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Capsids of hepatitis B virus e antigen with authentic C termini are stabilized by electrostatic interactions

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Abstract

The hepatitis B virus e antigen, an alternative transcript of the core gene, is a secreted protein that maintains viral persistence. The physiological form has extended C termini relative to Cp(–10)149, the construct used in many studies. To examine the role of the C termini, we expressed the constructs Cp(–10)151 and Cp(–10)154, which have additional arginine residues. Both constructs when treated with reductant formed capsids more efficiently than Cp(–10)149. These capsids were also substantially more stable, as measured by thermal denaturation and resistance to urea dissociation. Mutagenesis suggests that electrostatic interactions between the additional arginine residues and glutamate residues on adjacent subunits play a role in the extra stabilization. These findings have implications for the physiological role and biotechnological potential of this protein.

Keywords

conformational rearrangement; e antigen; encapsidation; furin cleavage; HBeAg; hepatitis B virus

Hepatitis B virus (HBV) is a small, partially double-stranded DNA virus which poses a major global health threat [1,2]. The infectious virion, known as a Dane particle, is a spherical double-shelled particle 42 nm in diameter. The outer layer consists of a phospholipid membrane with an embedded glycoprotein known as the surface antigen (HBsAg) which mediates virus entry into liver cells by binding to the sodium taurocholate cotransport polypeptide receptor [3]. Formulated HBsAg is the basis of an effective vaccine against HBV and was the first recombinant vaccine produced [4]. The lipid envelope surrounds a nucleocapsid which consists of the core antigen (HBcAg) and encloses the viral nucleic acid and DNA polymerase. HBcAg is a 183-residue multidomain protein consisting of the N-terminal capsid assembly domain (1–149) and an arginine-rich C-terminal region (150–183) which binds viral DNA at the inner surface of the capsid [5]. Recombinant forms of HBcAg (especially 1–149) are readily expressed in *Escherichia coli* which has facilitated

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Author contributions

PTW and ACS conceived of the study. PTW, NRW, IWP, and EE performed experiments. PTW and NRW analyzed data. PTW and NRW wrote and revised the paper. All authors gave final approval to the paper.

structural studies by X-ray diffraction and cryo-EM of the assembled capsid and the dimeric assembly unit [6–11]. Closely related to HBcAg is the e antigen (HBeAg), which is also derived from the viral C gene but initiated from an upstream start codon. HBeAg is expressed as a preprotein with a 29-residue signal sequence which targets the protein to the endoplasmic reticulum [12]. Following N-terminal processing of the signal sequence and additional processing of the C-terminal region, the secreted HBeAg retains 10 residues from the preprotein (–10) and at least 149 residues of the HBcAg sequence [13]. More recent studies with cell cultures have shown that C-terminal processing occurs in the Golgi by furin generating an e antigen with a C-terminal sequence extending to R154 and may involve more than one cleavage cycle [14,15]. This is in contrast to HBeAg isolated from patient sera by affinity chromatography where the C terminus was identified as R151 [16].

Processing in the ER of HBeAg and other secreted proteins is not efficient, and 15% or more of HBeAg is recycled to the cytoplasm [17]. We have previously shown that reduction of oxidized dimeric HBeAg results in conformational changes which shift it to an HBcAg-like structure which is now assembly-competent [18,19]. In the reducing environment of the cytoplasm, the recycled HBeAg appears to be able to form DNA deficient capsids which may play a role in maintaining viral persistence [20,21]. In the oxidizing environment of the endoplasmic reticulum [22], the dimeric e antigen will remain in the assembly-incompetent conformation.

In this study, we asked whether C-terminal extensions to HBeAg beyond residue 149, which correspond to forms detected *in vivo*, affect the physical properties of the antigen. Specifically, we made two HBeAg constructs, one terminating at R151 (V149-RR) and one at R154 (V149-RRRGR) (Fig. 1). Both Cp(–10)151 and Cp(–10)154 were expressed in *E. coli*, and the purified proteins were compared to the Cp(–10)149 construct using biophysical techniques. Important differences were observed which may have physiological significance as well as biotechnological application.

