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Epitopes Exposed on Hepatitis Delta Virus Ribonucleoproteins

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A total of 17 antibodies, raised in several nonhuman species and specific for different regions on the delta antigen (δAg), were used to map, via immunoprecipitation, those domains exposed on the surface of the viral ribonucleoprotein (RNP). These studies showed that the domains for the nuclear localization signal and the C-terminal extension, unique to the large form of δAg, are exposed. Also exposed is the C-terminal region of the small form of δAg. In contrast, reactivity was not found with the coiled-coil domain needed for protein dimerization. When the hepatitis delta virus (HDV) RNA was released by treatment of viral RNP with vanadyl ribonucleoside complexes, no change in the pattern of δAg epitope presentation was detected, consistent with the interpretation that a multimeric protein structure persists in the absence of RNA. These RNP studies have implications not only for understanding of the process of HDV assembly but also for evaluation of the immune responses of an infected host to HDV replication.

During the replication of hepatitis delta virus (HDV), the major proteins produced are the small and large forms of the delta antigen (δAg). These δAgs are 195 and 214 amino acids (aa) long and differ in that the large form has an additional 19 aa at its C terminus (11). In vitro experiments have shown that a shared property of both forms of the δAg is the ability to bind RNA, with a specificity for the rod-like folding of the HDV genome and antigenome (5, 15). Consistent with this binding is the ability to isolate, by a variety of procedures, ribonucleoprotein (RNP) complexes from virions (vRNP) and from the nuclei of infected cells (nRNP) (21). There are two major structural differences between the vRNP and the nRNP. First, the vRNP contains predominantly genomic RNA while the nRNP contains both genomic and antigenomic RNAs. Second, the relative amount of δAg per molecule of HDV RNA is higher for vRNP than for nRNP, upon the basis of equilibrium density sedimentation (21). The aim of the present study was to use a panel of antibodies which recognize epitopes spanning the δAg and determine which regions are exposed on the two forms of RNP and whether or not there are additional structural differences that can be detected. These findings, particularly in terms of the exposed epitopes, have relevance for the understanding of both the steps of HDV assembly and the immune responses of an infected host to HDV replication.

MATERIALS AND METHODS

Plasmids. Construction of plasmid pSVL(D3), containing a replication-competent trimer of HDV cDNA, was reported previously (8). Plasmid pSV45H, carrying the pre-S1-pre-S2-S gene of hepatitis B virus under the control of the simian virus 40 early promoter, was kindly provided by Don Ganem (9).

Viruses. HDV was obtained from an experimentally infected woodchuck. The animal's serum, containing about 1.2×10^{11} virions per ml, was clarified by low-speed centrifugation, followed by pelleting of the virus through a 20% sucrose cushion (Beckman SW27 rotor, 23,000 rpm for 16 h at 4°C). The virus-containing pellet was resuspended in STE buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA), and the virus was pelleted through another 20% sucrose cushion (SW60 rotor, 42,000 rpm for 6 h at 4°C) and resuspended in STE buffer.

Virus-like particles. Preparation of particles secreted by HDV cDNA-transfected Huh7 cells (16) was done essentially as previously described (20), with one alteration: plasmid pSV45H, for expression of all three hepatitis B surface antigen (HBsAg) proteins, was used for cotransfection with HDV cDNA.

Virion RNP. Virions from two sources were used for the preparation of vRNP: either those purified from the serum of infected woodchucks or those secreted by transfected cells. Such virions were disrupted in STE buffer containing 0.5% Nonidet P-40, 10 mM dithiothreitol (DTT), and 1 U of RNasin (Promega) per μl for 15 min at room temperature.

Antibodies. An antibody (rab α-δAg) reactive to both the small and large forms of δAg was raised in a rabbit by inoculation of the small form of δAg prepared in *Escherichia coli*. Production of guinea pig antipeptide sera, rabbit antipeptide sera, and mouse monoclonal antibodies (MAbs) to recombinant δAg was done as previously described (7, 19, 23). MAbs reactive to hepatitis B core antigen (HBcAg) (10C6) and HBsAg (S14) were also described previously (3). S14 was shown to efficiently recognize the nondenatured woodchuck hepatitis virus surface proteins (unpublished observation). MAbs 3.92, 5.60, and 7.79 were produced by using standard procedures and synthetic peptide 12-60(Y), synthesized as previously reported (19).

