A Protease-Sensitive Hinge Linking the Two Domains of the Hepatitis B Virus Core Protein Is Exposed on the Viral Capsid Surface

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Core particles of hepatitis B virus are assembled from dimers of a single 185-residue (subtype adw) viral capsid or core protein (p21.5) which possesses two distinct domains: residues 1 to 144 form a minimal capsid assembly domain, and the arginine-rich, carboxyl-terminal residues 150 to 185 form a protamine-like domain that mediates nucleic acid binding. Little is known about the topography of the p21.5 polypeptide within either the p21.5 capsids or dimers. Here, using site-specific proteases and monoclonal antibodies, we have defined the accessibility of p21.5 residues in dimers and capsids assembled from wild-type and mutant hepatitis B virus core proteins in *Xenopus* oocytes and in vitro. The data reveal the protamine region to be accessible to external reagents in p21.5 dimers but largely cryptic in wild-type capsids. Strikingly, in capsids the only protease target region was a 9-residue peptide covering p21.5 residues Glu-145 to Asp-153, which falls largely between the two core protein domains. By analogy with protease-sensitive interdomain regions in other proteins, we propose that this peptide constitutes a hinge between the assembly and nucleic acid binding domains of p21.5. We further found that deletion or replacement of the terminal Cys-185 residue greatly increased surface exposure of the protamine tails in capsids, suggesting that a known disulfide linkage involving this residue tethers the protamine region inside the core particles. We propose that disruption of this disulfide linkage allows the protamine region to appear transiently on the surface of the core particle.

The 21.5-kDa core protein (p21.5) of human hepatitis B virus (HBV) is the major structural protein of the viral nucleocapsid, or core particle, and also plays a key role in virion morphogenesis. Upon expression in a variety of heterologous systems (4, 7, 8, 14, 15, 32), p21.5 assembles into simple nucleocapsid-like core particles containing only p21.5 and RNA. Work on core particle assembly in *Xenopus* oocytes established that the building blocks for capsid assembly are p21.5 dimers (34) that exhibit a highly cooperative aggregation into capsids, requiring a threshold concentration of dimers (27). Studies in bacterial systems showed that the first 144 residues of p21.5 form a domain that directs capsid assembly (4, 8). The arginine-rich carboxyl terminus of p21.5, residues 150 to 185 (HBV subtype adw) or 183 (ayw), forms a functionally distinct protamine-like domain that is dispensable for particle assembly but mediates interactions between p21.5 and nucleic acid and has been implicated in viral RNA encapsidation and DNA synthesis steps (4, 8, 12, 18).

Much less is known about how core particles incorporate the viral polymerase and pregenome RNA or acquire their coat of viral envelope proteins to generate virions, but it is likely that p21.5 interacts directly with these components (for reviews, see references 9 and 28). Unfortunately, we have limited knowledge of the p21.5 polypeptide topography in the absence of a core particle crystallographic structure; hence, we are unsure which p21.5 peptide segments might be available on the outside or inside of the capsid to mediate these interactions. Although recombinant HBV capsids should facilitate topographical studies, success with this approach has been limited to the localization of part of an exposed epitope (HBc α) to the

vicinity of the p21.5 residue 80 (21, 22, 24). Conflicting conclusions have been reached concerning the location of other parts of the protein (1, 3, 5, 8).

In this study, we set out to perform a more systematic study of p21.5 topography by using p21.5 expressed in *Xenopus* oocytes and in reticulocyte lysates. As topographical probes, we used a panel of site-specific endoproteases plus monoclonal antibodies (MAbs) directed against subsegments of the p21.5 protamine domain. The results clarify our picture of p21.5 topography and reveal previously unreported structural features of p21.5.

MATERIALS AND METHODS

Expression plasmids. The SP6 plasmids used for expression of wild-type (wt) p21.5 (subtype adw) (pSP64T-C) and mutant core proteins with truncated protamine regions (pSP64T- Δ 172, - Δ 162, - Δ 157, - Δ 149, and - Δ 149R4) have been described earlier (12, 32). The construction and properties of p21.5 mutants in which either the terminal Cys-185 residue or else all four Cys residues have been replaced by Ala (A-185 and Cys⁻, respectively) have been reported elsewhere (33, 35).

Antibodies and ELISAs. Polyclonal rabbit anti-HBc/e serum was purchased from DAKO Corp. The properties of the mouse MAbs 904 (13) and 3120 (30) as well as their applications in HBe- and HBc-specific enzyme-linked immunosorbent assays (ELISAs) have been described previously (26, 27). MAb 19-27 (16) reacts with a determinant referred to as HBcAg that localizes to residues 150 to 159 of the core polypeptide. MAb T-2212 (17) recognizes an epitope that is dependent on phosphorylation and is located between p21.5 residues 165 to 175. All MAbs were a gift of M. Mayumi (Jichi Medical School, Tochigi-Ken, Japan). Briefly, ELISAs were performed on serial dilutions of pooled oocyte-expressed dimers and capsids

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which were captured with the appropriate solid-phase bound MAb (100 ng) and then disclosed with the polyclonal anti-HBc/e serum. A pure preparation of bacterial HBV capsids supplied by M.-A. Selby and P. Valenzuela of Chiron Corporation (Emeryville, Calif.) provided a native standard for the quantitation of HBc-bearing capsids, and an HBe-positive standard was generated by denaturing these capsids.