Materials and methods

Expression and purification of HBV Cp(–10)149, Cp(–10)151, and Cp(–10)154

The dimeric HBeAg constructs with varying C-terminal extensions were all expressed in *E. coli* as previously described [16]. All constructs contained the C48A and C107A mutations (Fig. 1). Briefly, cell pastes were resuspended in 50 mM Tris/HCl, 50 mM NaCl, and 1 mM EDTA to a final concentration of 3 mg·mL^{–1}. The cells were lysed with two passes through a French press at 12 000 psi. The suspension was sonicated for 1 min after each pass to reduce viscosity and then centrifuged in a Beckman JA-14 Rotor at 12 000 r.p.m. (22 500 g) for 1 h. Pellets were extracted (50 mL per 40 g of initial cell paste) with 100 mM NaCO₃, pH 9.6, containing 3 M urea, stirred for 30 min, and then centrifuged in a Beckman Ti-45 Rotor at 35 000 r.p.m. (100 000 g) for 1 h. The clear supernatant (up to 50 mL) was applied to a Superdex 200 column (60 mm dia. × 600 mm), and the fractions corresponding to the main peak containing dimeric protein were collected. The protein was loaded onto a Q-Sepharose column (26 mm dia. × 20 mm) equilibrated with 50 mM Tris/HCl, pH 8.0, containing 2 M urea and washed with column buffer until UV absorbance was back to baseline. Then, a 1-L linear gradient of 0–0.5 M NaCl in column buffer was applied and the fractions

corresponding to the main peak were collected. The urea was removed by dialysis against various buffers for further analyses.

Analytical centrifugation

A Beckman Optima XL-I analytical ultracentrifuge (Beckman Coulter, Brea, CA, USA), absorption optics, an An-60 Ti Rotor, and standard double-sector centerpiece cells were used. Sedimentation velocity measurements at 20 °C were made at either 45 419 or 116 272 *g* with data collection every 8 min to 2–3 h. Data analysis was done using DCDT+ 2.4.3 [23]. Correction of the sedimentation coefficient was made using protein partial specific volumes (v -bar), calculated from the amino acid compositions, and solvent densities estimated using the program SEDNTERP (<http://www.jphilo.mailway.com/download.htm>).

Protein concentrations

The concentrations of the purified proteins were determined by UV absorbance at 280 nm using the absorbance values calculated from their amino acid compositions.

Circular dichroism

Thermal denaturation studies were done using a Jasco-715 spectropolarimeter (Jasco North America, Easton, MD, USA) equipped with a PTC-343W1 Peltier-type thermostatic cell holder. Circular dichroism was monitored at 222 nm using a 1-cm path-length cell with a Teflon stopper (Hellma, Hellma USA Inc., Plainview, NY, USA). Cooling circulating water was supplied using a Neslab RTE-100 thermostatic circulator (Cole-Parmer, IL, USA). Proteins (~ 0.1 OD 280 nm) were heated at 1–2 °C per minute with a temperature slope of 20–90 °C. The step resolution was 1 °C, the response time 1 s, the bandwidth 2 nm, and the sensitivity 100 mdeg. Temperatures at the transition midpoints, that is, the melting temperature (T_m), were estimated from first derivative plots of the melting curves.

Negative stain electron microscopy

Carbon-coated grids were rendered hydrophilic prior to use by glow discharge (Fischione, Fischione Instruments, Export, PA, USA) in a plasma of 25% oxygen and 75% argon. The grids were then coated with 0.1 % poly-lysine to prevent capsid aggregation. Samples (~ 0.1 mg·mL⁻¹) were applied, and the grids were rinsed with distilled water and negatively stained with 1% uranyl acetate. Micro-graphs were recorded at 16 500× magnification on a Tecnai T12 electron microscope (FEI, Hillsboro, OR, USA) operating at 120 kV and equipped with a postcolumn energy filter (in zero-loss mode with a 20-eV energy slit width) and a 2kx2k CCD camera (Gatan, Pleasanton, CA, USA).

Structure modeling

The structure of dimers in reduced HBeAg capsids was modeled using the HBcAg structure 1QGT. The lengths of the C-terminal extensions were determined by modeling them as 8- and 11-residue long beta-strands with a 3.3 Å rise per residue. The figure was prepared with UCSF CHIMERA [24].

