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Intrinsic disorder and oligomerization of the hepatitis delta virus antigen

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Abstract

The 195 amino acid basic protein (δAg) of hepatitis delta virus (HDV) is essential for replication of the HDV RNA genome. Numerous properties have been mapped to full-length δAg and attempts made to link these to secondary, tertiary and quaternary structures. Here, for the full-size δAg, extensive intrinsic disorder was predicted using PONDR-FIT, a meta-predictor of intrinsic disorder, and evidenced by circular dichroism measurements. Most δAg amino acids are in disordered configurations with no more than 30% adopting a α-helical structure. In addition, dynamic light scattering studies indicated that purified δAg assembled into structures of as large as dodecamers. Cross-linking followed by denaturing polyacrylamide gel electrophoresis revealed hexamers to octamers for this purified δAg and at least this size for δAg found in virus-like particles. Oligomers of purified δAg were resistant to elevated NaCl and urea concentrations, and bound without specificity to RNA and single- and double-stranded DNAs.

Keywords

hepatitis delta virus; delta antigen; intrinsic disorder; protein oligomerization; nucleic acid binding

Introduction

The replication of the RNA genome of hepatitis delta virus (HDV) requires the translation of a single 195 amino acid basic protein known as the delta antigen (δAg) (Chao et al., 1990). As reviewed elsewhere (Casey, 2006; Lai, 2006; Taylor, 2009), different properties have been attributed to the δAg, some of which have been associated with domains on the δAg primary sequence. These include a nuclear localization signal (NLS) and an RNA binding domain (RBD)(Fig. 1). Also, during HDV replication an RNA editing event occurs, which allows the translation of a δAg that is 19 aa longer. This larger δAg acts as a dominant negative inhibitor of the smaller form (Chao et al., 1990), and has an essential role in recruitment of HBV envelope proteins for assembly of progeny virus (Chang et al., 1991).

Early studies with altered forms of δAg revealed that regions near the N-terminus were required to make dimers and showed that this ability was essential for functionality in viral

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RNA replication (Lazinski and Taylor, 1993). A peptide corresponding to amino acids 12-60, and spanning the region thought necessary for dimerization readily formed crystals; the structure was determined as a dimer of anti-parallel α-helices (Zuccola et al., 1998) that became known as the coiled-coil domain (CCD). From this structural analysis it was also argued that the δAg might form octamers. In addition, mass spectrometry after cross-linking, confirmed that some of the purified δAg did form octamers (Zuccola et al., 1998). Amino acids in the CCD structure that might be critical for the formation of dimers and higher multimers were chosen and tested (Moraleda et al., 2000). Some agreement with the structural models was obtained by mutating these residues (Cornillez-Ty and Lazinski, 2003).

Studies of δAg in virus particles have also been carried out. Associations with HDV genomic RNA were demonstrated by both equilibrium centrifugation (Ryu et al., 1993), rate zonal sedimentation, and agarose gel electrophoresis under non-denaturing conditions (Dingle et al., 1998b). Interestingly, δAg behaves as a high MW complex, even when the associated RNA is released by prior treatment with either RNase or vanadyl ribonucleosides, consistent with the idea that δAg has an intrinsic ability to form and maintain multimers (Dingle et al., 1998b).

Several studies indicate that δAg is also present in high MW complexes within cells. Wang and Lemon applied prior cross-linking to HDV infected liver tissue and showed that δAg was in complexes with a sedimentation value of 15 S (Wang and Lemon, 1993). Chang *et al.* obtained similar results using extracts of tissue culture cells transfected with HDV (Chang et al., 2008). Interestingly, similar-sized complexes were found when δAg was expressed in the absence of the full-length viral RNA, and no size change was detected when these complexes were first treated with RNase. An extensive mass spectrometry study identified >100 protein partners of δAg in cells undergoing HDV replication (Cao et al., 2009). Thus, while the majority of δAg expressed in cells seems to be in high MW complexes it remains unclear to what extent these represent multimers of δAg and/or association with host proteins. In terms of the intrinsic secondary, tertiary and quaternary structures of the δAg, most studies have been limited to fragments of δAg or complexes that include δAg but, in other respects, were of uncertain origin. Therefore, the present studies focus on the use of δAg that was of full-length, with no modifications, and purified in such a way as to maintain native structure. Furthermore, several experimental approaches have been used to quantitate intra- and inter-molecular interactions.

Theoretical approaches have been used to predict secondary structure (Deny, 2006; Taylor, 2009) but the tertiary structure for full-length δAg remains unknown; at least three labs have purified the full-length δAg and yet been unable to determine a crystal structure. A reason for this latter difficulty might be what is referred to as intrinsic protein disorder. Based on experimental evidence and the development and application of neural network predictors it is now believed that ~50% of natural eukaryotic proteins contain at least one region of disorder (Dunker et al., 2000). It is thought that disorder can offer an evolutionary benefit, making possible protein-protein and protein-nucleic acid interactions in which disordered regions fold into more structured domains (Dyson and Wright, 2004; Fuxreiter et al., 2008).

In the following study we have made use of structure predictors along with experimental strategies to detect secondary, tertiary and quaternary structures of δAg, and its interactions with nucleic acids.