Expression of p21.5 in *Xenopus* oocytes and in vitro. The synthesis of SP6 mRNAs used for core protein expression, the microinjection and handling of *Xenopus laevis* oocytes, and the gradient fractionation of capsids and dimers are described elsewhere (27, 29, 32, 36). Briefly, microinjected oocytes were incubated in the presence or absence of [³⁵S]Met and -Cys (~0.25 to 1 mCi/ml of medium; Expresslabel, NEN-Dupont) for 40 to 60 h and then lysed in 20 μ l of phosphate-buffered saline (PBS; 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 2.7 mM KCl, 137 mM NaCl) per oocyte. Separation of capsids from unassembled p21.5 dimers was accomplished by sedimenting lysate from ~5 oocytes through a minigradient of 10 to 60% (wt/vol) sucrose in 50 mM Tris-HCl (pH 7.5)–100 mM NaCl–1 mM EDTA (TNE) to yield the characteristic profile of core protein dimers at the top of the gradient (fractions 1 through 4 of 14) and capsids in fractions 7 through 12. The gradient fractions containing either the p21.5 dimers or core particles were pooled for protease digestion experiments and most ELISA analyses. Capsids were collected by centrifugation at 100,000 rpm (~500,000 $\times$ g) for 1.5 h at 4°C and resuspended in PBS (for ELISAs) or in 50 mM NH₄-bicarbonate buffer (pH 8.5) (for protease digests). For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analyses, metabolically ³⁵S-labeled core proteins were immunoprecipitated as described previously (29), by employing either the polyclonal anti-HBc/e serum or MAb T-2212 (see above). Relative molecular weights of the various core polypeptide species and proteolytic fragments (see below) were estimated from scans of the dried gels obtained with a Phosphorimager (Molecular Dynamics), with ¹⁴C-labeled molecular weight marker proteins (Bethesda Research Laboratories) used for calibration.

In vitro translation of core proteins in the presence of [³⁵S]Met (translation grade; NEN-Dupont) was accomplished with a coupled transcription-translation kit (SP6 TNT) as directed by the manufacturer (Promega).

Protease digests. Sequencing-grade-quality endoproteases Asp-N, Lys-C, and Glu-C were purchased from Boehringer Mannheim, and trypsin (essentially chymotrypsin-free) was obtained from Sigma. Solutions of these enzymes were prepared in 50 mM NH₄-bicarbonate buffer (pH 8.5) just before use. Typically, aliquots of ³⁵S-labeled core protein dimers or capsids (one-half to one oocyte's worth) in 50 mM NH₄-bicarbonate (pH 8.5) were mixed with (i) 500 ng of Asp-N, (ii) 500 ng of Lys-C, (iii) 4 to 10 μ g of Glu-C, or (iv) 1 to 6 μ g of trypsin in a final volume of ~25 μ l and incubated at 37°C for 8 min to 3 h depending on the experiment (see figure legends). For the longer incubations, samples were overlaid with light mineral oil (PCR quality; Sigma). All reactions were terminated by boiling with 1% SDS (wt/vol) for 2 min immediately after adding protease inhibitors as follows: 50 mM benzamide for the serine proteases Glu-C, Lys-C, and trypsin and 10 mM EDTA for the metalloprotease Asp-N. The core protein species were recovered by radioimmunoprecipitation and analyzed as described above. In one particular experiment, native or denatured (i.e., boiled in 1% SDS for 2 min) p21.5 dimers and capsids were immunoprecipitated with rabbit anti-HBc/e serum and a protein G-immunoabsorbent (Omnisorb; Calbiochem) prior to trypsin digestion. After three washes, the immunoprecipitates were suspended in 25 μ l of 0.1 M Tris-

HCl (pH 8.0); one-half of the sample was incubated with 6 μ g of trypsin; the other was incubated without enzyme for 8 min at 37°C. Reactions were stopped by boiling for 4 min with an equal volume of 2 $\times$ SDS-Laemmli sample buffer.

RESULTS

Digestion of HBV capsids by site-specific proteases. In a prior study, we showed that *Xenopus* oocyte-expressed p21.5 core particles are resistant to proteinase K hydrolysis, whereas the Arg-rich protamine tails were efficiently removed from unassembled p21.5 dimers (36). We interpreted these results to mean that the protamine regions are exposed on the surface of dimers but, upon formation of capsids, become sequestered within the capsid interior. However, others have reported that the p21.5 subunit protamine regions are at least partially accessible to trypsin in bacterially expressed core particles (8, 19), implying that at least part of this region is close to the capsid surface.