Results

Protein purification

Sequences of the various constructs are shown in Fig. 1. The Cp(–10)149 construct is normally extracted from *E. coli* as dimeric protein only; however, the longer construct Cp(–10)151 was expressed as dimeric protein together with assembled capsids and the Cp(–10)154 as dimeric protein with highly associated protein. The *E. coli* extract from Cp(–10)151 was examined by negative stain electron microscopy, and capsids and larger aggregates were observed (not shown). Further analysis by sedimentation velocity at 45 419 *g* indicated capsids with sedimentation coefficient (*s*) values of ~ 50 and 111 (Fig. 2). The 50 *s* capsids are close in size to HBcAg capsids [25]. No assembled capsids were observed in extracts of the Cp (–10)149 and Cp(–10)154 constructs. The yield of dimeric protein in extracts followed the series: Cp(–10) 149 > Cp(–10)151 > Cp(–10)154. After gel filtration with Superdex 200, the dimeric protein pools from Cp (–10)151 and Cp(–10)154 both contained small amounts of bound nucleic acid which was removed by the subsequent anion exchange step (Q-Sepharose). The purified dimers were analyzed by SDS/PAGE under reducing (plus DTT) and nonreducing conditions (Fig. 3). All the proteins were monomeric under both conditions with the oxidized proteins exhibiting slightly lower apparent molecular weights due to formation of intramolecular disulfide between cysteines at (–7) and 61 [26]. Also included is the mutant Cp(–10) 149.G123A; we previously showed that this mutation specifically blocks the assembly of Cp149 capsids [27].

Antigenic switching under reducing conditions

The HBcAg proteins were purified in an alkaline buffer (pH 9.6) under oxidizing conditions where they exist as stable dimers. In this buffer system, Cp149 is also a stable dimer but assembles into capsids when transferred to a neutral pH buffer with a moderate ionic strength [25]. Following dialysis of the HBcAg constructs into capsid assembly buffer (PBS, pH 7.2, containing 0.25 M NaCl), they were analyzed by sedimentation velocity at 116 272 *g* to assess their size distribution (Fig. 4, panels A, C, and E). All three constructs were monodisperse with *s* values of ~ 2.5, as expected for dimeric forms of these proteins. No assembly occurred consistent with the view that the HBcAg is a soluble low molecular weight entity under physiological conditions. We previously demonstrated that the Cp(–10)149 construct under reducing conditions can undergo a conformational change to a HBcAg-like structure which is assembly-competent at neutral pH [18]. Analysis of the three reduced HBcAg constructs by sedimentation velocity at 45 419 *g* is shown in Fig. 4 (lanes B, D, and F). The fast-moving species correspond to assembled capsids and the slower species to dimeric protein, and the height of the boundaries approximates to the amount of each species. The Cp(–10)149 construct exhibits the least efficient assembly (< 50%), whereas the Cp(–10)151 and Cp(–10)154 constructs are both > 85% assembled. When we introduce the G123A mutation into the Cp(–10)149 construct, it remains dimeric under reducing conditions (Fig. 5). We assume that the conformation shift was induced by reduction but that the dimeric core-like conformation was assembly blocked by the mutation. Direct observation of the capsid-like structures produced by the dimeric HBcAg constructs is shown in Fig. 6. The abundance of particles reflects the varying efficiency of assembly with Cp(–10)154 (panel D) showing the densest field of particles. Interestingly, all particles

appeared to have $T = 3$ icosahedral symmetry which differs from HBcAg where dimers assemble into mainly (90–95%) $T = 4$ capsids [28].

Stability of HBeAg capsids

The complete dissociation of HBcAg capsids can be effected by treatment with 2 M urea and adjustment of pH to > 9.0 (usually 9.5). Although urea is a protein denaturant at higher concentrations, at the concentration used in this study (2 M) it dissociates dimer–dimer contacts without global protein unfolding. The HBeAg constructs were dissociated to dimers by this treatment to assess the relative stability of their capsids. They were dissociated to dimers to varying extents: Cp(–10) 149 (100%); Cp(–10)151 (25%); and Cp(–10)154 (17%) (Fig. 7). Clearly, the longer constructs are more resistant to dissociation.