Results

Predictions of intrinsic disorder

In order to predict disordered regions in proteins, neural network strategies have been applied in programs that are then educated using experimental data on regions of proteins known to be either ordered or disordered. Recently Xue *et al*. have combined six of these programs to produce PONDR-Fit (Xue et al., 2010), a meta-predictor which provides significantly improved accuracy. We applied this program to the full-length δAg, as seen in Fig. 1, where a score of 1.0 indicates a region that is disordered and 0 indicates a highly ordered region. According to this meta-predictor most of the δAg is disordered. For the CCD, which spans positions 12-60, only a small patch is predicted to have less disorder. This is in rough agreement with studies of the corresponding peptide that crystallizes and has been solved as an anti-parallel coiled-coil (Zuccola et al., 1998). Also shown are the results for three of the component predictors.

The same meta-predictor was applied to the δAg for all 8 clades of HDV with results as summarized in Fig. 2. Maybe the only conserved feature was the prediction of partial order within the CCD. There were no regions with comparable levels of predicted order, and even regions with reduced order, were not conserved between clades. In summary, other than for part of the CCD region, the shared feature was predicted disordered. As a reference a comparison was made with the 125 amino acid apoptin protein of the chicken anemia virus. As reviewed in the Discussion, this protein has many similarities to δAg, including extensive disorder and yet, according to the meta-predictor, is not as disordered as the δAg. For comparison we also show the highly α-helical ammonia channel protein of *E. coli.*

Circular dichroism of purified δAg

We next evaluated the secondary structure of the full-length δAg, experimentally, via circular dichroism, which assesses the average secondary structure content of a protein in solution. As shown in Fig. 3, the spectrum of recombinant δAg exhibits a strong negative band around 208 nm and a somewhat weaker negative band around 222 nm. Assuming a molar mean-residue ellipticity of −34,100 mdeg cm^2 $dmol^{-1}$ at 222 nm for a fully α-helical peptide (Scholtz and Baldwin, 1992), the data indicate that δAg contains approximately 35% α-helical secondary structure. Alternatively the data were analyzed using the K2D2 program (PerezIratxeta and Andrade-Navarro, 2008) giving a best fit with 29% α-helix, 14% β-sheet, and 57% other (mainly disordered).

Previous studies have used CD to detect α-helical structure in the 24-50 peptide (Cheng et al., 1998; Lin et al., 1999; Lou et al., 2000) and in the larger 12-60 fragment of (Rozzelle et al., 1995). X-ray crystallography confirmed that the 12-60 peptide is largely α-helical, adopting a dimeric anti-parallel coiled-coil structure (Zuccola et al., 1998). If we accept that the 12-60 peptide is 90% α-helical structure (Zuccola et al., 1998) it could contribute 20% α-helical structure to the full-length protein. This is less than predicted for δAg by CD, implying that a small amount of additional α-helical structure exists within the full-length protein. This conclusion is consistent with previous theoretical predictions of α-helical structure within δAg (Deny, 2006).

Dynamic light scattering to detect δAg multimers

As mentioned in the Introduction a number of approaches have been used to evaluate the quaternary structure of δAg, using the full-length protein as well as truncated and/or tagged forms of δAg. For the recombinant full-length protein used in this study, there is already evidence from mass spectrometry for the occurrence of octamers, detected following protein

cross-linking (Zuccola et al., 1998). However, it was unclear if these were stable structures or transient structures that could be stabilized by the cross-linking protocol.

Dynamic light scattering (DLS) provides a measure of translational diffusion constants, which together with assumptions on protein shape can yield insight into the oligomerization state of protein solutions of non-cross-linked proteins that are sufficiently monodisperse (Schurr and Schmitz, 1986). The DLS data for δAg (Table S1 and Fig. S1) showed a major species with a hydrodynamic radius R_h = 6.44 nm (accounting for 98 % of the amplitude and essentially 100% of the total protein mass). Assuming the complex can be approximated as a sphere, the corresponding molecular mass is ~263 kDa, consistent with a multimer of twelve 22 kDa subunits. This is an upper limit of the subunit number, since a non-spherical structure could exhibit a similar R_h with fewer subunits. Very similar DLS parameters were obtained after addition of 150 mM NaCl (MW = 279 kDa) or lowering the temperature to 15 °C (275 kDa; Table S1), suggesting that the size of the δAg oligomer was quite insensitive to solvent conditions.

Gel electrophoresis to detect δAg multimers

To obtain a more precise estimate of oligomer subunit number, we used SDS polyacrylamide gel electrophoresis of purified δAg, with and without prior glutaraldehyde cross-linking. Un-cross-linked protein was almost homogeneous. There was a single major band with a minor band (<10%) of slightly slower mobility (Fig. 4A). Relative to MW markers, the major band is ~26 kDa, significantly larger than the predicted size of 21.8 kDa and as detected by mass spectrometry (Zuccola et al., 1998). However, SDS gel electrophoresis is known to overestimate the molecular size of the δAg (Bergmann et al., 1987;Gerlich et al., 1987). Following cross-linking of δAg at 2 μM, it migrated, relative to protein MW standards, as a broad band at about 160 kDa, consistent with aggregates of 6- to 8-mers. However, in addition to the anomalous mobility of the monomer, it might be that cross-linked multimers also migrate aberrantly relative to protein standards.

Identical samples were also analyzed by immunoblot to detect δAg. Without prior cross-linking, the δAg again gave a major band at ~26 kDa (Fig. 4B). This confirmed both the purity of the δAg preparation and the lack of hydrolytic protein fragments.