To clarify the topographical arrangement of the p21.5 protamine region in capsids, as well as to identify additional surface-exposed segments of the p21.5 polypeptide, we probed oocyte-derived capsids with proteases specific for the C-terminal bonds of lysine (Lys-C and trypsin), arginine (trypsin), and glutamic acid (Glu-C), or the N-terminal bonds of aspartic acid and oxidized cysteine (Asp-N). The ~50 potential p21.5 target residues for these enzymes are tabulated (Fig. 1A, top) and shown schematically aligned with the p21.5 polypeptide (bottom); the two Lys-C cleavage sites (K-7 and K-96) are not listed separately as they are also substrates for trypsin. Although many of these sites are in the protamine region (amino acids [aa] 150 to 185), the remainder are distributed fairly evenly throughout the p21.5 assembly domain.

We initially tested the four proteases on partially purified ³⁵S-labeled core particles generated from wt p21.5 and also from a p19.8 core protein truncated down to residue 172 at the C terminus (mutant Δ 172). The core protein fragments were immunoprecipitated, resolved by SDS-PAGE, and visualized by autoradiography (Fig. 1B); molecular mass estimates were obtained by comparing Phosphorimager profiles of the core fragments versus ¹⁴C-labeled protein standards included on the same gel.

Unexpectedly, the two types of particles were cleaved with different efficiencies by Asp-N and particularly by trypsin, though they reacted equivalently with Glu-C and Lys-C. Only Glu-C fully cleaved both capsid substrates (lanes 5 to 8), yielding a major ~16.5-kDa and a minor ~14-kDa species. The capsids were not digested by Lys-C (lanes 9 to 12), in keeping with a report that this protease does not cleave liver-derived HBV core particles (2). Asp-N (lanes 13 and 14) and trypsin (lanes 1 and 2) cleaved roughly 30 and 50%, respectively, of the p21.5 subunits present in wt cores, yielding an ~16.9-kDa Asp-N fragment and a main trypsin fragment of ~16.7 kDa and minor species of ~20.2 to ~17 kDa; analogous trypsin digestion data reported for recombinant bacterial capsids (8, 25) suggest that these closely resemble oocyte-derived capsids in structure. The same major Asp-N (lanes 15 and 16) and trypsin (lanes 3 and 4) digest products were seen with p19.8 capsids, but the cleavage efficiency increased to >50 and ~100% of the subunits, respectively. We are confident that the observed cleavages, though partial in some instances, represent true end-point digestions: time course experiments reveal that maximal cleavage of p21.5 capsids by trypsin, Glu-C, and Asp-N occurs after only a few minutes of digestion and that longer digestion (up to 8 h), or the addition of fresh protease, does not significantly increase the extent of specific cleavage

FIG. 1. Cleavage pattern of oocyte-expressed HBV capsids. (A) Target residues for trypsin, Glu-C, and Asp-N in the HBV p21.5 core polypeptide (subtype adw) are tabulated (top) and are displayed schematically (bottom) aligned with the 185 residues of p21.5 (hatched box). Also shown is the position of the HBc epitope. Symbols represent the target sites as follows: $\uparrow$, trypsin; $\blacklozenge$, Glu-C; and $\downarrow$, Asp-N. (B) Aliquots of ^{35}S -labeled wt and mutant $\Delta 172$ core particles were incubated for 3 h at 37°C with (+) 6 μg of trypsin, 10 μg of Glu-C, 0.5 μg of Lys-C, or 0.5 μg of Asp-N as indicated on top of the panel prior to radioimmunoprecipitation, SDS-PAGE, and autoradiography. In control reactions, proteases were omitted (-). The positions of ^{14}C -labeled molecular mass marker (M) proteins are given in kilodaltons (kDa) at the left; the positions of full-length wt (p21.5) and $\Delta 172$ (p19.8) core proteins are shown at the right.

(data not shown). Curiously, the yields of recovered core proteins vary, although care was taken to analyze comparable amounts of digested versus undigested core proteins. Recovery of Glu-C-cleaved core fragments was particularly low: we suspect that Glu-C may be giving secondary cleavages that yield undetectable fragments; alternatively, the fragment comprising residues 1 to 145 is not efficiently immunoprecipitated. In contrast, trypsin digestion appears to increase the recovery of core proteins, perhaps by removing contaminating proteins that reduce the immunoprecipitation efficiency.