PEPSCAN analyses. Mapping of the epitopes recognized by MAbs and antipeptide antibodies to δAg was carried out as previously described (24), by using a set of 209 plastic pins on which were synthesized nested hexapeptides overlapping each other by five residues and spanning the entire large δAg protein (residues 1 to 214). Rabbit and guinea pig antisera were tested at a dilution of 1:1,000. Murine MAbs (hybridoma supernatant fluids) were tested at a dilution of 1:250. Pins were considered reactive if they generated an absorbance value (optical density at 405 nm) of greater than 0.7 with guinea pig antisera, 0.45 with rabbit antisera, or 0.2 with murine MAbs. These cutoff values were established from the absorbance obtained with pins bearing peptides outside of the range of the peptide or δAg expression product used to raise antisera.

Immunoprecipitation. M-280 superparamagnetic beads conjugated with streptavidin (Dynal) were loaded with biotin-labeled protein A and then with rabbit or guinea pig antibodies to different epitopes of δAg, as recommended by the manufacturer. Mouse MAbs were bound to the protein A-loaded beads by using rabbit anti-mouse immunoglobulin G (Fc fragment) polyclonal antibodies (Sigma). Beads with antibodies were incubated with vRNP in STE buffer containing 0.5% Nonidet P-40, 10 mM DTT, and 1 U of RNasin per μl for 2 h at room temperature with rocking. After magnetic separation, the beads were washed five times with STE buffer and then the beads and supernatant were immediately subjected to Northern (RNA) blot and immunoblot analyses.

Northern blot analysis. After immunoprecipitation, HDV RNA was extracted from the beads and supernatant by using a sodium dodecyl sulfate-pronase procedure (17). After glyoxalation, the RNA was subjected to electrophoresis in 1.5% agarose gels, followed by RNA blotting to detect genomic HDV RNA sequences. A genomic full-length HDV RNA probe was synthesized in vitro in the presence of [α-^{32}P]UTP and used as previously described (6). Quantitation was achieved by using a Fuji Bio-Imaging system (Fujix BAS1000).

Immunoblot analysis. After immunoprecipitation, both the beads and supernatant were subjected to electrophoresis in 12% polyacrylamide gels by the method of Laemmli (10). After electrotransfer of proteins to a nitrocellulose

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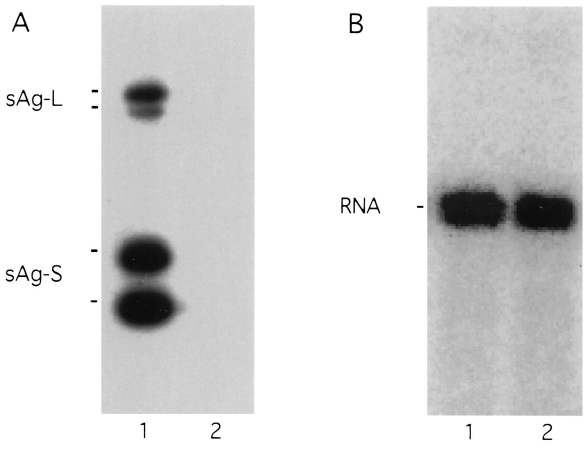

FIG. 1. Disruption of HDV. As discussed in the text, particles were assayed by immunoblotting for human hepatitis B virus surface proteins (A) and by Northern analysis (B) for HDV genomic RNA both before (lane 1) and after (lane 2) disruption and RNP isolation. Viral proteins and RNA are indicated at the left. sAg-L, large surface antigen; sAg-S, small surface antigen.

filter, δAgs were detected with rab α-δAg antibodies and ^{125}I-labeled protein A (Du Pont). Quantitation was achieved with a Fuji Bio-Imaging system (Fujix BAS1000).

Immunofluorescence. Monolayer cultures of Huh7 cells on glass coverslips were transfected with HDV cDNA [pSVL(D3)]. After 6 days, the cells were fixed and stained as previously described (2). Cells were stained by using mouse anti-δAg MAbs, guinea pig antipeptide sera, and rabbit antipeptide sera and corresponding species-specific polyclonal goat anti-immunoglobulin G antibodies conjugated with rhodamine (Sigma). After being mounted, the samples were viewed with a Zeiss Axiophot microscope with a 40× objective and specific filter blocks.