We next asked if reducing the extent of prior cross-linking altered estimates of oligomer size. As shown in Fig. 4B, with quantitation presented in Fig. 4D, we detected small amounts of species consistent with 1, 2, 3, 4 and 5 δAg. (Note that the immunoblot assay could give signals that increase in linear proportion to the size of a multimer.) We also note that with reduced cross-linking the major species near the top of the gel, indicated as 6- to 8-mers, gave a stronger immunoblot signal. This is consistent with less cross-linking producing multimers that even after SDS gel electrophoresis, are less compact and more readily reactive with the polyclonal antibody.

We next asked if we could use cross-linking to detect δAg multimers in virus-like particles. As shown in Fig. 4C, we obtained results similar to those obtained using purified δAg (c.f. Fig. 3B). By reducing cross-linking, we again detected species corresponding to 2, 3, 4, 5, and 6-8 molecules of δAg.

As a test of the natural stability of the complexes present in the purified δAg, we incubated purified δAg in various concentrations of NaCl and urea prior to cross-linking. As shown in Fig. S2, the complexes were largely resistant to these treatments.

Binding of nucleic acids to δAg multimers

The above studies support the conclusion that complexes of 6, 8 or more δAg molecules are formed in solutions of purified protein and in virus-like particles associations of 6 to 8 or more δAg molecules are formed. Based upon this similarity, and the reports that δAg binds RNA in virus-like particles, we asked whether the multimers made from purified protein were able to change the electrophoretic mobility in non-denaturing agarose gels of different species of ^{32}P-labeled RNA and DNA.

First we tested the ability of increasing concentrations of δAg to bind to relatively small amounts of ^{32}P-labeled unit-length genomic RNA. As shown in Fig. 5A, δAg at 0.2 μM did not affect RNA mobility, but with increasing concentration mobility shifts were observed, and by 1.6 μM the majority of the RNA was retained at the top of the gel.

In order to test whether rearrangement of δAg multimers was required for binding to RNA, we subjected the δAg to prior cross-linking followed by quenching of the excess cross-linker with ammonium acetate. As shown in Fig. 5B, the cross-linked δAg was still able to bind RNA with almost equal efficiency as the non-cross-linked δAg. To confirm the completeness of the quenching procedure, glutaraldehyde was pre-mixed with quencher and then added to δAg; no cross-linking was detected (data not shown). We conclude that preformed δAg multimers can bind directly to the RNA.

Next we tested other nucleic acids for their ability to bind to δAg. Figs. 6A and B show results with two different HDV RNA species. The first was 1,679 nt unit-length linear antigenomic RNA, while the second was a 419 nt antigenomic RNA which is not predicted to contain any of the HDV rod-like folding. As shown, as the concentration of the δAg was increased both of these RNAs underwent a mobility shift, ultimately collecting at the origin of the gel. Thus, rod-like folding is not needed for binding.

Two non-HDV RNAs were also tested in the mobility shift assay. RMRP is a 267 nt non-coding host RNA recently shown to bind to telomerase (Maida et al., 2009), and potato spindle tuber viroid RNA is 359 nt long. As shown in Figs. S2A and S2B respectively, both RNAs bound to δAg.

DNA species were also tested. For this we 5′-end labeled a DNA ladder with double-stranded species ranging from 100 to 12,000 bp. As shown in Fig. 5C all species were shifted towards the top of the gel. There was no significant difference related to the length of the DNA. Denatured DNAs were also shifted by the same concentrations of δAg (data not shown).

In summary these mobility studies allow two conclusions: (i) Multimers of δAg are able to bind many different forms of RNA and DNA. This is in contrast to previous reports from this and other labs and will be considered in the Discussion. (ii) Multimers can form first and then bind nucleic acids. As previously reported the δAg multimers assembled into virus-like particles and into serum virus retain their integrity when the RNA is removed (Dingle et al., 1998b). While this is consistent with the RNA binding to preformed multimers, it is equally consistent with the RNA assisting in the formation of multimers that no longer need the RNA for their integrity.

Since δAg is a basic protein with an expected net charge of +12 at neutral pH, a multimer will be even more positively charged, and this could facilitate binding to the negatively charged nucleic acid. Therefore we asked if high concentrations of NaCl could disrupt the binding of ^{32}P-labeled genomic HDV RNA to δAg. As shown in Fig. 7A, at 2.4 M NaCl, the RNA was released. In parallel we tested whether NaCl was able to disrupt δAg multimers.

As shown in Fig. S2, even at 3.6 M NaCl, this did not occur. We also showed that increasing concentrations of vanadyl ribonucleosides (VRC) released the RNA from the δAg multimers (Fig. 7B).

Discussion

This study advances our understanding of the secondary, tertiary, and quaternary structures of δAg that are essential for HDV replication. Previous studies have identified a dimerization domain (CCD) spanning amino acids 12-60 (Fig. 1). In particular, the crystal structure of a synthetic 12-60 peptide revealed an anti-parallel coiled-coil dimer in which over 90% of the residues (45 out of 49) are α-helical (Zuccola et al., 1998) consistent with earlier CD data (Rozzelle et al., 1995). From our CD measurements on full-length δAg we deduced that the remainder of the protein contains very little additional secondary structure (Fig. 3). We arrived at a similar conclusion using the recently developed meta-predictor PONDR-Fit (Xue et al., 2010) which combines six neural network programs trained on a large number of regions of known protein disorder. Analysis of the δAg sequence studied here (Fig. 1) as well as for all 8 clades of HDV (Fig. 2) predicted high levels of disorder (P-Fit scores >0.75) throughout the protein, except for parts of the CCD region. There was no evidence for additional ordered regions outside the CCD that are conserved between clades. PONDR-VLXT, one of the six components of PONDR-Fit, predicts somewhat higher levels of order, both in the CCD and two or three segments in the C-terminal half of δAg (especially for clades 5-8). This algorithm recognizes short regions experimentally known to be disordered that become structured when they are bound to other proteins (Obradovic et al., 2005). This supports the possibility that δAg multimerization promotes helix formation in the CCD while isolated segments within the initially disordered C-terminal region are poised to become ordered upon interaction with other cellular proteins.

