Internal Core Protein Cleavage Leaves the Hepatitis B Virus Capsid Intact and Enhances Its Capacity for Surface Display of Heterologous Whole Chain Proteins*

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Virus capsids find increasing use as nanoparticulate platforms for the surface display of heterologous ligands, including as multivalent vaccine carriers. Presentation on the icosahedral hepatitis B virus capsid (HBcAg) is known to strongly enhance immunogenicity of foreign sequences, most efficiently if they are inserted into the dominant c/e1 B cell epitope, a surface-exposed loop in the center of the constituent core protein primary sequence. Even some complete proteins were successfully inserted but others, e.g. the outer surface protein A (OspA) of the Lyme disease agent Borrelia burgdorferi, impaired formation of capsid-like particles (CLPs). This difference can be rationalized by the requirement for the termini of the insert to fit into the carrier. We reasoned that cleavage of one of the two bonds connecting insert and carrier should relieve these constraints, provided the cleaved protein fragments remain competent to support the particle structure. Indeed, HBcAg CLPs containing a recognition site for tobacco etch virus (TEV) protease in the c/e1 loop remained intact after cleavage, as did CLPs carrying a 65-residue peptide insertion. Most importantly, in situ cleavage of a core-OspA fusion protein by coexpressed TEV protease strongly enhanced CLP formation compared with the uncleaved protein. These data attest to the high structural stability of the HBcAg CLP and they significantly widen its applicability as a carrier for heterologous proteins. This approach should be adaptable to any protein-based particle with surface-exposed yet sequence-internal loops.

Because of their highly symmetric architectures, viral capsids are increasingly utilized as nanoprotocols for the multivalent display of surface ligands, with diverse applications in materials science and biomedicine (1, 2), including vaccine carriers (3). The icosahedral capsid of hepatitis B virus (HBV), a small enveloped DNA virus that replicates via protein-primed reverse transcription (reviewed in Refs. 4, 5), is particularly well characterized in this respect (6, 7). Authentic nucleocapsids, serologically defined as hepatitis B core antigen (HBcAg), are exceptionally immunogenic (8, 9). They are formed by 120 dimers (triangulation number \( T = 4 \)) of a single 183-residue core protein (for review see Ref. 10); a minor class of 90 dimer particles \( (T = 3) \) is also observed (11, 12). Recombinant core protein self-assembles into genome-less, structurally very similar (13) capsid-like particles (CLPs); this requires minimally the first 140 amino acids (aa) of the protein (assembly domain) (14, 15) but not its nucleic acid binding (16) Arg-rich C-terminal domain (CTD). A model for the fold of the assembly domain (17, 18), derived by electron cryomicroscopy (cryo EM) and based on biochemical data (19, 20), was confirmed at about 3.5-Å resolution by x-ray crystallography (Refs. 21, 22; see Fig. 1). Two long \( \alpha \)-helices in the center of the sequence (\( \alpha 3 \) and \( \alpha 4a + b \)) form a hairpin structure; the hairpins from two monomers associate into stable four-helix bundles that protrude as spikes from the particle surface. The connecting loop is exposed on the spike tips, and comprises the immunodominant c/e1 B cell epitope that covers approximately aa 74 – 84 (23, 24). In addition, the capsid surface harbors various, in part highly complex, conformational epitopes (25, 26).

Display on the surface of HBcAg CLPs can potently enhance the immunogenicity of heterologous molecules; for genetic fusions this is most efficiently achieved by insertion into the c/e1 loop. While well established for short peptides (6, 7), we have demonstrated that even some complete proteins can be inserted into this site, without compromising folding of the insert or the carrier part (27). Insertion of the green fluorescent protein (GFP) yielded, in Escherichia coli, highly fluorescent CLPs that evoked a strong anti-GFP antibody response (28); efficient CLP formation and strong, including neutralizing, antibody responses were also achieved with the outer surface protein C (OspC) of the Lyme disease agent Borrelia burgdorferi (29). With the recent exception of the Flock House virus capsid (30), HBcAg is the only carrier for which such native whole chain protein display has been accomplished.