RESULTS

RNP preparation. The following experiments were undertaken to monitor the extent of virus disruption by nonionic detergent and DTT and the ability to recover the released RNP free of associated or contaminating complexes of surface proteins. Virus-like particles secreted by Huh7 cells cotransfected with HDV cDNA [pSVL(D3)] and an HBsAg expression plasmid were collected and partially purified by sedimentation through a 20% sucrose cushion. After disruption with 0.5% Nonidet P-40 and 10 mM DTT for 15 min at room temperature, RNP was sedimented through a 20% sucrose cushion. As shown in Fig. 1, the undisrupted particles (lane 1) and the RNP pellet fraction (lane 2) were assayed by immunoblotting to detect HBsAg (Fig. 1A) and Northern analysis to detect HDV genomic RNA (Fig. 1B). Quantitation of these data showed that <2% of the HBsAg was in the pellet fraction while 96% of the HDV RNA was recovered. Our interpretation is that disruption with Nonidet P-40 and DTT released RNP from associated HBsAg. The same fraction (95%) of HDV RNA was found in RNP after disruption of serum-derived HDV virions under the same conditions.

Immunoprecipitation of vRNP. We next measured the ability of the vRNP to be immunoprecipitated by using 17 antibodies recognizing different epitopes of δAg. The ability of the vRNP to be precipitated was assayed via its two components: HDV genomic RNA and δAg. This quantitation, along with some of the characteristics of the antibodies used, are summarized in Table 1. The four functional regions that have previously been defined for δAg are represented in Fig. 2A, and in parallel with these are definitions of the specificities of the 17 antibodies used (Fig. 2B). As can be seen from Table 1 and as represented in Fig. 2B by the shaded boxes, we found that 9 of the 17 antibodies tested were able to efficiently precipitate the vRNP under our experimental conditions. For five of these nine antibodies, only partial shading is presented to reflect the fact that we were able by PEPSCAN procedures to obtain a more precise delineation of the epitopes recognized (Fig. 3). Such detailed epitope mapping was not obtained for the eight antibodies, shown by open boxes, that failed to immunoprecipitate the vRNP.

In previous studies, it was shown that vanadyl ribonucleoside complexes (VRC) were able to disrupt HDV RNA and δAg in the vRNP (21). Thus, we examined whether the spectrum of epitopes exposed on the vRNP would be changed as a result of VRC treatment. As expected, the results summarized in Table 1 show that the RNA was no longer precipitable by any of the antibodies tested. However, to our surprise, we found that the ability to immunoprecipitate δAg was essentially the same as for the untreated vRNP. In other words, with the antibodies used we were not able to reveal δAg epitopes that were affected by removal of RNA from the vRNP.

As a control for the ability of nonionic detergent plus DTT to disrupt HDV and release the vRNP, we made use of an anti-HBsAg antibody. This antibody was able to precipitate both RNA and δAg before, but not after, disruption of HDV virions (Table 1, antibodies 18 and 19). (The observed relatively low efficiency of virion precipitation by the anti-HBsAg antibody was not due to lack of specificity of the antibody. It was probably due to the presence in our HDV preparation of a large excess of empty surface particles, which compete for the antibody in this assay.) For a negative control, we tested the unrelated antibody 10C6 to HBcAg; this gave a background level of 4% for vRNP immunoprecipitation (Table 1, antibody 20). Finally, as a positive control, we used a rabbit polyclonal antibody raised against the entire δAg; as expected, this gave efficient immunoprecipitation (Table 1, antibody 21).

Antibody specificity. The inability of certain antipeptide sera to immunoprecipitate RNP can have multiple causes. One cause is that the relevant domain is not exposed on the RNP structure. Another cause could be a critical amino acid sequence variation between the peptide used for immunization and the corresponding antigen found in virions. To exclude this second possibility, we used alternative assay procedures to characterize such antibodies. As summarized in Table 1, we confirmed the ability of the antibodies to recognize δAg in an enzyme-linked immunosorbent assay (ELISA), immunoblotting, and immunofluorescence microscopy with Huh7 cells transfected with replicating HDV cDNA (see Materials and Methods).

DISCUSSION

The aim of these studies was to use multiple antibodies with known specificity for regions on the δAg to determine which of these epitopes are exposed on the surface of the HDV RNP. Several controls were used to verify the complete disruption of virions with nonionic detergent plus DTT and the quantitative release and subsequent recovery of the vRNP (Fig. 1 and Table 1); it was shown that after disruption, surface proteins were no longer associated with RNP, so they could not mask any of the δAg epitopes and thereby affect the results of our immunoprecipitation studies. The results of immunoprecipitation studies with 17 different antibodies are summarized in Table 1 and Fig. 2B. (i) Results obtained with antibodies 7 and 8 revealed that the so-called core sequence of the bipartite nuclear localization signal is exposed; this indirectly supports the speculation that in the initial stages of infection, following uncoating, the viral RNA is delivered to the nucleus as an RNP. (ii) Results