Intrinsic disorder is increasingly recognized for its relevance in understanding the intra- and inter-molecular interactions of important proteins (Dunker et al., 2008). In the case of viral proteins that are expressed in cells in relatively large amounts, disordered regions may increase the capacity to structurally adapt for engagement in a wide variety of homo- and heteromultimeric interactions (Goh et al., 2008a, b); during HDV replication δAg interacts with more than 100 different host proteins (Cao et al., 2009). It is interesting to compare δAg with apoptin, a protein of chicken anemia virus. Like δAg this protein is small (125 amino acids), basic, essential for replication, binds nucleic acids, and has not yielded to crystal-structure determination. Apoptin forms homomultimers of 30-40 molecules and is known to interact with several different host proteins (Leliveld et al., 2003a; Leliveld et al., 2003b; Los et al., 2009; Teodoro et al., 2004). Comparison of the PONDR-Fit profiles of apoptin with that of δAg (Fig. 2) show somewhat higher degrees of order centered around residue 45 with flanking regions of predicted disorder, similar to the patterns observed for the N-terminal 120 residues of δAg. (Incidentally, the N-terminal 69 amino acids of apoptin have been demonstrated to account for its multimerization behavior (Leliveld et al., 2003b).) However, a much higher fraction of the residues in δAg are predicted to be highly disordered suggesting that intrinsic disorder might be even more relevant to understanding the properties and functions of δAg in the HDV life cycle.

Qualitative insight into δAg quaternary structure was obtained by denaturing gel electrophoresis. Prior cross-linking revealed the existence of multimers formed both by the purified protein and that present in virus-like particles. With the purified δAg the complexes were detected when the proteins were at 0.2 and 2 μM during cross-linking (Fig. 4 and data not shown). Much higher concentrations are present in cells during HDV replication, suggesting that multimers should also arise *in vivo*; for example, the observed accumulation

of 2.4 million copies of δAg in a nucleus of ~9 μm diameter represents an average concentration of 24 μM (Chang et al., 2005).

Relative to protein standards the majority of cross-linked multimers appeared to contain 6 - 8 molecules of δAg (Fig. 4A-C). This confirms and extends previous mass spectrometry studies in which octamers were detected although not quantified (Zuccola et al., 1998). In addition, the hydrodynamic dimensions observed in our dynamic light scattering measurements, were consistent with an oligomer of ≤12 subunits. The δAg multimers we observed were remarkably stable even in the presence of high NaCl or urea concentrations (Fig. S2).

The multimers of full-length δAg did bind nucleic acids but without specificity; HDV RNAs with and without rod-like folding and of different lengths were bound, as were non-HDV RNAs, and double- and single-stranded DNAs (Figs. 5A, 6 and S3). This binding is consistent with simple electrostatic interactions. The δAg has a predicted net charge of +12 at neutral pH (Kuo et al., 1988) and multimers will have a greater total charge and are thus expected to have enhanced affinity for nucleic acids that have one negative charge per nucleotide. Similar electrostatic interactions with nucleic acids have been reported for the apoptin mentioned earlier, which is also basic and forms large oligomers (Leliveld et al., 2003a). We also found that nucleic acid binding was achieved when the protein was first cross-linked (Fig. 5B), indicating that δAg rearrangement was not necessary during binding. Observations that the binding was reversed with increasing concentrations of NaCl (Fig. 7A) or vanadyl ribonucleosides (VRC)(Fig. 7B), agrees with the interpretation of an electrostatic interaction. The findings are also consistent with our earlier studies on natural HDV ribonucleoprotein complexes where VRC released the HDV RNA but left the δAg complexes intact (Dingle et al., 1998b). However, unlike the natural situation, the purified δAg did not demonstrate specificity for rod-like HDV RNA.

The ability of δAg to bind non-specifically to nucleic acids might explain its behavior as a chaperone. In a series of papers Wang *et al*. showed that δAg would facilitate strand exchange reactions between short DNA oligonucleotides (Wang et al., 2003). We confirmed their results including evidence that the chaperone effect can be achieved with N-terminal and C-terminal fragments of the δAg lacking the oligomerization domain (G. Moraleda, H.J. Netter, and J.M. Taylor, unpublished observations). Thus, the chaperone activity of δAg appears to be independent of its ability to oligomerize. It seems to be a property of a positively charged protein independent of multimerization.

The present findings of non-specific binding of δAg to nucleic acids also appear to disagree with earlier studies from this lab by Chao *et al*. which indicated that a delta antigen would bind to HDV RNAs but only if they had the ability to form rod-like folding structures (Chao et al., 1991). However, the assay used in the previous study was quite different: the protein tested was a fusion protein and the detection method was a northwestern. The present findings also disagree with recent studies that employed a δAg with an N-terminal fusion and a C-terminal deletion in a mobility shift assay and concluded that binding to HDV RNA required a minimum of ~311 nt of rod-like folding (Defenbaugh et al., 2009; Lin et al.). However, it should be noted that when a non-truncated form of fusion protein was used, no specificity was detected in the RNA binding assay.