However, analogous insertion of OspA, another important B. burgdorferi antigen (31, 32), caused insolubility and pre-native agarose gel electrophoresis; OspA, outer surface protein A; OspC, outer surface protein C; TEV, tobacco etch virus; VLP, virus-like particle; aa, amino acid; PDB, protein data bank.

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‡1 The abbreviations used are: HBV, hepatitis B virus; CB, Coomassie Blue; CLP, capsid-like particle; EM, electron microscopy; GFP, green fluorescent protein; HBcAg, hepatitis B core antigen; mAb, monoclonal antibody; NAGE, tobacco etch virus; VLP, virus-like particle; aa, amino acid; PDB, protein data bank.
vented CLP formation, unless the insert was flanked by very long connecting linkers (10 and 22 aa on the N and C proximal side, respectively); even then the major products were non-
regular multimers (33). Though they still induced potent, pro-
tective anti-OspA antibodies, the extra linker sequences may have an antigenic potential on their own and thus not be desir-
able for vaccine applications (33).

This and additional work (34) strongly suggested that the structure of the inserted protein is key to CLP formation (Fig.
1); in both GFP (35) and OspC (36) the N and C termini are closely juxtaposed, naturally fitting into the acceptor sites in the HBCag carrier; OspA, by contrast, has an extremely elongated structure with far apart termini (37). Hence the use of HBCag as a particulate protein carrier appeared to be inherently restricted to heterologous proteins with compatible structures. Similar constraints apply to insertions into surface-exposed but sequence-internal loops (38, 30) of any protein-based nanoparticle carrier.

We reasoned that the steric strain imposed by the two-sided fixation might be overcome if one of the two bonds connecting the insert to the N-terminal (coreN) and C-terminal (coreC) core protein segments were cleaved, provided the intra- and intersubunit interactions in the particle remain intact even if the polypeptide chain is interrupted. As shown below, this was indeed the case. Moreover, the approach was also applicable to core protein fusions carrying a 65-residue heterologous peptide and, most importantly, the entire 256-aa OspA ectodomain, with strongly enhanced CLP formation compared with the continous chain coreOspA fusion (33). Hence single-sided protease cleavage of an insertion is a promising novel means of broadening the applicability of HBCag CLPs, and by inference also other carrier systems, to the display of whole proteins regardless of their three-dimensional structure. Furthermore, cleavage in the c/e1 loop creates new functional groups at the most exposed site of the particle enabling numerous further derivatizations.

**EXPERIMENTAL PROCEDURES**

*Plasmid Constructs—*All core protein constructs are based on plasmid pET28a-HBc149H6 (34), which carries a synthetic core gene (39) encoding the first 149 aa of the core protein plus a C-terminal His6 tag under control of the T7 RNA polymerase promoter. The TEV protease recognition sequence ENLYFQ-G (the dash indicates the scissile bond) was introduced into this and into previously described pET vectors encoding core fusions with the ACID peptide (40, 41) and OspA (33) by conventional PCR-mediated mutagenesis, using prim-
ers encoding in addition appropriate restriction sites. For co-
expression of the TEV protease, the MBP-TEV fusion gene in pRK793 (42) essentially as described (42). The specific activity on c149_TEV_H6 pro-
tein as substrate was similar to that of a commercial TEV pro-
tease preparation (Invitrogen), as indicated by the comparable amounts of either preparation required to achieve about 50% cleavage in 50 mm Tris-Cl, pH 8.0, 0.5 mm EDTA as reaction buffer. Core protein derivatives were expressed in *E. coli* BL21(DE3) Codonplus cells (Stratagene) as previously described (43, 34); for TEV protease co-expression, BL21(DE3) cells were instead co-transformed with the pET vector plus the modified pRARE plasmid. In brief, cleared lysates from induced *E. coli* cultures were subjected to sedimentation in 10–60% sucrose step gradients in a TST41.14 rotor run for 2 h at 20 °C and 41,000 rpm (34); analytical sedimentations (44) were per-
fomed in sucrose mini gradients (1.4 ml; TLS-55 rotor; 45 min
55,000 rpm at 20 °C); fourteen fractions (860 µl each for TST41.14 and 100 µl each for TLS-55 runs) were collected from the top.

