	pREP77/93+pHBc3	+++	++	10–20	ND	ND
	#86/93-long	p86/93	+++	+++	100	–	–
7	#86/93-long*+wt-short	p86/93*+pREPpT	+++	+++	35–45	5–15/30–40	50–60
8	#86/93-long+wt-long	p86/93+pREPc3	+++	+++	ND	ND	ND
9	#86/93-long*+#77/93-short	p86/93*+pREP77/93pT	++/++	++	50–60/40–50	ND	ND

Asterisk (*) indicates the aa exchange Cys183Ser in the appropriate HBc. ND, not determined.
[a]Determined by immunoblotting as: +++, more than 50% of specific protein in soluble form; ++, 25–50% of protein soluble; +, 10–25% of protein soluble; –, no protein detected in soluble *E. coli* fraction.
[b]Determined by electron microscopy of soluble fraction as: +++, level similar to wt, useful for dimer analysis; ++, low amount, useful only for analysis of monomer content; +, only few particles detected; –, no particles detected.
[c]Determined by immunoblotting as an approximate percentage of variant HBc protein in the particle preparation after disruption of particles under reducing conditions.
[d]Determined by immunoblotting as an approximate percentage of two homodimer forms after disruption of particles under non-reducing conditions.
[e]Determined by immunoblotting as an approximate percentage of heterodimers after disruption of particles under non-reducing conditions.

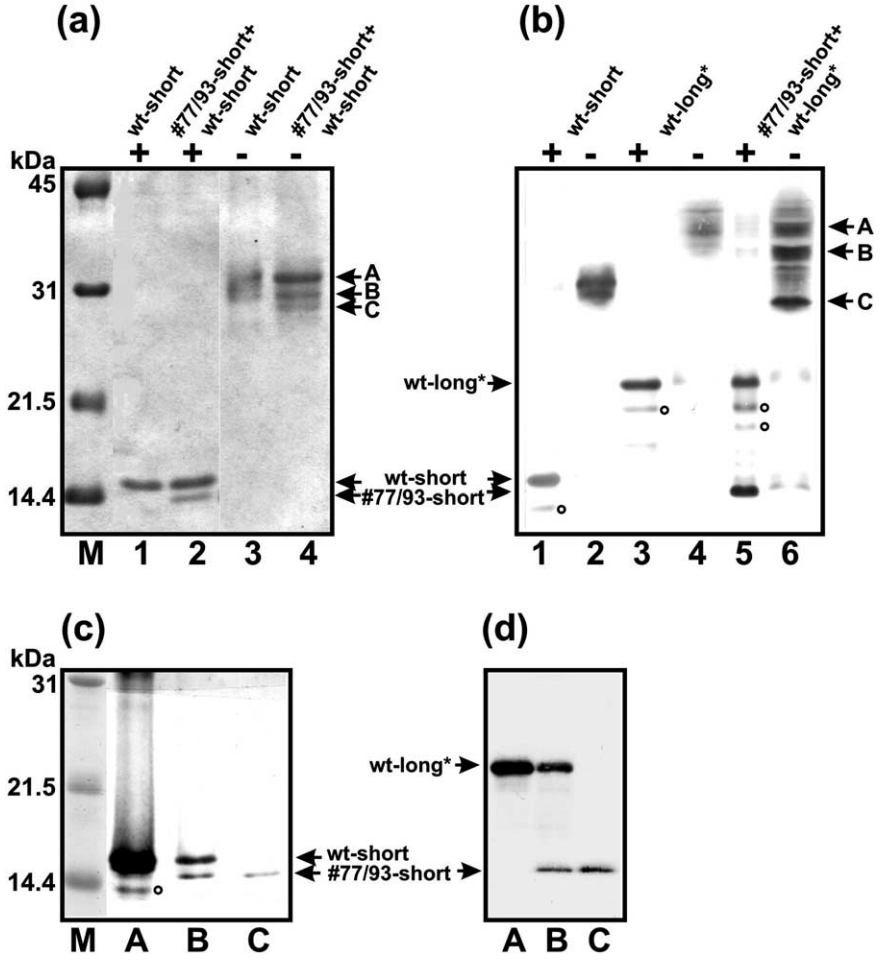

Fig. 1. SDS–PAGE analysis of mosaic particles formed by co-expression of the #77/93-short variant with the wt HBc. Purified particles were disrupted under reducing ('+') or non-reducing ('−') conditions after $CuSO_4$ treatment (100μM) and analyzed in PAAG by (a) Coomassie blue staining and (b) immunoblotting with 13C9 antibody. A, B, and C show putative dimer bands in co-expressed particles. The content of the bands from gel (a), lane 4, and gel (b), lane 6, after elution, treatment with Laemmli buffer containing β-ME, and immunoblotting with 13C9 antibody is shown in (c) and (d), respectively. Degradation products of wt HBc proteins are marked with (°). M, molecular mass marker.

long* was purified when wt HBc was expressed from the high-copy number vector pHBc3* and the variant from the medium-copy number vector pREP77/93pT (Table 1, No. 4). The reverse combination led to insoluble proteins (Table 1, No. 3).