TABLE 1. Immune detection of epitopes on vRNP with specific antibodies

Antibody no., designation	Immunogen	PEPSCAN epitope(s)[a]	vRNP immunoprecipitation (%)[b]				ELISA for liver δAg[c]	Immunoblotting[d]	Immunofluorescence[e]
			RNA		δAg				
			−	+	−	+			
1, MAb 4A5	δAg-S[f]	None detected	5	3	3	4	<100	+	+
2, rab 1066	aa 12–60	ND[g]	4	2	2	3	>400	+	−
3, rab 1067	aa 12–60	ND	2	1	2	3	>400	+	−
4, MAb 3.92	aa 12–60	ND	3	ND	2	ND	<100	−	ND
5, MAb 5.60	aa 12–60	ND	3	ND	3	ND	<100	−	ND
6, MAb 7.79	aa 12–60	ND	4	ND	3	ND	<100	−	ND
7, gp3	aa 58–78	aa 58–63, 67–73	67	3	95	90	6,250	+	+
8, gp4	aa 58–78	aa 66–72	65	ND	92	ND	6,250	+	ND
9, gp5	aa 82–102	aa 93–98	58	ND	53	ND	156,000	ND	ND
10, MAb 3G3	δAg-S	None detected	62	ND	36	ND	1,600	+	+
11, gp8	aa 123–143	None detected	9	3	5	5	6,250	−	ND
12, rab-LP2	aa 156–184	aa 156–160, 167–183	96	2	57	60	6,250	+	+
13, gp10	aa 156–184	aa 167–182	73	ND	66	ND	6,250	ND	ND
14, gp12	aa 167–184	ND	56	ND	73	ND	6,250	ND	ND
15, MAb 9E4	δAg-S	None detected	61	3	66	71	<100	+	+
16, MAb 1C4	δAg-S	None detected	9	4	2	3	<100	−	+
17, rab-LP3	aa 197–211	aa 200–210	71	2	47	41	<50	+	+
Controls									
18, MAb S14[h]	HBsAg		14		16				
19, MAb S14	HBsAg		3		4				
20, MAb 10C6	HBcAg		4		4				
21, rab α-δAg	δAg-S		56	2	62	57			

[a] The epitopes indicated were determined by PEPSCAN analysis (see Fig. 3), by using techniques previously described (25). In addition, antibody 1 was strongly reactive in an ELISA with aa 12 to 60 Y. Also, antibodies 10, 15, and 16 were characterized by reactivities in a Western blot (immunoblot) analysis with fragments of δAg. Antibody 10 reacted with aa 89 to 164, and antibodies 15 and 16 reacted with aa 164 to 195 (7).

[b] Each vRNP sample was tested both before (−) and after (+) treatment with vanadyl ribonucleosides (10 mM). We estimated that a value of <5% represents the background and a value of >10% is positive.

[c] Most of these values were as previously reported (23), except those for antibodies 1, 10, 15, and 16, which were subsequently derived by the same method (23), and those for antibodies 2 to 6, which were assayed with an Abbott Delta-EIA diagnostic kit.

[d] The immunoblot data are results either from this study (antibodies 2, 3, 7, 8, and 11) or from reports by Wang et al. (23) and Hwang and Lai (7).

[e] The immunofluorescence data are results either from this study (antibodies 2, 3, 7, 12, and 17) or from a report by Hwang and Lai (7).

[f] δAg-S, small δAg.

[g] ND, not done.

[h] This control was done with intact HDV rather than isolated vRNP.

obtained with antibody 17 indicated that the C-terminal extension unique to the large δAg is exposed; this is consistent with the essential role of this region in virus assembly, presumably by promotion of an interaction with the envelope proteins of the helper virus (4, 20). (iii) Four of five antibodies, 12 to 15, specific for the C-terminal region of the small δAg were also reactive; it has been speculated that this region is an assembly domain (13); however, more recent studies argue that the 19-aa C-terminal extension on the large δAg is both necessary and sufficient for assembly into particles of individual protein molecules (14). Our studies reopen the possibility that for RNP, this region is exposed and thus might have a role in some process, such as assembly, which involves not just individual proteins but RNP. (iv) Antibody 9 interacted with the region adjacent to one side of the bipartite RNA-binding domain (15). Antibody 10 was also found to be reactive, but lack of epitope mapping data makes this result less informative. The antibody failed to react with any of the synthetic peptides utilized in the PEPSCAN or peptide ELISA; thus, it remains unclear whether the corresponding epitope resides with the RNA-binding domain or is adjacent to it, as with the epitope for antibody 9. Antibody 11 failed to bind to the RNA-binding domain. Taken together, these studies are not sufficient to conclude whether or not the RNA-binding domain is exposed. (v) Finally, six antibodies directed against the coiled-coil domain, which is necessary and sufficient for dimerization and oligomerization (12, 25, 26), failed to recognize the RNP; these results support but do not prove the interpretation that the coiled coil is not exposed, for example, because it is involved in intermolecular δAg interactions essential for the structure of the RNP.