Material from the center gradient fractions (fr. 7 to 9) was used further, usually without further purification. Western blotting was performed as previously described (34), using mAbs directed against linear epitopes (45, 46) in the N-terminal coreN (mAb 10E11) or C-terminal coreC part (mAb 10F10), or the particle-specific mAb 3120 (25). Native agarose gel electro-
phoresis (NAGE) was performed in 1% agarose gels; proteins were stained with Coomassie Blue (CB) for direct detection, or the gel contents were transferred onto polyvinylidene fluoride membrane and immunologically detected as described (34).

**Succinylation of c149OspA_TEV_H6 Proteins—**Gradient-enriched c149OspA_TEV_H6 protein expressed without or with TEV protease was dialyzed against phosphate-buffered saline. 50 µl containing about 50 µg (1 nmol) of the respective proteins were mixed with 200 µl of 50 mm sodium carbonate buffer, pH 8.5, plus 50 µg (about 500 nmol) of succinic anhydride in 20 µl of DMSO. After 60 min at room temperature, the reactions were concentrated (Amicon Centricon cartridges, cut-off 10 kDa), diluted with 500 µl of phosphate-buffered saline, and concentrated again. This procedure was repeated three times. To the final concentrate (about 100 µl), 50 µl of 50 mm Tris-Cl, pH 7.5 was added to quench unreacted anhydride, and aliquots of this solution were used for NAGE.

**Electron Microscopy—**Negative staining EM using 2% uranyl acetate was performed as previously described (44).

**RESULTS**

**HBCag CLPs Remain Intact after Virtually Complete Proteolytic Cleavage in the c/e1 Epitope—**To ensure site-specific cleavage, we engineered a TEV protease recognition sequence (ENLYFQ-G (42); dash indicates the scissile bond) flanked by G4T and TG4 linkers between aa 78 and aa 81 of a C-terminally truncated (aa 1–149) core protein with a C-terminal His6 tag (Fig. 1C). When expressed in *E. coli* (yield about 20 mg per liter of culture), the corresponding protein, c149TEV_H6, sedi-
mented in sucrose gradients (Fig. 2A) into the particle-typical (34) center fractions (fr. 7 to 9 of 14). An aliquot from fraction 9 was incubated with recombinant TEV protease (core protein: protease molar ratio about 5:1), and aliquots were withdrawn over a time course of 20 h to analyze specificity and efficiency of cleavage; unmodified c149_H6 CLPs served as control (Fig. 2B). The band corresponding to the intact 19 kDa c149TEV_H6
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Figure 1. Structural aspects. A, x-ray structures of HBV core protein, GFP and OspA. In the side view of the HBc monomer (PDB: 1QGT; aa 1–143), α-helices are represented as cylinders. The c/e1 loop connecting helix α3 and the bi-partite helix α4+α5 harbors residues Pro-79 and Ala-80 (gray spheres indicating Cγ positions), which were replaced by various inserts; acceptor residues Asp-78 and Ser-81 are shown as space-filled models. The view of the HBc dimer on the right is perpendicular to the four-helix-bundle, the positions of Pro-79 and Ala-80 are indicated by the gray spheres. For GFP (PDB: 1GFL) and OspA (PDB: 1OSP), the first and last ordered residues (GFP: Ser-2 and Ile-229; OspA: Ser-23 and Lys-273) are shown as space-filled models. All representations are drawn approximately to scale. B, schematic illustration of the cleavage approach. The GFP structure fits naturally into the acceptor sites at the tip of the helical hairpin (cylinders marked α3 and α4+α5), and its insertion has no major structural impact. The extended OspA structure imposes steric strain that could affect monomer folding (of the core and/or OspA parts), dimerization and CLP assembly. This strain may be relieved if one of the two connecting bonds is cleaved (lightning symbols). In this study, a TEV protease recognition site (double-cone symbol) was engineered into the C-terminal linker, C, schematic view of the constructs used. All constructs are based on core protein 1–149 with a C-terminal His6 tag (c149_H6). Inserts were fused to Asp-78 on one, and Ser-81 on the other side. Linker sequences are shown in lowercase lettering, the TEV protease recognition site is shown in capital letters; the slash indicates the scissile bond.