3.4. *The wt HBc helper influences behavior of the #77/93-long, but not of the #86/93-long variant*

Co-expression with both forms of wt HBc helper markedly enhanced accumulation and solubility of the #77/93-long variant (Table 1, Nos. 5, 6). Although the wt-short protein appeared to be slightly more efficient in its helper activity, the amount of the #77/93-long variant was insufficient for the dimer analysis (Table 1, No 5).

In regard to expression level, solubility, and self-assembly, the #86/93-long* variant, when expressed alone, was similar to the wt HBc (Table 1). Co-expression with wt HBc proteins, short or long, resulted in a synthesis of both proteins in comparable amounts, as well as self-assembly. For dimer analysis, particles from co-expression with wt-short (Table 1, No 7) were selected, since this combination enabled good separation of both proteins in PAGE (Fig. 2).

3.5. *The amount of the HBc variants within the mosaic particles depends on amount and form of co-expressed wt HBc protein*

The amount of assembled #77/93-short protein increased, when wt-short protein was expressed from high-copy number plasmid pT31 (Table 1, Nos.1 and 2). High-level expression of the wt helper was absolutely necessary to form mosaic particles, when the wt-long was used in co-expression with the #77/93-short protein (Table 1, Nos. 3, 4); in this case up to 55% of the #77/93-short were incorporated into mosaic particles (Table 1, No. 4).

A slightly higher incorporation level was observed for the #77/93-long variant, when the wt-long helper was used, in spite of less particles found in soluble *E. coli* fraction compared to co-expression with the wt HBc-short (Table 1, Nos. 5, 6). It appears therefore, that full-length wt-HBc is more reactive in interactions with its 'analogue' of low assembly competence.

Mosaic particles were easily formed in co-expression of the wt and the assembly-competent #86/93 variant. The variant #86/93-long* was found incorporated in capsids at a share of 35–45% even when wt protein was expressed in short form

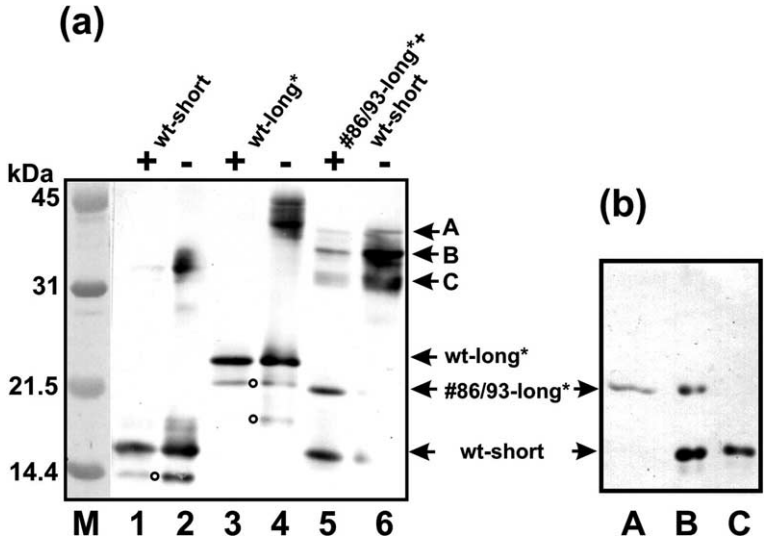

Fig. 2. Immunoblotting with 13C9 antibody of mosaic particles formed by co-expression of the #86/93-long* variant with the wt-short HBc. (a) Purified particles were disrupted under reducing ('+') or non-reducing ('−') conditions. A, B, and C in lane 6 represent bands of putative dimers in co-expressed particles. The content of the bands after elution, treatment with Laemmli buffer containing β-ME, and immunoblotting with 13C9 antibody is shown in (b). Degradation products of wt HBc are marked with (°). M, molecular mass marker.

from the medium-copy number pREP plasmid (Table 1, No. 7).