There was a discordance between peptide ELISA values and vRNP and δAg precipitation results obtained with some antibodies. Thus, antibodies 15 and 17 (which were both raised against determinants located in the carboxy third of δAg) were nonreactive by ELISA but demonstrated high activity in vRNP and δAg precipitation assays. On the other hand, polyclonal antibody 11 (raised against a peptide representing residues 123 to 143) was moderately reactive by ELISA but nonreactive in the immunoprecipitation assays. This suggests a possible difference in physical structure between the δAg utilized in the ELISAs and that present in immunoprecipitation assays, with resulting differences in the accessibility of the relevant epitopes. This may reflect the fact that the liver-derived δAg utilized in the ELISA was extracted from infected tissue under denaturing conditions (6 M guanidine-HCl) followed by dialysis against phosphate-buffered saline (24).

Our previous studies showed that treatment of vRNP with VRC led to the release of RNA (21). We confirmed this and found, in addition, that the constellation of exposed and unexposed δAg epitopes was not detectably changed by treatment with VRC (Table 1). Apparently, the δAg multimer was unchanged by the release of the RNA. However, this interpretation must be qualified in that separate studies have indicated

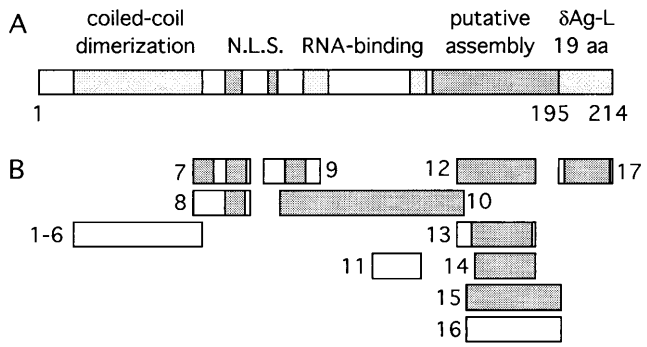

FIG. 2. Functional domains on δAgs and epitopes exposed on RNPs. (A) Summary of the functional domains, as discussed in the text, that have been mapped on the small and large forms of δAg (11, 13). δAg-L, large δAg. (B) Specificities of the 17 antibodies, as described in Table 1, that were tested for the ability to immunoprecipitate the HDV vRNP. The open boxes represent antibodies that failed to bind vRNP. The shaded boxes represent antibodies that were positive for binding. The partially shaded boxes represent not only antibodies that were positive for binding but also those for which the relevant epitopes were further defined by PEPSCAN analyses, as illustrated in Fig. 3. N.L.S., nuclear localization signal.

that the small and large forms of δAg can assemble into homo- and heterodimers and higher-ordered multimers (12, 19, 24, 25). It is thus possible that individual δAg molecules within dimers or multimers have different contact points with the viral RNA and that an epitope recognized by an antibody can be in contact with the RNA in one molecule but available for antibody binding and immunoprecipitation in its dimerization partner. This may partially explain the inability to unmask epitopes of δAg following release of RNA from vRNP complexes by treatment with VRC.

We were able to extend our results obtained with the vRNP isolated from virions to the nRNP isolated from the nuclei of transfected cells (Table 1 and Fig. 2B). In terms of the epitopes exposed to the antibodies used in this study and in terms of the consequences of treatment with VRC, there was no significant difference (unpublished observations). Apparently, the structural differences between vRNP and nRNP, as mentioned in the introduction, do not extend to δAg epitope presentation.