HBeAg capsid stability was also examined by thermal denaturation studies where helix–coil transitions were monitored by circular dichroism at 222 nm (Fig. 8). The samples used were derived from the assembly experiments shown in Fig. 4 (panels B, D, and F). The Cp(–10)149 construct exhibits two transitions at 47 °C and 71.5 °C, corresponding to dimeric and assembled capsid protein, respectively. The Cp(–10)151 and Cp(–10)154 capsids are more stable, both melting at ~ 74 °C. Both of these constructs also exhibited (poorly resolved) minor transitions at 47–50 °C corresponding to the small number of unassembled dimers. Interestingly, all the HBeAg capsids are more stable than $T = 4$ Cp-149 capsids which melt at 70 °C ($T = 3$ capsids also melt at this temperature). However, dimeric HBcAg is significantly more stable (by this analysis) than HBeAg dimers [27].

Using the structural model of HBcAg capsids, it was of interest to see how the addition of the basic arginine residues to the C-terminal region might enhance capsid stability. Figure 9 shows two dimers from inside the capsid, with chains A (green) and B (yellow) represented as ribbons, and chains C and D represented as a charged surface. Two prominent regions of positive charge, corresponding to residues E46 and (E113 + E117), are observed on this surface of each monomer. The model suggests that a charge interaction could occur between 144-PETTVVRR-151 or 144-PETTVVRRRRGR-154 and (E113 + E117), and that there could be an additional interaction between 144-PETTVVRRRRGR-154 and E46. This additional interaction could explain the greater yield and greater stability of the Cp(–10)154 capsids.

To test the potential electrostatic interaction involving E113, we made the following construct Cp(–10) 154.C48A.C107A.E113A where the negatively charged glutamic acid residue was substituted by the neutral alanine. Protein expression and purification proceeded as normal, and the purified oxidized protein was dimeric under capsid assembly condition at neutral pH. Reduction of the protein resulted in production of $\sim 20\%$ capsids with the remainder dimeric protein with small amounts of aggregates. This contrasts with the wild-type sequence which as shown above exhibits $\sim 85\%$ capsid assembly upon reduction (Fig. 4, panel D).

Discussion

The recombinant version of the HBV e antigen used to solve the structure [18] was Cp(–10)149, and the C-terminal sequence was based on earlier work [13]. This was also consistent with the HBcAg construct Cp149 used for many structural studies [5]. More recent work shows that HBeAg C-terminal processing occurs in the Golgi apparatus by the transmembrane protein convertase furin. The minimal sequence for furin cleavage is RXXR together with two longer alternatives RXRXXR and RXXXRR [29]. The arginine-rich HBcAg C-terminal domain has six potential furin cleavage sites. Messageot *et al.* [14], using cell culture, concluded that mature HBeAg terminates at R154 *via* either a single or multiple processing events. Ito *et al.* [15] showed that there were genotype differences in processing and suggested furin processing at R154 for type B and at R159 for type A. We recently isolated the HBeAg from human sera using affinity chromatography and used mass spectrometry to determine the C-terminal sequence for several genotypes. The results showed that the C terminus was V¹⁴⁸V¹⁴⁹R¹⁵⁰R¹⁵¹ indicating a Cp(–10)151 sequence. When the full-length HBcAg is processed directly with trypsin, cleavage occurs very rapidly (< 5 min) at R150 and this is also the processing site of a small percentage of HBcAg when expressed in *E. coli* [25]. These results highlight the fact that the arginine-rich C-terminal domain is a hot spot for processing by serum protease-like enzymes and that the R151 C terminus detected in serum samples may be derived from multiple processing.

We wished to see if Cp(–10)151 and Cp(–10)154 behaved differently from Cp(–10)149, which was used in many of our previous studies. First, we observed during extraction and purification that both new constructs carried 260 nm absorbing material which we assumed was nucleic acid (bound nonspecifically to C-terminal arginine residues). In addition, we saw direct capsid formation of the Cp(–10)151 construct, analogous to HBcAg [25]. The Cp(–10)151 capsids were heterogeneous (Fig. 2) and appeared to contain significant amounts of nucleic acid, similar to HBcAg (Cp183) capsids [25]. The reducing environment of the *E. coli* cytoplasm [30] presumably mediates the structural switch of e antigen to assembly-competent forms.