3.6. *Heterodimers along with homodimers are found within mosaic particles for all co-expression combinations*

Three combinations: #77/93-short+wt-short, #77/93-short+wt-long*, and #86/93-long*+wt-short (Table 1, Nos. 2, 4, 7) were selected for further analysis of dimer composition. The latter two were chosen, since they enabled good separation of both co-expressed proteins in SDS–PAGE (see Fig. 1b and Fig. 2a, lanes 5).

As shown in Fig. 1a (lane 4), 1b (lane 6), and Fig. 2a (lane 6), dimers were represented by three distinct bands (marked as A, B, and C) of different molecular mass for all three combinations. Calculation of the molecular mass of the expected HBc dimers suggested that the A and C bands represented homodimers formed by the wt HBc or the particular deletion variant, whereas the B band represented the wt/variant heterodimers.

3.7. *Content of heterodimers is not lower than that of variant homodimers in mosaic particles*

Although wt HBc homodimers strongly dominated in the mosaic particles formed by the wt-short and #77/93-short proteins (Fig. 1a, lane 4, band A), the heterodimer content (15–25%) was found slightly higher than the variant homodimer content (5–15%), suggesting that the variant monomers prefer to interact with the wt partners (Table 1, No. 2).

The heterodimer content in mosaic particles containing the #77/93-short was markedly increased (to 30–40%) when the wt-short helper was exchanged by the wt-long one (Table 1, No. 4). Notably, increased heterodimer formation in this case correlated with higher incorporation of the variant homodimers (30–40%, compared to 5–15% incorporated in the co-

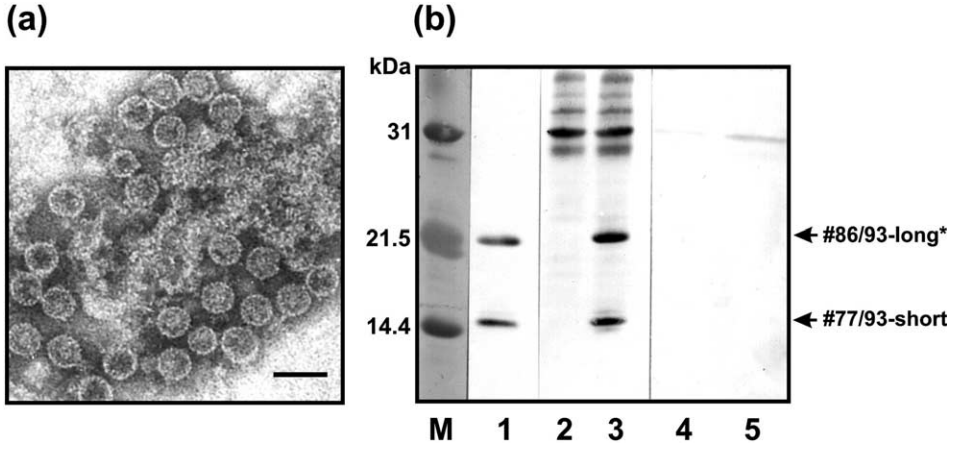

Fig. 3. Electron microscopy (a) and immunoblotting (b) of mosaic particles formed by co-expression of the #86/93-long* and #77/93-short variants. Mosaic particles were bound on protein A-Sepharose CL-4B beads coupled with monoclonal anti-HBc antibody C1-5 recognizing the aa residues 78–83 [12] as described previously [8]. a: Electron microscopy of soluble *E. coli* fraction. Bar, 50 nm. b: Immunoblotting with 13C9 antibody of C1-5 antibody-bound and unbound proteins: 1, monomer content of particles used for precipitation; 2, negative control: CL-4B Sepharose-bound C1-5 antibodies without adding of particles; 3, monomer content of C1-5 antibody-bound particles; 4 and 5, ammonium sulfate concentrated Sepharose-unbound material. M, molecular mass marker.

expression with the wt-short), suggesting that heterodimers may act as mediators attracting the variant homodimers to the particle assembly.

Strong heterodimer dominance over variant homodimers was observed in the co-expression of the #86/93-long* and wt-short proteins (Fig. 2a, lane 6). This finding was quite surprising, since the #86/93-long variant is assembly competent and we expected a high proportion of the variant homodimers in mosaic particles. We speculate that the #86/93-long plays a role of an assembly helper and initiates heterodimer formation with the truncated wt-HBc.

Therefore, self-assembly competence and composition of mosaic particles depend widely on the length and localization of the deletion within the HBc protein.