Some comment must be made about the relationship between the epitopes found in this study to be exposed on the RNP (Fig. 2 and Table 1) and previous reports of δAg domains found to be immunodominant in the course of natural and experimental HDV infections. Four such studies have been reported (1, 18, 22, 24), and the predominant B-cell epitopes were mapped to four regions, three of which correspond to the nuclear localization signal, the putative assembly domain, and the 19-aa C-terminal extension unique to the large δAg, all of which were found to be exposed in our study. The fourth domain, at the N terminus, aa 2 to 17, was also tested and found to be negative, but corresponding antipeptide antibody gp2 failed to recognize δAg in ELISA, immunoprecipitation, immunoblotting, and immunofluorescence microscopy (23 and unpublished observations). Therefore, this antibody was considered to be specific for the peptide but not for the δAg protein and no conclusions could be drawn about the exposure of the domain between aa 2 and 17.

In conclusion, the studies described here have provided a better understanding of the structure of the two RNPs of HDV. The immunological data obtained will be useful in future studies of HDV genome replication and also in studies of

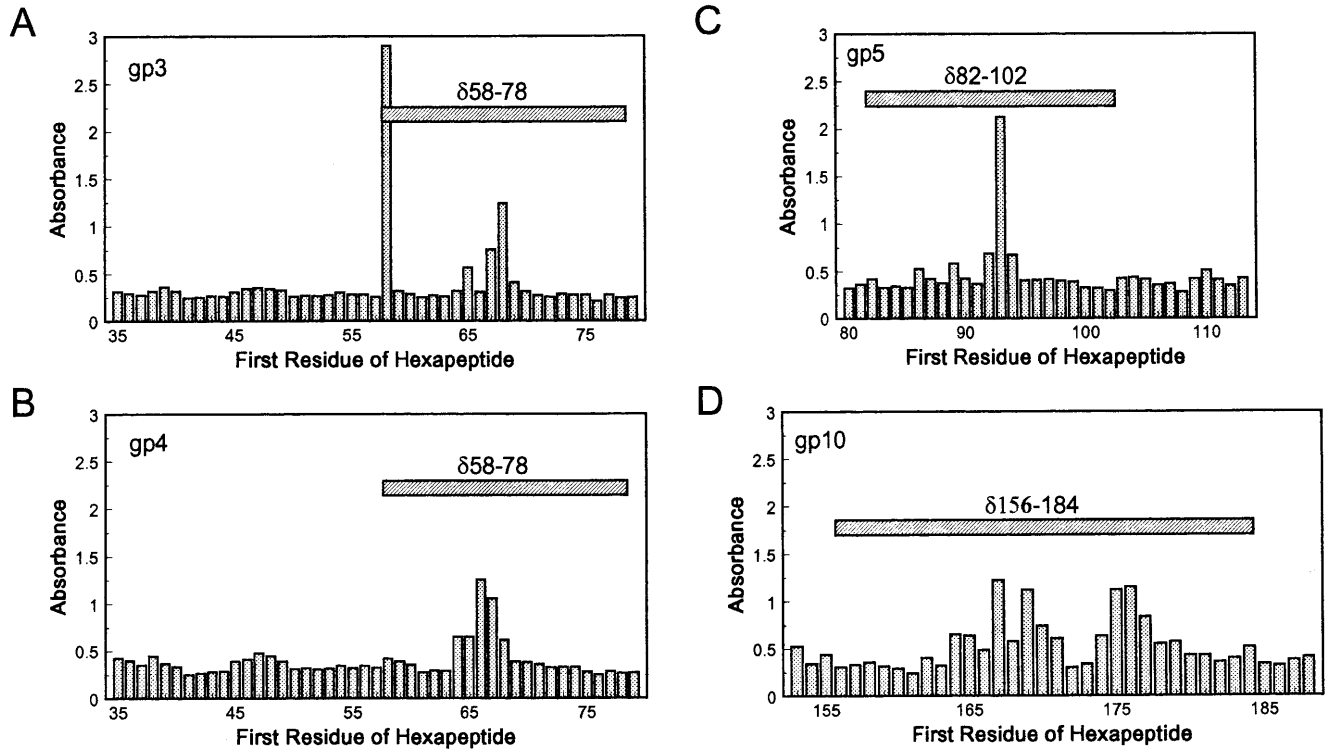

FIG. 3. PEPSCAN analyses to define epitopes recognized by representative guinea pig antisera. Vertical bars indicate the absorbance obtained with each individual pin (hexapeptide). The pins are labeled numerically according to the first amino acid residue of the synthetic hexapeptide. The horizontal bars indicate the locations of the synthetic peptides used as immunogens for the antibodies being tested within the δAg domains scanned in the assays.

the ability of RNPs to interact with the envelope proteins of the helper virus to achieve assembly and release of progeny virus particles.

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