Cleavage—To demonstrate that cleavage with concomitant preservation of the particulate structure could be applied to HBcAg carrying a larger insertion, we introduced a TEV protease site into an HBcAg construct carrying a 32-aa peptide termed ACID, flanked by long flexible linkers, in the c/e1 loop (total insert length including the TEV recognition sequence 65 aa). ACID can adopt a coiled-coil structure with the complementary BASE peptide (40) but on its own it is largely disordered (41). Protein c149ACID-TEV_H6 was also well expressed in E. coli (about 40 mg per liter of culture), sedimented into the particle-typical fraction (Fig. 3A), and was cleaved by TEV protease with similar kinetics as c149_TEV_H6 (Fig. 3B). Due to the extra peptide sequence, the N-terminal cleavage product is now discernably larger than the C-terminal product (16 versus 9.1 kDa), in full accord with the band pattern observed in SDS-PAGE (Fig. 3B). Sedimentation behavior, including exact cosedimentation with the uncleaved protein when both were mixed (not shown), and mobility during NAGE (again distinctly reduced compared with the uncleaved sample) were also fully compatible with an intact CLP structure (Fig. 3, C and D), and the presence of particles was corroborated by EM (Fig. 3E). Hence the cleavage approach was also applicable to HBcAg carrying a medium-sized heterologous insertion.

In Situ Cleavage Enhances CLP Formation by a Core Protein Fusion with a Large, Unfavorably Structured Whole Chain Protein—Even with long flanking linkers the major products of the previously described contiguous chain core-OspA fusion protein were nonregular, though soluble, multimers, leading to a broad distribution in sucrose gradient sedimentation (33). Into the C-terminal linker of this construct, the TEV recognition sequence was introduced (Fig. 1C). Upon sedimentation of crude bacteria lysates the 48-kDa c149_OspA_TEV-H6 protein, expressed at about 7 mg per liter of bacterial culture, was similarly broadly distributed from fractions 5 to 12 (Fig. 5A, top panel); its identity was confirmed (Fig. 5B, top panel) using the OspA-specific mAb LA-2 (48, 49). Upon negative staining EM, material from fraction 8 revealed mostly aggregates, some appearing, by curvature, like incomplete CLPs (Fig. 5, C and D; panels -TEV). In vitro incubation with TEV protease yielded the expected 39-kDa and 9-kDa fragments; however with distinctly slower kinetics than with the previous constructs. Cleavage remained incomplete even after 20 h of incubation at a 1:1 ratio of protease:substrate (not shown), probably because the large OspA domains hindered protease access, especially in the observed multimeric aggregates. We reasoned that co-expression of the protease might increase cleavage efficiency as it would potentially allow for cleavage prior to folding and/or
Sequence-internal Cleavage Enhances Display Capacity of CLPs

![Diagram A](image1)