3.8. *Simultaneous expression of the #86/93-long* and #77/93-short variants leads to formation of mosaic particles*

As the #77/93 and #86/93 variants have emerged in the same renal transplant recipient [2], we were intrigued to investigate the possible interaction of both variants. The combination of #86/93-long*+#77/93-short was used for co-expression in *E. coli* (Table 1, No 9). Although only a minor part of the expression products was found soluble, particle formation was detected (Fig. 3a, Table 1). Since the amount of particles was found insufficient for dimer analysis, purified particles were analyzed for their monomer composition. Immunoprecipitation of particles with the C1–5 antibody, which recognizes the #86/93, but not the #77/93 variant, resulted in co-precipitation of the #77/93-short variant. Approximately equal amounts of both proteins were found in the precipitated material, confirming the mosaic structure of the particles (Fig. 3b, lane 3). The mutant variants like #86/93 can interact therefore with other, assembly non-competent HBc deletion variants in the infected organism, stabilizing and thus rescuing them from degradation.

3.9. *Structural and functional significance of mosaic HBc particles*

In-frame C-gene deletions are found frequently within the HBc MIR, suggesting an underlying B cell immune escape mechanism for their occurrence [1,2]. Many HBc gene deletions are too large to conceive formation of a functionally active protein [3–5]. However, one can imagine that the MIR-shortened HBc variants may be rescued by the wt HBc and involved in viral and/or cellular processes in HBV-infected hepatocytes. Structurally, the heterodimers should be able to tolerate the shortage of the HBc spikes after rearrangement of their α-helices in the MIR-shortened HBc monomers (Fig. 4).

Appearance of heterodimers in our co-expression experiments seems to be in conflict with the earlier finding that the HBc monomers differing only by an epitope tag show a strong preference for forming homodimers rather than heterodimers in *Xenopus* oocytes [13]. It cannot be excluded that differences in the experimental systems and the local concentrations of the different monomers could have contributed to these varying findings.

In hepatocytes, the HBc variants could be involved in the replication of HBV genomes by influencing the pregenome encapsidation. Further, the HBc variants may disturb the transcription regulatory function of HBc, which was demonstrated recently [14], with possible fatal consequences for cellular processes.

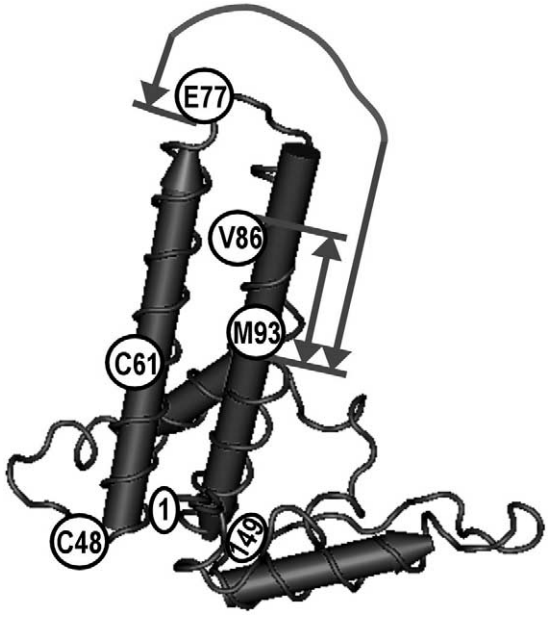

Fig. 4. Location of MIR deletions on the 3D map of the HBc monomer. The map of the HBc monomer (aa 1–149) is taken from the X-ray structure of the HBc particle [6]. α-Helices are shown by filled arrows. Locations of important aa residues (positions 1 and 149, cysteines 48 and 61, first and last aa of MIR deletions) are marked by white circles. Deletions 77–93 and 86–93 are shown by arrows.

3.10. *Perspectives of mosaic HBc particles in protein engineering and vaccine development*

In *E. coli*, recombinant mosaic HBc particles were employed, when the wt HBc helper enabled incorporation of assembly incompetent HBc derivatives carrying C-terminally fused foreign sequences [15]. However, the most promising site for foreign insertions is represented by the HBc MIR (for review see [16]). Artificial deletions within the MIR are able to abrogate the intrinsic HBc antigenicity/immunogenicity and to enhance immunogenicity of inserted foreign sequences. Therefore, naturally occurring HBc deletion variants could be considered as candidates for insertion of epitopes and construction of mosaic particles with desired properties. Additional investigations are needed to prove if the presence of the wt helper and formation of mosaic particles might overcome problems of poor solubility, instability, and assembly competence of the HBc fusion proteins.

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