In summary, proteolysis of core proteins in intact capsids is restricted to a few select sites: the rapidity of these cleavages

FIG. 2. Digestion of in vitro-translated core proteins. (A) ^{35}S -labeled core proteins with wt and truncated protamine regions were translated in reticulocyte lysates and then recovered by radioimmunoprecipitation under native conditions either without incubation (Undigested, lanes 1 to 6) or after incubation for 8 min at 37°C with 6 μg of trypsin (lanes 7 to 9) or 10 μg of Glu-C (lanes 10 to 12). The various core protein species were analyzed by SDS-PAGE and autoradiography. Arrows at the left indicate the positions of wt (p21.5) and $\Delta 149$ (p17) core proteins. (B) Protease-accessible residues in the C terminus (aa 140 to 185) of p21.5. Arrows indicate their relative positions and degrees of protease susceptibility: filled, fully cleaved; open, partially cleaved; small open, little cleaved. Shown below are the approximate locations of epitopes recognized by MAbs 19-27 and T-2212.

(see above), together with further evidence presented later in this report, establishes that the proteases are indeed acting on intact capsids. Since the p21.5 assembly domain is believed to be intrinsically protease resistant (28), we tentatively assigned the observed cleavages to sites at the protein C terminus. Consideration of the Glu-C, trypsin, and Asp-N target sites (Fig. 1A) together with the sizes of the major proteolytic fragments (16.5, 16.7, and 16.9 kDa, respectively) suggests that the observed specific cleavages likely occur at Glu-145, Arg-150, and Asp-153, respectively. Data reported below support these assignments. We note that only N-terminal fragments are seen in our analyses: C-terminal fragments are unlikely to be seen as they are (i) very weakly labeled under our experimental conditions and (ii) may be poorly immunoprecipitated. The intriguing question of why only Glu-145 is completely accessible in capsids, while trypsin and Asp-N digestion is limited to some 50% of the p21.5 capsid subunits, is further explored and discussed later in this report.

Cleavage of in vitro-translated HBV core proteins. To exclude the formal possibility that the observed fragments arise by N- as opposed to C-terminal cleavage of p21.5, we analyzed (Fig. 2A) the effects of trypsin (lanes 7 to 9) and Glu-C (lanes 10 to 12) on in vitro-translated wt p21.5 as well as two C-terminally truncated core proteins: mutant $\Delta 149$ encodes a p17 core protein ending at aa Val-149, and the $\Delta 149\text{R4}$ core protein is identical to p17 except for an additional four Arg residues at its C terminus. We note that in vitro translation in

reticulocyte lysates produces core protein dimers but not capsids in our hands (data not shown); we anticipated that N-terminal residues were even more likely to be exposed to proteases in dimers than in capsids. To obtain molecular mass estimates, we compared the electrophoretic mobilities of the resultant proteolysis fragments with those of (i) ^{14}C -labeled protein molecular mass standards (data not shown) and (ii) undigested wt and C-terminally truncated core proteins bearing defined endpoints at p21.5 residues 185, 172, 162, 157, and 149 (Fig. 2A, lanes 1 to 6). The estimated sizes of the latter were in good accord with the theoretical values: 21.6 versus 21.38 kDa (wt); 20.0 versus 19.76 kDa ($\Delta 172$); 18.6 versus 18.55 kDa ($\Delta 162$); 18.1 versus 18.11 kDa ($\Delta 157$); 17.4 versus 17.47 kDa ($\Delta 149\text{R4}$); and 17.0 versus 16.84 kDa ($\Delta 149$).

The data reveal that Glu-C and trypsin cleaved all three ^{35}S -labeled core proteins into fragments that appeared identical to those obtained from native capsids (cf. Fig. 1). Thus, trypsin liberated a single immunoprecipitable 17-kDa fragment (lanes 7 to 9), which was electrophoretically indistinguishable from untreated $\Delta 149$ -derived p17 (lane 6); these data confirm cleavage at the Arg-150 site and rule out the use of the Lys-7 site. Likewise, Glu-C released a major proteolysis fragment of ~ 16.5 kDa from all three core proteins (lanes 10 to 12), verifying the Glu-145 cleavage site; alternative cleavages at N-terminal sites (e.g., Glu-8 or -14) would result in different digestion patterns for the three core proteins and are thus ruled out. The minor Glu-C cleavage fragment of ~ 13.8 to 14 kDa seen in Fig. 1 and 2 appears to be due to digestion at a secondary site (probably Glu-117, less likely Glu-40) which is used at high Glu-C concentrations and/or long reaction times (data not shown).

Taken together, the data in Fig. 1 and 2 provide compelling evidence that N-terminal sequences in p21.5 are protease resistant and that the main p21.5 cleavage sites (summarized in Fig. 2B) localize to Glu-145 (Glu-C), Arg-150 (trypsin), and Asp-153 (Asp-N) near the start of the p21.5 protamine region. Minor trypsin sites seen in Fig. 1 were assigned to Arg residues 159, 166, and 174, on the basis of the sizes of the respective proteolytic fragments; since data presented later in this report suggest that these Arg residues are not surface exposed on wt p21.5 capsids, we suspect that the minor sites are used after the partial cleavage at Arg-150 has weakened the capsid structure.