**Figure 2.** Protein c149TEV_H6 CLPs remain intact after site-specific cleavage with TEV protease. A, enrichment by preparative sucrose gradient sedimentation. Crude lysate from bacteria-expressing protein c149TEV_H6 was sedimented through a preparative 10–60% sucrose step gradient; 14 fractions of 860 µl each were harvested from the top. Aliquots of 8 µl each were analyzed by SDS-PAGE and Coomassie Blue (CB) staining; M, marker proteins with their molecular masses (in kDa) indicated on the left. The bulk of protein c149TEV_H6 (arrow) was found in fractions 8 and 9, as is typical for intact CLPs (34). B, TEV protease cleavage. CLPs from fraction 9 in A were incubated, at a 5:1 molar ratio of core protein subunits to protease, with TEV protease (apparent molecular mass about 27 kDa). Aliquots withdrawn at the indicated time points were analyzed by SDS-PAGE and CB staining; Ø, sample aliquot before TEV protease addition. Protein c149_H6 CLPs served as control. No change was seen after 20 h of incubation. C–E, cleaved c149TEV_H6 remains particulate. C, sucrose mini gradient sedimentation. The material incubated for 20 h with TEV protease (B) was sedimented through a 10–60% sucrose minigradient (44), collected in 14 100-µl fractions from the top, and 15-µl aliquots of the indicated fractions were analyzed by SDS-PAGE and CB staining; uncl, sample of the uncleaved protein. D, NAGE. Aliquots of the indicated proteins before (+TEV) or after 20 h of incubation with the protease (+TEV) were run in 1% agarose gels, and subsequently stained with CB, E, EM, aliquots of protein c149ACID_TEV_H6 (+TEV) from fr. 8 of the gradient in A, or after TEV protease digestion (+TEV; from fr. 7 of the gradient in C) were negatively stained with uranyl acetate. Panel D is a composite of two micrographs from the same grid (black separation line).

association of the substrate protein subunits (in situ cleavage). We therefore cloned the TEV protease gene, also under control of a T7 RNA polymerase promoter, into the PET-compatible rare tRNA providing pRARE plasmid, such that IPTG addition induces expression of both substrate and protease. Determination of the relative plasmid concentrations in co-transformed cells (not shown) indicated that the copy number of the PET plasmids exceeded that of the pRARE plasmid by ~1.5-fold.

Initial experiments with the c149TEV_H6 protein showed that the protease was relatively efficiently expressed (Fig. 4A); its distribution in the gradient suggested it may aggregate, in accord with its known low solubility (42), or that it remained partly associated with the substrate. However, a similar distribution was seen upon coexpression with core proteins lacking a TEV recognition site (c149_H6, c183_H6); as in vitro, no evidence for cleavage of these proteins was observed (not shown). Importantly, however, for protein c149TEV_H6 a band corresponding in size to the two expected 9-kDa cleavage products peaked in fractions 7–9 (Fig. 4A), and reacted on Western blots with both a mAb recognizing the coreN (10E11) and the coreC part (10F10) (45, 46). Because no (mAb 10E11) or only very weak bands (mAb 10F10) of identical mobility were detectable in the upper gradient fractions, this indicated efficient CLP formation (Fig. 4B), as confirmed by NAGE analysis of the gradient fractions and immunoblotting using mAb3120 (Fig. 4C). This mAb recognizes a complex conformational epitope involving N proximal (region 20–29) and C proximal (region 126–132) residues from two different core protein dimers (25), an arrangement typically present in intact particles. Analogous results were obtained with protein c149ACID_TEV_H6; only the NAGE blot with mAb3120 is shown (Fig. 4C, bottom panel).

Based on these data, we applied the co-expression approach to the c149OspA_TEV_H6 protein. Though the yield of recombinant protein was 4–6-fold lower than without protease co-
expression (also observed for various other core protein derivatives), two bands corresponding in size to the expected cleavage products formed a relatively narrow peak in fractions 8–10 (Fig. 5A, bottom panel); TEV protease was again detected in these and further-down fractions. Specific cleavage at the TEV site was further supported by expressing the original coreOspA protein without TEV site either without, or with TEV site was further supported by expressing the original core protein, as demonstrated by sedimentation velocity (Fig. 5C, panel + TEV) revealed relatively abundant, mostly spherical or close to spherical particles; although irregular aggregates were also present, they constituted a drastically smaller fraction than in the uncleaved sample (Fig. 5C, panel – TEV). CLP diameters ranged approximately between 40 and 45 nm, clearly exceeding those observed for c149TEV_H6 CLPs (Fig. 5D). To obtain information on the bulk status of the cleaved protein, we finally exploited a modified version of the NAGE assay. OspA is a basic protein, hence migration toward the anode would be expected to be slow even for properly formed CLPs; manipulation of surface charge via the pH of the electrophoresis buffer is limited by particle dissociation and/or denaturation (14, 50, 51). We therefore treated the cleaved and uncleaved c149_OspA_TEV_H6 preparations with succinic anhydride which is expected to preferentially react with the ε amino groups of exposed lysine side chains, reversing their charge. Even before derivatization, the cleaved material migrated more distinctly than the uncleaved; after succinylation one major new band of distinctly increased mobility was detectable by CB staining for the cleaved protein, suggesting a relatively uniform composition. For the uncleaved protein, a general increase in mobility but no distinct bands were observed (Fig. 5E).

