Tree squirrel hepatitis B virus: Antigenic and structural characterization

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ABSTRACT

Tree squirrel hepatitis B virus (THBV)-associated particles isolated from the livers of naturally infected animals share one or more antigenic determinants with hepatitis B surface antigen in solid-phase immunoassays. Characterization of THBV-associated polypeptides by sodium dodecyl sulfate/polyacrylamide gel electrophoresis reproducibly demonstrated major components with apparent sizes of 15.5 and 17 kDa. Peptide mapping of these components shows that they are related to the peptide maps of the major surface antigen polypeptides associated with hepatitis B virus and like viruses. Sodium dodecyl sulfate/polyacrylamide gel analysis also demonstrated discrete bands at 14.5, 19, 20, and 35 kDa. Upon blotting of THBV-associated polypeptides with sera containing antibodies to hepatitis B core antigen or hepatitis B x antigen, only the 35-kDa band became detectable, suggesting that this component is core related. These results establish the presence of both surface and core antigen-related polypeptides associated with purified THBV and better define the relationship of THBV to the family of hepatitis B virus and like viruses.

Recently the existence of another member of the hepatitis B virus (HBV) class has been documented in tree squirrels (Sciurus carolinensis pennsylvanicus) having histological evidence of hepatitis (1). Although viremia or antigenemia was not detected, some of the squirrel sera tested bound hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBCAg), or both, suggesting the presence of cross-reacting antibodies in many sera, and by implication, a high rate of infection. Liver extracts from three animals with hepatitis possessed surface and core antigen activities that purified with DNA polymerase-positive virus-associated particles (1). This study presents antigenic cross-reactivity between HBsAg and tree squirrel hepatitis B virus (THBV) measured with several sera. The THBV-associated polypeptides are enumerated by NaDdSO4/PAGE, and a number of bands are further characterized by a combination of protein blotting and tryptic peptide mapping. By these techniques, the relationship between THBV and the hepatitis B family of viruses has become more firmly established.

MATERIALS AND METHODS

Animal Studies. Animal collection and the acquisition of sera and livers have recently been described (1). THBV Isolation. Isolation of THBV-associated particles for these studies included preparation of a crude liver extract, followed by equilibrium density gradient centrifugation in CsCl and finally by rate zonal sedimentation in CsCl exactly as described (1, 2). THBV-positive fractions were detected by testing appropriate samples in the Auszyme and DNA polymerase assays (1, 3). Purity was assessed by electron microscopy.

Cross-Reactivity Assays. To determine whether THBV and HBsAg particles share one or more antigenic determinants, the binding of THBV by sera containing antibodies to HBsAg (anti-HBs) and the binding of HBsAg by sera containing anti-THBV were tested. Immulon 2 Removawells (Dynatech, Alexandria, VA) were coated with increasing dilutions of sera containing anti-HBs or with identical dilutions of sera from THBV-positive squirrels. All washes and dilutions were carried out in phosphate-buffered saline, pH 7.3 (1), containing 10% fetal calf serum. After overnight adsorption at 4°C, the wells were washed six times in the phosphate-buffered saline/fetal calf serum, and 1 μg of either purified HBsAg (4, 5) or THBV was added per well. After overnight binding at 4°C, the wells were washed and then incubated 1 hr at 37°C with goat anti-HBs conjugated to horseradish peroxidase, obtained directly from the Auszyme II kit (Abbott, North Chicago, IL). After washing, binding was detected colorimetrically by addition of o-phenylenediamine (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

NaDdSO4/PAGE and Silver Staining of THBV-Associated Polypeptides. Purified THBV-associated particles obtained by rate zonal sedimentation were used for polypeptide characterization by NaDdSO4/PAGE. Samples of THBV material were dialyzed in colloidon bags (25-kDa cutoff; Schleicher & Schuell) against 0.1 M sodium borate, pH 8.2, containing 0.1% NaDdSO4. After three changes of buffer within 24 hr, the samples were lyophilized, reduced with dithiothreitol, and alkylated with iodoacetamide (4, 5). NaDdSO4/PAGE slab gels were run (5, 6) and individual components were visualized by silver staining (7).

Protein Blotting. Reduced and alkylated THBV separated by NaDdSO4/PAGE into individual polypeptides was electrophoretically transferred to nitrocellulose sheets (BA85, Schleicher & Schuell) as described by Towbin et al. (8) and blotted with two different antibodies. Antiserum made in sheep against Escherichia coli-produced HBCAg (a kind gift from Wolfram Gerlich) and having an ELISA titer of 1:320,000 was used diluted at 1:10,000 in phosphate-buffered saline/fetal calf serum for blotting. After the nitrocellulose had been washed as described (9), horseradish peroxidase-conjugated rabbit anti-sheep immunoglobulin (Cappel Laboratories, Cochrantville, PA) was added at a dilution of 1:100 in phosphate-buffered saline/fetal calf serum and incubated for 1 hr at 37°C. After washing, binding was detected by addition of 4-chloro-1-naphthol substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Nitrocellulose

Abbreviations: HBV, hepatitis B virus; THBV, tree squirrel hepatitis B virus; DHBV, duck hepatitis B virus; HBsAg, hepatitis B surface antigen; HBCAg, hepatitis B core antigen; GSHsAg, ground squirrel hepatitis surface antigen; WHsAg, woodchuck hepatitis surface antigen; DHBsAg, duck hepatitis B surface antigen; anti-HBs, antibodies to HBsAg.