Further *in vitro* experiments are thus needed to determine whether or not we can identify features of the full-length protein and/or the RNA substrates that will produce specific interactions. Recent *in vitro* studies with the full-length core protein of HBV have also encountered RNA interactions that are efficient but not specific for viral RNA (Porterfield et al., 2010). However, it remains to be seen how and to what extent the *in vivo* interactions

can be specific for viral RNA. Further work is needed to gain a more detailed understanding of the mechanism of *in vivo* self-associations of δAg and its interactions with HDV RNAs as well as host proteins and nucleic acids. We have reported that *in vivo* δAg is present in large complexes, even in the absence of HDV RNA genome replication or when HDV RNAs are present but released by treatment with VRC (Dingle et al., 1998b) or RNase (Chang et al., 2008; Dingle et al., 1998a). In addition, a recent study reported the identification by mass spectrometry of more than 100 host proteins that interact with δAg and began to test how these might contribute to supporting HDV replication (Cao et al., 2009). Sorting out which are the relevant associations is non-trivial because millions of copies of δAg are produced per cell during HDV replication, most of which are in the nucleus at concentrations exceeding 24 μM (Chang et al., 2005), much higher than the 0.2 - 2 μM used for the *in vitro* studies reported here. A further *in vivo* complication is that in the presence or absence of HDV RNA, δAg is found in either the nucleoplasm or nucleolus, respectively (Han et al., 2009). That is, the same high concentrations of δAg can assume different intranuclear associations depending upon whether or not HDV RNA species have been allowed to replicate.

Conclusion

A combination of biochemical and biophysical approaches have been used to show that extensive intrinsic disorder and multimerization are essential aspects of full-length δAg. However, the *in vivo* interaction between this essential protein and viral RNAs is more specific and no doubt more complex than we have been able to reproduce *in vitro* with purified protein.

Material and methods

Purified delta protein

As previously described, full-length δAg was expressed in *E. coli* and purified (Dingle et al., 1998a; Moraleda et al., 2000; Zuccola et al., 1998). The chromatography procedures used in the purification were chosen with the aim of obtaining a protein that was still in its native conformation. The protein was a generous gift from Harmon Zuccola and James Hogle and had been evaluated as >85% pure. The sequence corresponds to an American strain of HDV (M28267). From optical density measurements we deduce that the purified protein contains less than one nucleotide of RNA or DNA per molecule of delta antigen. The protein has been tested for its ability to support HDV genome replication: surprisingly transfection into cells of this protein and in vitro transcribed HDV RNA at as low as an equimolar ratio was sufficient to initiate replication (Dingle et al., 1998a). Nevertheless, this is not proof that the majority of the purified protein is native in conformation.

PONDR-FIT analyses

PONDR-Fit (Xue et al., 2010) is a meta-predictor which combines the results of 6 different programs each separately designed to predict disorder in proteins. It was applied here to the δAg sequence of the purified protein, as well as to the δAg for all eight clades of HDV: clade 1 (US-1), D01075; clade 2 (Taiwan-3), U19598; clade 3 (Vnzd8375), AB037947; clade 4 (Tokyo), AB118847; clade 5 (dFR2600, Togo), AM183326; clade 6 (dFR2627, Nigeria), AM183329; clade 7 (dFR2158, Cameroon), AM183333; clade 8 (dFR2072, Senegal), AM183330, the 125 amino acid apoptin (Noteborn et al., 1991) of chicken anemia virus (M55918), and the 428 amino acid ammonia channel protein of *E. coli* (P69681).

Circular dichroism analysis

The far-UV circular dichroism (CD) spectrum of a 5.3 μM protein solution (in 20 mM potassium phosphate buffer, pH 6.3) was acquired at 25 °C on an Aviv 62A spectropolarimeter (Aviv, Lakewood, NJ), using a 1 mm quartz cuvette. The CD spectrum is an average of five scans recorded in the far-UV region (195-250 nm) with a band pass of 2 nm.

Dynamic light scattering analysis

Dynamic light scattering experiments were performed at 15 and 25 °C on a DynaPro Molecular Sizing Instrument with Dynamics V6 data analysis software (Protein Solutions, Inc.) on 80 μl of a 10 μM solution of δAg (in 20 mM potassium phosphate buffer, pH 6.3, without or with 150 mM NaCl). Autocorrelation curves were acquired for a total acquisition time of 600 sec. Representative data are shown in Fig. S1A with deductions summarized in Table S1.

Virus-like particles

As previously described, Huh7 cells were transfected with plasmids to initiate HDV genome replication and to express the envelope proteins of hepatitis B virus (Gudima et al., 2007). Media were harvested at days 7-10, and clarified of cellular debris by low speed centrifugation. Virus-like particles were then collected by two cycles of ultracentrifugation through a cushion of 20% sucrose.

DNA-directed RNA transcription

Plasmids containing HDV and non-HDV sequences were transcribed *in vitro* using T7 RNA polymerase using a RiboMax large-scale transcription system (Promega) with the addition of [α-^{32}P] CTP (MP Biomedicals). Unit-length genomic HDV was transcribed from pSG253 precut with *Not*I (Gudima et al., 2004). Unit-length, 1,060 and 419 nt antigenomic RNAs were transcribed from pSG254 pre-cut with *Not*I, *Stu*I, and *Bgl*II, respectively (Gudima et al., 2004). Unit-length genomic PSTVd RNA was transcribed from pJC144 pre-cut with *Eco*RI (Chang et al., 2003). RMRP was transcribed using expression PCR (Chang and Taylor, 2002). Transcripts were gel purified prior to use in gel retardation assays.