Protease susceptibility of core protein dimers versus capsids. As mentioned above, we previously showed that p21.5 dimers, but not capsids, are susceptible to proteinase K—a difference that we ascribed to a structural change that accompanies capsid assembly, masking both proteolytic sites and epitopes (27). Here we sought further evidence for such a transition by comparing the protease digestion profiles of gradient-fractionated oocyte-expressed p21.5 dimers and capsids (Fig. 3A and B).

Consistent with the results shown in Fig. 1 and 2, trypsin digestion (of immunocomplexes in this experiment [see Materials and Methods]) completely converted the ^{35}S -labeled p21.5 subunits in dimers into p17 subfragments (Fig. 3A, lanes 1 and 2) but only $\sim 50\%$ of the p21.5 subunits in capsids (lanes 5 and 6); denaturation of the capsids (by boiling them with 1% SDS for 2 min) rendered 100% of the p21.5 chains trypsin sensitive (lanes 7 and 8). p17 remained the main tryptic fragment seen with denatured capsids and even denatured p21.5 dimers (lanes 3 and 4), indicating that denatured p21.5 polypeptides retain an intrinsic resistance to trypsin.

Asp-N (Fig. 3B, lanes 1 to 4) and Glu-C (lanes 5 to 8) digests confirmed that dimers are more protease sensitive than particulate p21.5. Asp-N digested capsids weakly (lanes 3 and 4) but largely eliminated p21.5 dimers (lanes 1 and 2), leaving

FIG. 3. HBV capsids are less protease sensitive than p21.5 dimers. Pools of ^{35}S -labeled p21.5 dimers or capsids were partially purified by sucrose gradient fractionation and pelleting and exposed to proteases. (A) Comparison of the cleavage patterns obtained by incubating immunocomplexed p21.5 dimers (lanes 1 to 4) and capsids (lanes 5 to 8) for 8 min at 37°C with (+) $6\text{ }\mu\text{g}$ of trypsin. In control reactions (–), no enzyme was added. Digests were performed either on native (Nat.) or on heat-SDS-denatured core proteins (Den.). The resultant core species were rapidly denatured by boiling in SDS-Laemmli buffer, resolved by SDS-PAGE, and detected by autoradiography of the dried gel. (B) Aliquots of native dimers and capsids were incubated with (+) or without (–) Asp-N ($0.5\text{ }\mu\text{g}$, lanes 1 to 4) or Glu-C ($4\text{ }\mu\text{g}$, lanes 5 to 8). Immunoprecipitated core proteins were analyzed by SDS-PAGE and autoradiography. (C) Oocyte-expressed HBV capsids were refracted through a 10 to 60% sucrose minigradient after trypsin digestion ($5\text{ }\mu\text{g}$, 3 h, 37°C). Portions from each of the 14 fractions (arranged from the top at the left to the bottom at the right) were subjected to radioimmunoprecipitation with a polyclonal anti-HBc/e serum and then analyzed by SDS-PAGE and autoradiography.

only traces of the ~16.9-kDa Asp-153 cleavage product. Glu-C essentially erased immunoprecipitable core proteins from the dimer pools (lane 5), whereas capsids gave the expected 16.5-kDa and ~13.5-kDa species (lane 8).

In summary, although it is difficult to compare the cleavage extents of dimers and capsids accurately, the data suggest that all three proteases are significantly more active with dimers than with capsids. This provides further support for our earlier conclusion that capsid assembly markedly reduces protease targets in p21.5, presumably because the exquisitely protease-sensitive protamine regions become (at least in part) sequestered within the capsid interior.

We next refractionated trypsinized ³⁵S-labeled p21.5 capsids through sucrose gradients to ask whether the p17 cleavage products remain in particulate form (Fig. 3C). The core protein sedimentation profile clearly reveals most of the immunoprecipitable p17 cosedimenting with undigested p21.5 in the core particle fractions (lanes 7 to 10) and very little migrating as nonparticulate p17 at the top of the gradient (lanes 1 to 4); the ~10-kDa species seen in the latter fractions is probably due to a secondary trypsin cleavage following release of p17. This result establishes that (i) cleavage of p21.5 subunits into p17 species occurs *in situ* in intact capsids and (ii) core particles are not disrupted by removal of some 50% of the p21.5 protamine tails.

Immunoprecipitation of core proteins by a protamine-region-specific antibody. Earlier in this report, we noted a differential trypsin sensitivity that implies a difference in the architecture of the protamine regions in wt versus Δ172 core particles. In light of this, we probed the protamine regions in core particles (and dimers) via an independent experimental strategy involving immunoprecipitation with MAb T-2212, which reportedly (17) recognizes a p21.5 phosphopeptide corresponding to residues 165 to 175 (Fig. 2B); thus, T-2212 reacts with phosphorylated p21.5 expressed in animal cells but not with unmodified p21.5 produced in bacteria (17).