Together these data demonstrate that cleavage of one of the two bonds connecting an insert to the core protein carrier can strongly enhance assembly capacity even for a large, unfavorably structured insert protein.

**DISCUSSION**

Our results firstly demonstrate, in accord with previous studies (14, 51, 41) yet in a completely different way, an enormous stability of the HBcAg CLP structure which remained intact even after essentially complete cleavage in the center of its constituent core protein primary sequence. Secondly, they provide proof-of-principle that deliberately cleaving one of the two connections to the carrier potently enhances particle formation with a structurally demanding protein insert that, without such cleavage, strongly impairs assembly. The approach provides a novel means to widen the usefulness of HBcAg CLPs as display platform for heterologous molecules, but should be generalizable to any protein-based nanoparticle carrier.

**High Stability of the HBcAg CLP Structure**—Viral capsids combine several features that make them attractive as nanoparticulate carriers for materials science, medicine and virology (1, 52, 2, 3). Many can assemble spontaneously, and the resulting particles are of defined composition and geometry. HBcAg CLPs stand out as their constituent capsid protein is one of the smallest known (less than 150 aa if only the assembly domain is used), its fold is very stable (51), and although the individual interdimer interactions are weak (51, 53), the assembled CLPs resist relatively harsh conditions of pH, temperature, and chaotropic agents (14, 50, 51). Our results extend these data by showing that the HBcAg CLP withstands even essentially complete cleavage of the core protein, as demonstrated by sedimentation velocity (Fig. 2C), NAGE (Fig. 2D), reactivity with a particle-specific mAb (Fig. 4C), and negative staining EM (Fig. 2E). This combination of assays addressing bulk properties as well as individual particles strongly suggests that the ability to
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FIGURE 5. In situ TEV protease cleavage strongly enhances CLP formation by protein c149OspA_TEV_H6. A, sedimentation profiles for uncleaved versus in situ cleaved c149OspA_TEV_H6 protein. Lysates from bacteria expressing only the core-OspA fusion protein (upper panel), or in addition TEV protease (lower panel), were subjected to sucrose gradient sedimentation, and individual fractions were analyzed by SDS-PAGE and CB staining. The uncleaved about 48-kDa protein was broadly distributed between fractions 5 and 12, whereas co-expression of TEV protease yielded two products (arrows) with the expected mobilities for the specific cleavage products that accumulated in fractions 8–10. TEV protease was again detectable in these and further down fractions. B, Western blotting confirms product identities and reveals complete cleavage by co-expressed TEV protease. Aliquots from the gradient fractions shown in A were analyzed using the indicated mAbs. Note the cosedimentation of the large cleavage product (reactive with anti-OspA and anti-coreN mAb) and the small product (reactive with anti-coreN mAb) into distinct fractions, and the absence of signals at the position of the uncleaved protein (lane uncl). C, EM. Samples of the uncleaved (−TEV) and cleaved (+TEV) protein were negatively stained with uranyl acetate. Without cleavage non-regular, often elongated and sometimes curved (arrows) aggregates were visible. The cleaved sample showed mostly spherical, or close to spherical particles, the majority with diameters of around 40 and 45 nm. D, comparison of particles without and with OspA insert. Typical large and small particles from protein c149TEV_H6 and c149OspA_TEV_H6 coexpressed with TEV protease (+TEV), and aggregates, including an apparently incompletely closed CLP, from uncleaved c149OspA_TEV_H6 (−TEV) are shown at the same magnification. E, NAGE indicates that the bulk of the cleaved, but not the uncleaved, c149OspA_TEV_H6 protein is present as CLPs. Samples of uncleaved protein (fr. 9 of the gradient in A, upper panel) and of the cleaved protein (fr. 9 in A, lower panel) were treated with succinic anhydride (SU +), or not (SU −), subjected to NAGE and stained with CB. Note the distinct band in the cleaved versus smear in the uncleaved sample. The staining in the loading slots is not due to protein as it was also observed when no sample was loaded (lane Ø).