Gel electrophoresis analyses

Gel retardation assays with ^{32}P-labeled nucleic acids, were performed using gels of 1.5% agarose (in 1xTBE) followed by drying onto charged paper (DE81, Whatman) and bioimager detection (Fuji). ^{32}P-labeled RNAs were prepared as described above. ^{32}P-labeled DNA was obtained by phosphorylating a 1 kb Plus DNA Ladder (Invitrogen) using [γ-^{32}P] ATP (MP Biomedicals) following the manufacturer's instructions.

Association of δAg with nucleic acids was performed in 150 mM NaCl - 5 mM HEPES (pH7.5) for 10 min at room temperature. RNA concentrations were at 2 nM and δAg concentrations were as indicated in the figure legends.

Protein samples, without and with prior glutaraldehyde cross-linking, were examined on 4-12% acrylamide NuPAGE gels (Invitrogen). Glutaraldehyde was typically added to 0.1% final concentration, and after 10 min at room temperature it was inactivated by the addition of 100 mM ammonium acetate. Total protein in gels was stained with SimplyBlue SafeStain (Invitrogen) and detected using an Odyssey laser scanner (LI-COR). For immunodetection δAg proteins were electrotransferred to a cellulose nitrate membrane and reacted with a rabbit polyclonal anti-δAg followed by infrared dye-labeled goat anti-rabbit antibody (LI-COR), with detection using the Odyssey scanner.

Research highlights

Majority of hepatitis delta antigen exhibits intrinsic disorder; Purified delta antigen forms stable multimers; Delta antigen multimers bind without specificity to nucleic acids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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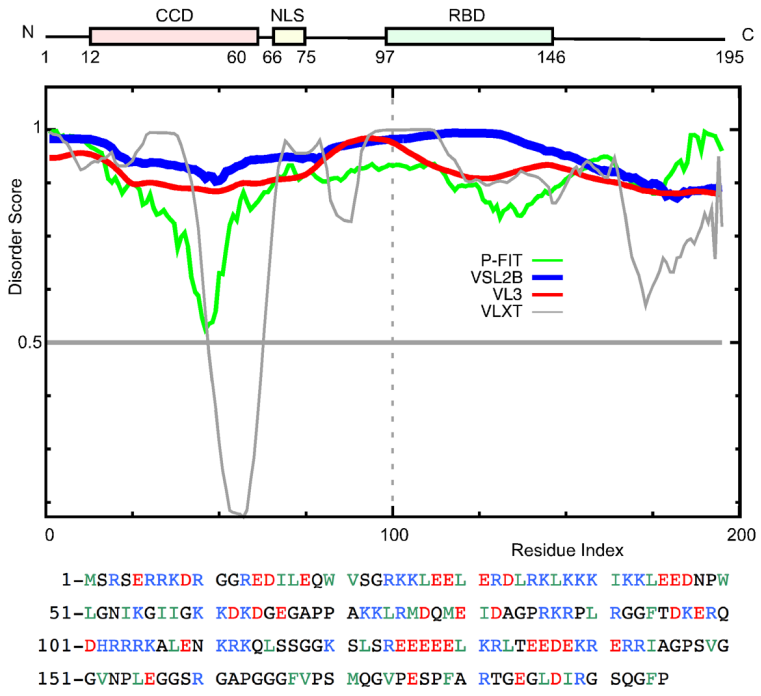

Fig. 1.

Primary and secondary structure features of the 195 amino acid δAg. The upper panel indicates the coiled-coil domain (CDD), nuclear localization signal (NLS) and the RNA binding domain (RBD) (Han et al., 2009). The middle panel shows predictions of disorder using the meta-predictor PONDR-Fit (Xue et al., 2010), and three of its component programs (Obradovic et al., 2003; Obradovic et al., 2005), as indicated. The disorder score is a measure of the certainty that a region of the protein is disordered; a score of 1 indicates 100% certainty. The lower panel shows the primary sequence with basic, acidic and hydrophobic amino acids indicated in blue, red and green, respectively.

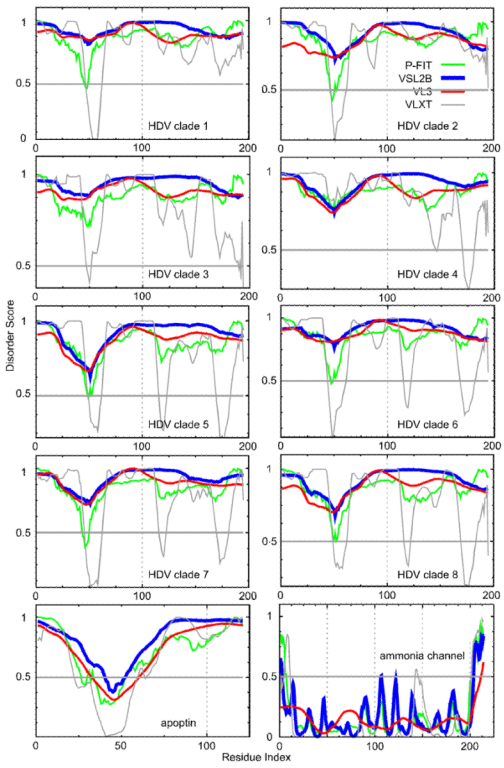

Fig. 2.