³⁵S-labeled dimers (lanes 1 to 4) and capsids (lanes 5 to 8) corresponding to either wt p21.5 or else C-terminally truncated Δ157, Δ162, and Δ172 core polypeptides were immunoprecipitated under native conditions with MAb T-2212 (Fig. 4A, top), or with an anti-HBe/c control serum (bottom). Surprisingly, T-2212 failed to immunoprecipitate any of the dimer species, although all were strongly captured by anti-HBe/c (lanes 1 to 4). The reasons for this are unknown: it is conceivable that the T-2212 epitope is either latent or not correctly phosphorylated in dimers, but there may be trivial reasons (e.g., low-affinity binding) why this immunoprecipitation failed. The capsid immunoprecipitations gave a very different result. As expected, anti-HBe/c antiserum precipitated all the capsids effectively (lanes 5 to 8). T-2212 was not reactive against Δ157 capsids (lane 5) and only weakly reactive against Δ162 capsids (lane 6), neither of which contain the putative epitope, but precipitated Δ172 capsids more effectively than the polyclonal antiserum (lanes 7). However, only a minor fraction (<20% as judged by phosphorimaging) of wt core particles were captured by MAb T-2212. Since immunoprecipitation of a capsid likely requires surface exposure of only a few (perhaps one) epitopes, these data suggest that p21.5 residues 165 to 175 are essentially inaccessible from the exterior of most wt core particles. Deletion of the last 13 p21.5 protamine residues evidently enhances the presentation of this epitope on the Δ172 capsid surface.

The terminal p21.5 residue, Cys-185, is known to form a homologous disulfide bond with Cys-185 from an adjacent p21.5 subunit (19, 31, 35). We suspected that this disulfide linkage might be tethering the protamine region inside the

FIG. 4. Accessibility of the protamine region to MAbs. (A) Aliquots of ³⁵S-labeled dimers (lanes 1 to 4) and capsids (lanes 5 to 8) assembled from C-terminally truncated Δ157, Δ162, and Δ172 core polypeptides or from wt (W.T.) p21.5 were immunoprecipitated under native conditions with either MAb T-2212 (top) or a polyclonal anti-HBe/c serum (bottom). Core proteins were resolved by SDS-PAGE and visualized by exposure to X-ray film. (B) ³⁵S-labeled intact capsids formed from Δ172 core protein or mutant p21.5 (A-185) bearing an Ala residue in place of Cys-185 as well as wt (W.T.) core particles were immunoprecipitated with either T-2212 or polyclonal anti-HBe/c antibodies (Ab) and analyzed as described above.

capsid structure. To test this idea, we compared the efficiencies with which T-2212 immunoprecipitated capsids assembled in oocytes from (i) wt p21.5, (ii) the Δ172 core protein, and (iii) an A-185 core protein bearing an Ala residue in place of Cys-185. The experimental results (Fig. 4B) confirmed our hypothesis. Once again, T-2212 precipitated ³⁵S-labeled Δ172 capsids (lane 1) effectively and wt capsids (lane 2) poorly relative to the anti-HBe/c polyclonal antibody (lanes 3 and 4); phosphorimaging suggested a discrimination by T-2212 of ~20-fold in favor of Δ172 capsids in this experiment. Similarly, whereas the polyclonal antibody did not distinguish the A-185 and wt capsid preparations (lanes 7 and 8), MAb T-2212 showed an approximately fivefold preference for precipitating A-185 capsids (lanes 5 and 6). These data, together with the observed increase in susceptibility of Δ172 capsids towards trypsin and Asp-N (cf. Fig. 1B), argue strongly that deletion or replacement of the p21.5 terminal residue, Cys-185, causes an

TABLE 1. Relative immunoreactivities of protamine tail-specific MAbs against oocyte-derived HBV wt and mutant capsids^a

Construct	MAb T-2212 ^b	MAb 19-27 ^c
wt	1.6	14.0
Δ 172	48.8	100
Cys	12.2	11.1

^a T-2212 and 19-27-specific immunoreactivity values were assessed by ELISA and are expressed as the percentage of the HBc/ β -specific reactivity present in the same sample.

^b Epitope located between p21.5 residues 165 and 175 (17).

^c Determinant localized to aa 150 to 159 of p21.5 (16).

increased exposure of at least a fraction of the protamine tails which are normally buried in wt capsids.