The purified and nucleic acid-free Cp(–10)151 and Cp(–10)154 proteins were well-behaved soluble dimers at neutral pH in physiological-type buffers (Fig. 4). The *in vitro* transitions to capsid assembly-competent conformations are mediated by reduction [18,19]. The addition of the basic C-terminal residues in the Cp(–10)151 and Cp(–10)154 constructs enhances the extent of this transition, as judged by formation for both constructs of ~ 85% capsids (Fig. 4). The Cp(–10)151 and Cp(–10)154 capsids were also more stable than the Cp(–10)149 capsids as judged by both their resistance to disassembly (Fig. 7) and higher thermostability (Fig. 8). In terms of physiological significance, it is clear that any C-terminal processed versions of the e antigen cycling back to the reducing environment of the cell cytoplasm [31] will potentially be capsid formers. Moreover, the presence of multiple arginine residues beyond residue V149 will greatly enhance the efficiency and stability of capsid formation.

A variety of capsids is produced during viral infection; however, aside from the infectious virions, very little is known about their functions [32]. At least two types of capsids similar to the ones described here have been reported. The artificial construct p18 ((–10)–144) was

reported to form hybrid capsids with p21 and potentially inhibit viral replication [33]. More recently, it was shown that the ‘empty’ virions produced copiously during infection have capsids consisting of p22cr ((–28)–150), though this is now questioned [20,32]. It seems likely that greater stability conferred by C-terminal extensions, as described here, would favor the formation of such capsids and enhance their activity, whatever it might be.

Mutation of E113, to either D or more frequently Q, has been reported for almost 40 years [34,35]. A recent detailed genomic analysis of HBV variants in a large patient cohort identified numerous mutations strongly associated with both e antigen status and viral load [36]. A subset of these mutations was mapped onto the e antigen dimer, and it was proposed that they may disrupt the dimer interface [36]. While this is plausible for most of the mutations, the peripheral location of E113 in the e antigen dimer suggests that there may be a different mechanism for this residue. The e antigen dimer can convert to a core antigen dimer-like conformation and assemble into core-like capsids [18,19,27]. It may be that the E113Q mutation interferes with the assembly process by reducing the electrostatic interaction with the C termini of the adjacent subunit, much as described here. The frequent detection of the conservative E113D mutation suggests the possibility of a second, as yet undefined, mechanism in e antigen immune regulation.

Whether e antigen capsids play any part in the progression or maintenance of chronic hepatitis is unclear. Recent work indicates that drugs which misdirect HBcAg capsid assembly also reduce HBeAg levels [37]. The authors suggest this is due to drug-induced aggregation of HBeAg capsids formed from nonsecreted protein. We have shown that antibodies which target HBcAg assembly also block HBeAg capsid assembly [19]. Targeting HBeAg with drugs or antibodies, which may reduce its levels in patients with chronic hepatitis, may be of therapeutic value. We have seen during *E. coli* expression and during *in vitro* assembly in the presence of nucleic acid that e antigen capsids (both Cp(–10)151 and Cp(–10)154) do appear to encapsulate RNA, presumably mediated by the C-terminal arginine residues. It is not clear whether this could occur either specifically or nonspecifically during the viral production cycle. Potential electrostatic interactions involving the additional arginine residues may play a role in the extra stabilization. The switch from the HBeAg to the HBcAg-like conformation could potentially be used to encapsulate small cationic molecules such as nucleic acid aptamers, for cell delivery.

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Abbreviations

Cp(–10)149, Cp(–10)151, and Cp(–10)154

mean e antigen with the 10-residue propeptide followed by residues 1–149, 1–151, and 1–154 of core antigen, respectively

Cp149

core antigen with residues 1–149

HBcAg

HBV core antigen

HBeAg

HBV e antigen

HBsAg

HBV surface antigen

HBV

hepatitis B virus

 $T = 3$ and $T = 4$

icosahedral symmetry with triangulation numbers 3 and 4, respectively

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-10 1 10 20 30 40 50
 SKLCLGWLWG MDIDPYKEFG ATVELLSFLP SDFFPSVRDL LDTAAALYRD ALESPEHCSP
 60 70 80 90 100 110
 HHTALRQAIL CWGDLMTLAT WVGTLNLEDP SRDLVVS YVN TNVGLKFRQL LWFHISCLTF
 120 130 140 150 160 170
 GRETVLEYLV SFVWIRTPP AYRPPNAPIL STLPETTVV RRRGRSPRRRT PSPRRRRRSQS
 180
 PRRRRRSQSRE SQC

Fig. 1.