Predictions of δAg disorder for all 8 clades of HDV. Analyses were made as in Fig. 1. Also shown are controls of the highly disordered apoptin of chicken anemia virus, and the highly structured ammonia channel protein from *E. coli*.

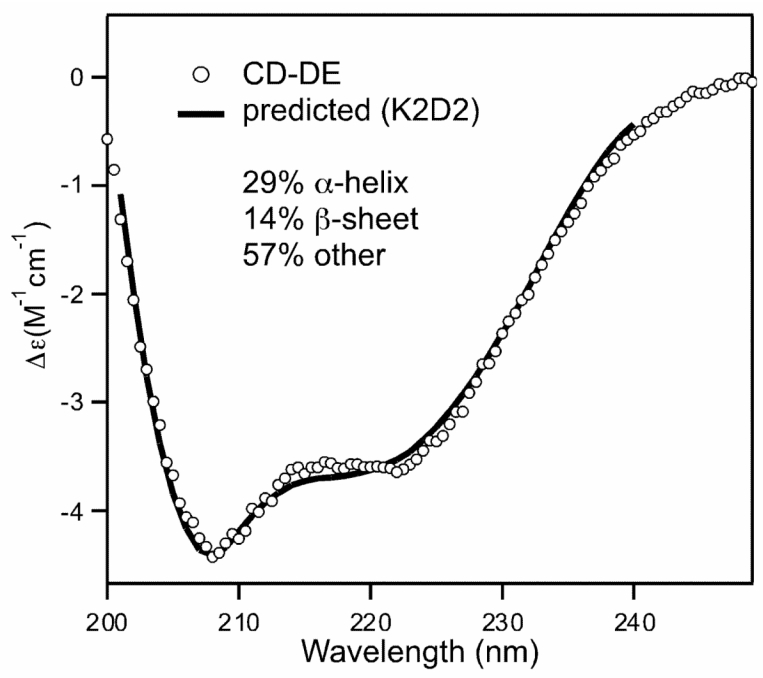

Fig. 3.

Circular dichroism of δAg. The purified protein was analyzed as described in Materials and Methods. The open circles show the data points and the solid line the fit using the K2D2 program (Perez-Iratxeta and Andrade-Navarro, 2008) and the indicated results for α-helix, β-sheet and other (disordered) structures.

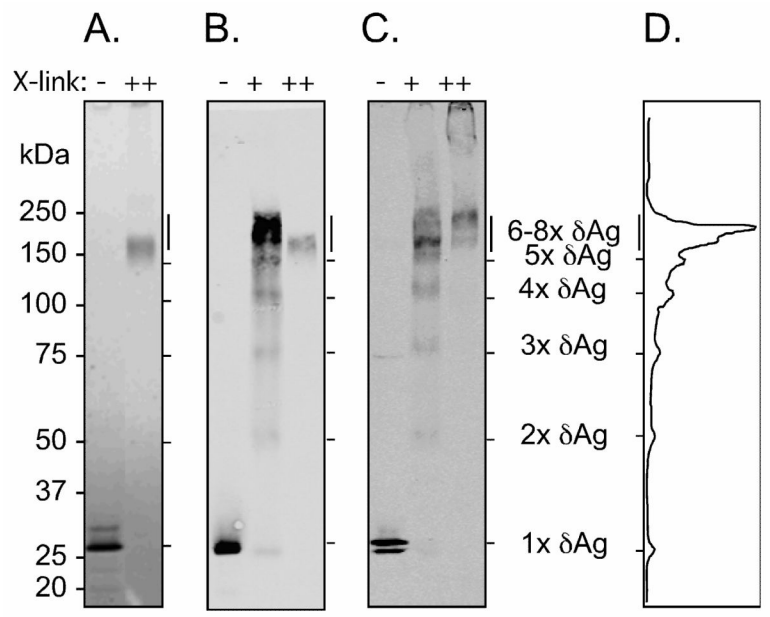

Fig. 4.

Electrophoretic mobility of purified δAg and of δAg assembled into virus-like particles. In panels A and B purified δAg, at 2 μM was treated without or with glutaraldehyde cross-linking, 0.01 or 0.1%, as indicated by + and ++ respectively. This was followed by SDS denaturation and electrophoresis on a gel of 4-12% polyacrylamide. In panel A, total protein was detected by SimplyBlue staining and in panel B, δAg was detected by immunoblot using specific antibody. In panel C, virus-like particles containing δAg, without and with prior cross-linking were examined as in panel B. The minor band migrating faster than the monomer might be a proteolytic fragment. At left are indicated MW markers. Panel D is the quantitation of panel B, lane 2. Indicated an interpretation of detected bands as multimers of δAg.

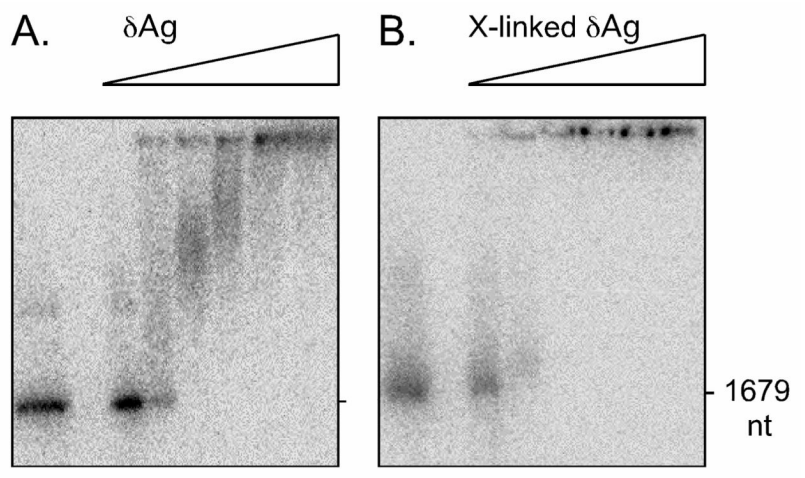

Fig. 5.