Analysis of the protamine region accessibility by ELISA. For a more quantitative assessment of protamine region exposure, we turned to protamine region-specific ELISAs to analyze serial dilutions of dimers and capsids assembled from wt p21.5, the Δ 172 mutant, and also a Cys⁻ mutant core protein in which the four p21.5 Cys residues were replaced by Ala. These species were captured with either MAb T-2212 or MAb 19-27; the latter recognizes an epitope within p21.5 residues 150 to 159 (16) (cf. Fig. 2B). As T-2212 cannot bind our unphosphorylated bacterial HBc- and HBe-core protein concentration standards, we were unable to obtain core protein concentration data from this assay. We therefore subjected each set of samples to parallel ELISAs with (i) a protamine region-specific test MAb and (ii) the capsid-specific anti-HBc/ β MAb 3120 or dimer-specific anti-HBe/a MAb 904; after correction for background and dilution, the data are presented as the ratio (%) of the test antibody A_{490} signal versus the HBe or HBc antibody A_{490} value.

Neither MAb differentiated significantly between wt and the mutant dimer species (data not shown), suggesting that the protamine regions are equally exposed in these dimers; T-2212 was consistently weakly reactive with all the dimeric core proteins, yielding $\sim$ 10-fold less immunoreactivity than the HBe-specific MAb 904; MAb 19-27 detected all three dimers with anti-HBe-like efficiency, establishing that residues 150 to 159 are fully exposed in each case. Discrimination was, however, observed at the level of capsids (Table 1). MAb T-2212 barely captured wt capsids (1.6% of the HBc signal), implying that the epitope is buried, but reacted significantly better with Cys⁻ core particles (12.2% of the HBc signal) and strongly with the Δ 172 capsids (48.8% of HBc). MAb 19-27 displayed a moderate ability to capture wt capsids (14% of HBc) and Cys⁻ (11.1%) core particles but, surprisingly, showed the same capture efficiency as the anti-HBc MAb with Δ 172 capsids: this cannot be due to dissociated core particles, since the samples exhibited no measurable HBe immunoreactivity.

In summary, the T-2212 ELISA data suggest that protamine region residues 165 to 175 are essentially inaccessible from the exterior of wt capsids but become progressively more surface exposed with (i) replacement of Cys-185 and (ii) deletion of the last 13 p21.5 residues. These results are certainly consistent with the proposed role of Cys-185 in tethering the protamine tails but imply that other p21.5 C-terminal residues contribute to this effect. Although the 19-27 epitope (residues 150 to 159) has been reported to be inaccessible in capsids (16), our results, which accord with the trypsin and Asp-N results (cf. Fig. 1B), suggest that this region is already partly surface exposed even in wt capsids and much more so in Δ 172 capsids.

DISCUSSION

Topographical features of the p21.5 polypeptide. In this report, we extended prior studies by performing a more systematic probing of p21.5 capsids with proteases of diverse cleavage specificities, thus scanning some 50 potential target residues covering the entire p21.5 polypeptide (Fig. 1). Our most striking finding is that protease attack on intact core particles is restricted to a single 9-aa segment of the p21.5 polypeptide, demarcated at one end by Glu-145 (rapidly and efficiently hydrolyzed by Glu-C) and at the other by Arg-150 and Asp-153, which are cleaved by trypsin and Asp-N, respectively, in roughly 50% of the p21.5 subunits in wt capsids (see below).

Our data establish that the p21.5 protamine regions are well exposed to external reagents in dimers but are more segregated towards the interior of assembled capsids (especially in oxidized p21.5 capsids [see below])—topographical arrangements which are fully consistent with the proposed role of these Arg-rich sequences in binding RNA and packaging it inside the capsid (4, 12). Detection of p21.5 residues 165 to 175 on the surface of wt capsids with MAb T-2212 was minimal, corroborating results of Mayumi's group (17). However, our data substantiate reports that the protamine region is partially accessible to trypsin in recombinant bacterial capsids (8) and authentic liver-derived cores (2) and further show that it is somewhat accessible to Asp-N and to MAb 19-27 (specific for p21.5 aa 150 to 159). It therefore appears that a rather short segment at the start of the protamine region is exposed to the external environment in wt capsids: an intriguing though unproven idea is that proteolytic cleavage within this short region might help promote nucleocapsid disassembly *in vivo*.

In contrast to the protamine region, the first 144 p21.5 residues were almost totally protease insensitive in capsids and at least somewhat resistant in dimers. Moreover, our protease scans of dimers failed to clearly identify a single residue that is completely exposed on dimers but buried in capsids. It therefore appears that all the potential cleavage sites present in the first 144 p21.5 residues either must lie buried inside the p21.5 tertiary or quaternary structure or else are masked by the folding of the p21.5 polypeptide. We note that our data do not bear out a report suggesting that the N terminus of p21.5 is exposed on the capsid surface (5): the absence of cleavage at two Glu-C sites and one trypsin site within the first 14 p21.5 residues (Fig. 2) suggests that the N terminus of p21.5 is probably buried in capsids. Likewise, we did not detect cleavage at any of four sites around aa 80, the location of a surface-exposed loop bearing the HBc epitope (Fig. 1A).