HBcAg and HBeAg sequences. Shown is the full sequence for HBcAg (residues 1–183) with the 10-residue HBeAg propeptide appended at the N terminus. For many studies, the HBcAg sequence was residues 1–149 and the HBeAg sequence was residues (–10)–149. In this study, the HBeAg sequences (–10)–151 and (–10)–154 were also used. The HBeAg propeptide is shown in red, Cysteine at positions (–7), 48, 61, 107, and 183 is shown in green (in this study, at positions 48 and 107, it was mutated to alanine), glycine at position 123 (which when mutated to Alanine blocks capsid assembly) is shown in magenta, and arginine at positions 150, 151, 152, and 154 is shown in blue.

Fig. 2.

Sedimentation velocity of Cp(-10) 151. Analysis of an *Escherichia coli* extract partially purified by gel filtration indicates resolution of capsid structures assembled during expression. The blue peak at ~ 50 s corresponds to normal-sized capsids and the broad green peak to larger capsid and protein aggregates. The sample was centrifuged at 45 419 *g*.

Fig. 3. SDS/PAGE of oxidized and reduced HBeAg. Analysis under oxidizing conditions (no DTT): Lanes 2, 3, 4, and 5 correspond to Cp(-10)149.G123A, Cp(-10)149, Cp(-10)151, and Cp(-10)154, respectively. Lanes 6, 7, 8, and 9 correspond to the same samples under reducing conditions (+DTT). All HBeAg constructs contain C47A and C107A mutations. Lane 1, standard proteins.

Fig. 4. Sedimentation velocity of oxidized and reduced HBeAg. Panels A, C, and E correspond to oxidized Cp(-10)149, Cp(-10)151, and Cp(-10)154 in PBS, pH 7.2, containing 0.25 M NaCl, centrifuged at 116 272 *g*. Panels B, D, and F correspond to Cp(-10)149, Cp(-10)151, and Cp(-10)154 in same buffer following treatment with 5 mM DTT, centrifuged at 45 419 *g*.

Fig. 5. Sedimentation velocity of oxidized and reduced Cp(-10)149 with the G123A mutation. Panel A, oxidized protein; and panel B, reduced (+DTT) protein in PBS, pH 7.2, containing 0.25 M NaCl. Both oxidized and reduced samples were analyzed at 116 272 g.

Fig. 6. Negative stain electron microscopy of HBeAg constructs. Samples were examined following reduction under assembly conditions. (A) Cp(-10)149, (B) Cp(-10)151, (C) Cp(-10)149.G123A, and (D) Cp(-10)154. The additional C-terminal arginine residues caused more capsids to form. The G123A mutant did not form capsids; however, it formed small quantities of linear polymers that the other constructs did not.

Fig. 7. Sedimentation velocity of HBeAg under capsid dissociation conditions. Capsids corresponding to those shown in Fig. 4 (panels B, D, and F) were treated with 2 M urea and then dialyzed against sodium bicarbonate buffer, pH 9.5. Samples were centrifuged at 116 272 g.

Fig. 8. Thermal denaturation of HBeAg capsids. The samples Cp(-10)149, Cp(-10)151, and Cp(-10)154 correspond to those shown in Fig. 4 (panels B, D, and F). Cp149 corresponds to assembled HBcAg capsids ($T = 4$). Samples were scanned at 222 nm using a 1-cm path-length cell over the temperature range indicated. The midpoints of the transitions are the melting temperatures (T_m) and were estimated from first derivative of the curves indicated. Units of the ordinate are mean residue ellipticity $[\theta]_{\text{MWR}}$ and have the dimensions: $\text{deg} \times \text{dmol}^{-1} \times \text{cm}^{-2}$.

Fig. 9. Capsid-stabilizing interactions of the HBeAg C termini. Interactions in reduced HBeAg capsids are modeled on the HBcAg structure 1QGT as viewed from inside the capsid. Chains A (green) and B (yellow) are shown as ribbons, and C and D as a charged surface. Regions of positive charge (shaded red) corresponding to residues E46, E113, and E116 on chain C are indicated. The N- and C termini are labeled. The circles, centered on the C terminus (L143) of the B chain, are drawn at radii corresponding to the lengths of peptides 144-PETTVVRR-151 (26 Å) and 144-PETTVVRRRGR-154 (36 Å) in the β -strand conformation. The circles pass approximately through the center of the set of alternative side-chain rotamers for the C-terminal arginine residues of the respective extensions (not shown).