maintain the integrity of the particle structure, despite cleavage, is an intrinsic trait of the HBV core protein. Though remarkable, this stability is well comprehensible in view of the capsid structure. Assembly proceeds by multimerization of dimers, through interfaces mainly located in the C proximal part of the assembly domain (17, 18, 20, 21), which do not interact with the c/e1 region; hence cleavage in the loop should have little effect as long as the dimer structure per se is intact. Notably, it has recently been reported that partial, but substantial, protease cleavage after R127 (at the end of helix α5) also does not induce particle dissociation (54). However, that site appears to only transiently be exposed, and it would not be well suited to accommodate heterologous sequences. Spontaneous internal scission with preservation of particle integrity has also been found in cowpea mosaic virus-like particles (VLPs) carrying small foreign peptides; in that case, however, cleavage occurred, inside the inserted sequence (38). Thus one added value of our study is that it proves stability of the HBCAg particle upon deliberate cleavage at a site that is highly useful for further modification.

Importance of Maintaining the Particulate Structure—Immunologically, the favorable protein-characteristic features of HBCAg are complemented by its ability to induce both T cell-dependent and T cell-independent immune responses (8), a property that relates to the repetitive epitope arrays on the particle surface, which probably allow direct B cell activation via B cell receptor crosslinking; such epitope-receptor interactions may be particularly effective at specific epitope spacings (9). Though not formally proven for HBCAg, the importance of pattern matching for other polyvalent ligand-receptor interactions has recently been demonstrated (55). Overall particle size also matters (56) because, due to the constant threat of viral infection, the mammalian immune system is highly attuned to recognizing virus-sized particles (20–200 nm). Hence preserving the multimeric, and probably better, the ordered particular structure of the carrier appears mandatory to achieve the desired immune enhancement for the displayed heterologous moiety. Short peptides have successfully been fused to, or inserted into, a variety of capsids from different viruses, including bacteriophages (57, 58), plant viruses (59), and insect (30) and mammalian viruses (60, 61). However, such peptide vaccines usually address a single epitope which may be recognized by only certain major histocompatibility complex (MHC)-T cell receptor (TCR) combinations, and which may be subject to genetic variation or escape on the pathogen side. Importantly, explanted epitopes may adopt irrelevant conformations that induce antibodies unable to recognize the genuine antigen, as impressively demonstrated for human rhinovirus epitopes presented on cowpea mosaic VLPs (38). Native presentation of the whole protein antigen overcomes these restrictions, yet properly accommodating a large, structured protein into the carrier structure is much more demanding. Except for the recent successful insertion of anthrax toxin receptor 2 into surface-exposed loops of the Flock House insect virus (30), HBCAg is the only system for which native display of complete foreign proteins has been clearly demonstrated (28, 29). However, all successfully inserted proteins had closely juxtaposed termini with a natural fit to the carrier acceptor sites.
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OspA Model for Difficult Protein Inserts—The results of this study demonstrate that this inherent restriction can be overcome, using OspA as a proven difficult model insert (33). The distance between its N and C terminus exceeds 60 Å, whereas that between the Asp-78 and Ser-81 acceptor sites in the carrier is less than 10 Å (Fig. 1); thus direct insertion is incompatible with maintaining the native structures of both insert and carrier. Only very long linkers allowed for multimerization, with little CLP formation. The uncleaved c149OspA_TEV_H6 fusion protein used here behaved very similarly, with a broad distribution in sucrose gradients (Fig. 5, A and B, top panels), smearable appearance in NAGE even after succinylation (Fig. 5E), and detectability of multimers, but few if any intact CLPs, in negative staining EM (Fig. 5, C and D, panels −TEV). In vitro cleavage was less efficient than with the c149TEV_H6 and c149ACID_TEV_H6 proteins, most likely for steric reasons; however, essentially complete cleavage was achieved by coexpression with the TEV protease (Fig. 5, A and B), which also proved effective with the two smaller fusion proteins (Fig. 4). Cleavage correlated with a sedimentation profile typically observed for intact CLPs (Fig. 5A, bottom panel), with clear cosedimentation of the coreN-OspA fragment and the small coreC fragment (Fig. 5B, bottom panels). Negative staining EM revealed a strongly enhanced proportion of spherical particles compared with the uncleaved sample (Fig. 5C, panel +TEV); their average diameters, though not accurately measurable due to imperfections in shape, the rough surface appearance, plus potential structural impacts of the staining procedure per se, were clearly larger (around 40–45 nm, similar to those observed for CLPs carrying GFP or OspC (28, 41, 29)) than for the CLPs from the two other proteins (around 31 and 34 nm, likely corresponding to T = 3 versus T = 4 architectures). Finally, the bulk of the cleaved protein migrated as a distinct band in NAGE, particularly well visible after succinylation (Fig. 5E). Thus, as conceptually expected, cleavage of one of the two bonds between insert and carrier strongly enhanced CLP formation with OspA as insert. In all likelihood, the same approach is applicable to a wide range of differently structured inserts as well as other carrier systems.