A protease-sensitive hinge joins the two domains of p21.5. Intriguingly, the p21.5 protease target region (aa 145 to 153) defined in this report falls between the two known functional domains of the p21.5 polypeptide. The minimal core protein "assembly domain," characterized by Nassal, ends at p21.5 residue 144; core proteins shorter than this are assembly incompetent in bacteria (4) and mammalian cells (18). The nucleic acid binding domain comprises residues 150 to 185, the proximal Arg motifs being crucial for RNA binding (4, 8, 12, 18).

Short, protease-sensitive peptides joining two globular polypeptide domains have been described for many proteins, a classic example being the peptide linking the Fab and Fc domains of immunoglobulins (6); structural studies suggest that this region forms a hinge-like loop which allows the two domains to move, thereby imparting flexibility to the protein. In the trimeric influenza virus hemagglutinin protein, a protease-sensitive hinge joins the stem and globular head domains

FIG. 5. Topographical model of the HBV core protein. The cartoon depicts two adjacent p21.5 dimers in a capsid lattice. The barrel-like globular assembly domain, the armlike nucleic acid binding domains (protamine regions), and the protease-sensitive hinges joining the two domains of the p21.5 subunits are all indicated. Residues Glu-145, Arg-150, and Asp-153 are shown in two of the hinges. Also shown are the homologous Cys-185–Cys-185 disulfide bonds (S-S) that tether protamine arms inside the capsid. We have attempted to draw the p21.5 monomer subunits and the p21.5 dimers with twofold symmetry. For further details, see text.

and allows a slight movement of the heads which is thought to initiate membrane fusion (10). Interestingly, flexion of a hinge in the tomato bushy stunt virus capsid protein reportedly introduces quasiequivalence into otherwise identical dimers, thus facilitating their packing into capsids (11). By analogy, we propose that p21.5 residues 145 to 150/153 act as a flexible hinge between the globular assembly domain and the nucleic acid binding domain: this may facilitate conformational changes that undoubtedly accompany nucleic acid binding and dimer packing during capsid assembly. Hinge-related quasiequivalence could plausibly explain why only a subset of the p21.5 protamine regions is available to trypsin or Asp-N; however, other factors could alternatively limit cleavage, such as hydrolysis-induced structural rearrangements or unfavorable thermodynamics.

Figure 5 presents a possible model which integrates the features described in the present study into our current view of p21.5 core particle structure. We depict a portion of the capsid lattice with two identical noncovalently packed p21.5 dimer units: the two interior-facing protamine regions are tethered by a homologous intradimer Cys-185–Cys-185 disulfide (35) (the assembly domains are joined by a homologous Cys-61–Cys-61 linkage which is not shown); others have proposed an alternative disulfide linkage pattern (19, 31). The putative hinge region (approximately aa 145 to 153) is envisioned as a loop pointing towards the capsid surface but positioned at the base of a fenestration in the capsid: this allows p21.5 residues 150 to 153 to be close enough to the capsid interior to mediate RNA binding yet still be accessible to external reagents. It is apparent that quasiequivalent dimers could be created by moving the hinge slightly downward so as to bury Arg-150 and Asp-153, while leaving Glu-145 accessible.

The p21.5 protamine region is tethered inside the capsid. A final finding from this work, confirmed by both antibody and protease probes, is that the p21.5 terminal Cys residue (per-

haps in conjunction with other C-terminal residues) is intimately involved in tethering the distal part of the protamine region inside the wt core particle—presumably via the disulfide bond depicted in Fig. 5. Removal of Cys-185 allows this segment more freedom to move to the capsid surface, perhaps aided by the proposed hinge. Even in $\Delta 172$ capsids, it seems probable that only a minor fraction of protamine regions faces the outside. This explains why $\Delta 172$ capsids are immunoprecipitated by MAb T-2212 (this requires only a few exposed protamine regions per capsid), yet are still able to package comparable amounts of RNA to wt capsids (12) (this must require many interior-facing protamine domains). We propose that a dynamic equilibrium between interior and exterior protamine domains may explain the ability of trypsin to cleave all the $\Delta 172$ protamine regions as they transiently appear at the surface. Presumably, such a dynamic equilibrium would also apply to wt p21.5 capsids within the reducing environment of the cytoplasm, where disulfide bonds are disfavored.

Intriguingly, the carboxyl-terminal domain of duck core protein is functionally (though not structurally) analogous to the p21.5 protamine region but lacks a tethering Cys residue. Thus, duck core particles may somewhat resemble the A-185 capsid: this would neatly explain why intact duck capsids can be immunoprecipitated by an antibody directed against the final 13 residues of the carboxyl-terminal region (23) and why treatment of the capsid with phosphatase enzyme strips modifying phosphate residues from the carboxyl-terminal regions (20).

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