Electrophoretic mobility shift of HDV genomic unit-length RNA by δAg. Increasing amounts of δAg, without (panel A) or with (panel B) prior cross-linking with 0.1% glutaraldehyde followed by quenching with ammonium acetate, were incubated with trace amounts of ^{32}P-labeled unit-length linear genomic HDV RNA. Samples were analyzed on non-denaturing gels of 1.5% agarose. After electrophoresis the gel was dried and radioactivity assayed using a bioimager. In each panel the first lane is the control in the absence of δAg. The triangle indicates use of increasing concentrations of δAg: 0.2, 0.4, 0.8, 1.6, 2 and 3.2 μM.

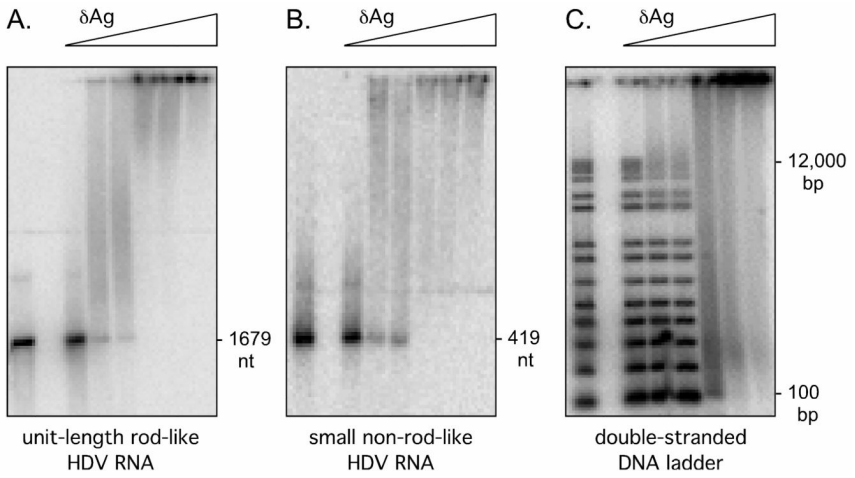

Fig. 6.

Electrophoretic mobility shift of RNA and DNA species by δAg. ^{32}P-labeled RNA species were gel purified and then incubated in the absence or presence of increasing amounts of δAg. Samples were then subjected to electrophoresis under non-denaturing conditions, as in Fig. 5. In panel A the RNA was the 1679 nt unit-length antigenomic linear HDV RNA. In panel B it was a 419 nt species of antigenomic linear HDV RNA, representing the region 714-224, using the numbering of Kuo *et al.* (Kuo et al., 1988), that is predicted to not include the rod-like folding of the RNA. In panel C, we used end-labeled double-stranded DNA with a ladder of sizes from 100 to 12,000 bp. In each panel the left lane is the control in the absence of δAg. The triangles indicate use of increasing concentrations of δAg: 0.2, 0.4, 0.8, 1.6, 2 and 3.2 μM.

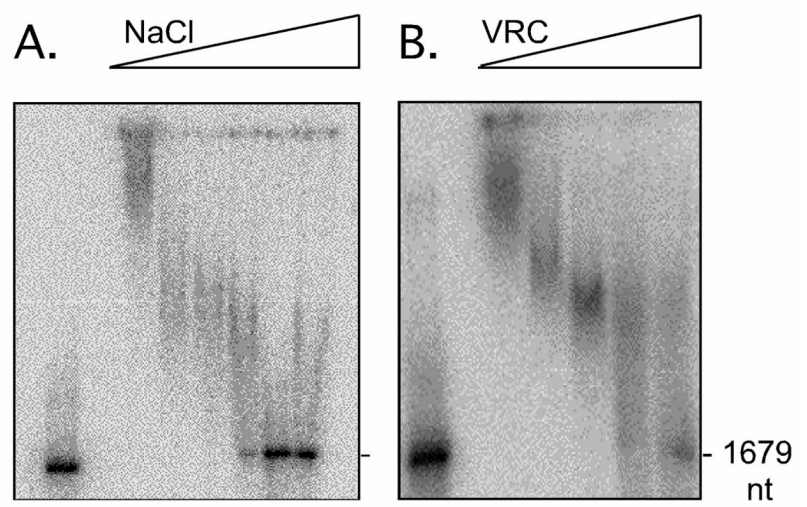

Fig. 7.

Binding of HDV RNA to δAg can be reversed by increasing concentrations of NaCl and VRC. ^{32}P-labeled unit-length genomic HDV was incubated with δAg at 2 μM, and then increasing concentrations of NaCl (panel A) or VRC (panel B), and then analyzed as in Fig. 5. In each case the first lane is the control in the absence of δAg. The triangles indicate use of increasing concentrations of NaCl: 0.15, 0.3, 0.6, 1.2, 2.4, and 3.6 M (panel A) or VRC: 0, 0.3, 1, 3, and 10 mM (panel B).