Future Practical Improvements—While our study documents the principal feasibility of the cleavage strategy, actual vaccine applications would require further technical improvements. Introduction of a few aa constituting a recognition site for a specific protease is inherent to the approach, but concerns on an undesired immunogenic potential of the extra sequences may be minimized by shortening the linkers, deliberately long in the current study to ensure efficient cleavage, in particular when combined with in situ cleavage. Removal of the TEV protease which, in part, cosedimented with the substrate proteins (Figs. 4A and 5A), should be achievable by adding suitable affinity tags and/or the use of more sophisticated systems that allow for temporal and quantitative control of protease expression. Such optimization may also raise the fraction of truly regular CLPs when the insert, without cleavage, impairs particle formation as does OspA. The higher efficiency of in situ versus in vitro cleavage implies that, beyond the mere size of OspA, also multimerization restricts protease access. The aggregates formed by the uncleaved protein are probably not in a denatured state (33), suggesting the contacts mediating multimerization are similar to those in regular CLPs. In the protease coexpressing cells, the assembly pathways toward such aggregates and ordered CLPs may therefore coexist and intersect, with formation of a mixed population of aggregates, regular, and less than perfect particles. Thus ordered CLP formation might benefit if the protease was already present before expression of the substrate protein is induced. Furthermore, TEV protease may be substituted by a variety of other site-specific proteases.

CONCLUSIONS

Native surface display of proteins on ordered nanoparticle scaffolds has numerous potential applications, including enhancement of immunogenicity. HBcAg is one of the most advanced such platforms yet, as the optimal attachment site for heterologous proteins locates to the center of the core protein sequence, the inherent two-sided insert fixation restrained the choice of insertable proteins to those with a matching structure. Our novel strategy relieves this restriction, and thus should allow the display of a large variety of proteins regardless of their structures. In addition, the newly created termini at the most exposed site of the particle should provide useful targets for further derivatization. Various other viral capsids bear sequence-internal surface loops that are potentially, or evidently, suitable for insertion of small flexible sequences. Appropriately adapted, our one-sided cleavage approach should also enable such other carriers to display large heterologous molecules.

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