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sheets were also blotted with rabbit sera containing antibodies against two synthetic peptides derived from the X gene region of HBV (a gift from Richard Lerner) (9). After washing, a 1:100 dilution of goat anti-rabbit immunoglobulin was added, and binding was detected by addition of substrate as outlined above. For the blocking assay, 200 μg of the appropriate X gene-derived synthetic peptide was incubated for 1 hr with corresponding antiserum and the mix was then added to the blot as described above.

\[125I] Labeling of THBV-Associated Polypeptides and Tryptic Peptide Mapping. Purified THBV dialyzed and lyophilized as outlined above was labeled with the Bolton–Hunter reagent (5, 10). The polypeptides were separated by NaDodSO4/PAGE, the gel was dried under reduced pressure, and the profiles were determined by screen-intensified autoradiography at −70°C. \[125I]-labeled polypeptides were further characterized by two-dimensional tryptic peptide mapping (4, 5, 11). Peptides were mapped by screen-intensified autoradiographic exposure at −70°C.

RESULTS

Antigenic Relatedness Among THBV and HBsAg Particles. The antigenic relationship between purified THBV and HBsAg was studied by using solid-phase assays in which the binding of THBV to several anti-HBs-containing sera and the binding of HBsAg to tree squirrel sera were tested. As shown in Fig. 1A, serum from an animal positive for THBV (308960) bound THBV-associated particles in several 5-fold dilutions. An equivalent amount of HBsAg did not bind, suggesting the presence of antigenic determinants on THBV that are not present on HBsAg. Using a serum from another infected tree squirrel (309017) (Fig. 1B) demonstrated the presence of one or more cross-reacting antigenic determinants. A serum derived from a THBV-negative animal free of hepatitis (309009) bound neither antigen (Fig. 1C). THBV-associated particles bound to an anti-HBs-containing serum derived from a chronic human carrier (Fig. 1D) as well as to guinea pig anti-HBsAg serum (Fig. 1E) but not to a pooled normal human serum (Fig. 1F). These results are consistent with the presence of both shared and unique antigenic determinants associated with THBV compared with HBsAg.

The presence of antigenic relatedness between THBV and HBV shown above and in previous work (1) suggests that structural relatedness might also exist. Accordingly, THBV-associated polypeptides were analyzed by NaDodSO4/PAGE and detected by silver staining (Fig. 2). Two major bands, at 15.5 and 17 kDa (p15.5 and p17, respectively), were observed on all gels. On some gels another major band at 14.5 kDa (p14.5) was also observed. Discrete minor bands at 19 (p19), 20 (p20), and 35 (p35) kDa, the latter of which was the most prevalent of the three, were observed on most polypeptide profiles. Since the number and size of bands associated with purified THBV were unlike the polypeptide profiles of HBsAg (Fig. 2) or HbcAg (2), further characterization of these bands was carried out by using protein blotting and tryptic peptide mapping.

Protein Blotting. The reactivity of one or more THBV-associated polypeptides presented in Fig. 2 was assayed with antiseras to surface or core antigens by protein blotting. Antiserum raised against a synthetic peptide derived from the S gene, spanning amino acid residues 48–81 (a gift from Richard Lerner), bound individual HBsAg-associated polypeptides but not THBV-associated components (data not shown). A similar experiment in which sera containing antibodies capable of detecting duck hepatitis B surface antigen (DHBsAg) p18.5 by protein blotting also failed to detect any THBV-associated components (data not shown). However, when an antiserum derived from sheep immunized with an E. coli-produced HBcAg was used for blotting, a single band of 35 kDa showed reactivity (Fig. 3). The recent finding of X gene-derived antigenic determinants in core-associated polypeptides (9) suggests that if p35 is a core-related protein, then it may bind antibodies made against HBV gene X determinants. Lane C of Fig. 3 demonstrates that this is the case and that the reactivity is blocked by incubation of antiserum with the appropriate synthetic peptide (Fig. 3, lane D). Together these results suggest that the

![Fig. 1. Antigenic determinants shared among THBV and HBsAg were determined by assaying the binding of purified HBsAg or THBV particles to anti-THBV-containing sera from infected tree squirrels and to anti-HBs-containing sera. Starting with undiluted sera, each succeeding assay well was coated with 5-fold dilutions (1/5, 1/25, 1/125, and 1/625) of serum. Serum from one THBV-positive animal (308960) was used in A and that from another positive animal (309017) was used in B. Serum from an apparently uninfected animal (309009) was used in C. Serum from a human carrier (negative for HBsAg) containing anti-HBs was used in D. Guinea pig anti-HBsAg was used in E, and pooled normal human serum (Sigma) was used in F. Quantitation of binding was obtained by optical density measurements at 492 nm. The positive-to-negative (P/N) ratios plotted reflect the quantitative binding of antigen in panels A or B compared to panel C for tree squirrel sera, the binding in D compared to that in F for human sera, and the binding in E compared to that with a normal guinea pig serum sample. The binding of purified antigens to wells coated with normal sera or with only the diluent (phosphate-buffered saline/fetal calf serum) resulted in similar optical density readings and P/N ratios less than 2. ●, THBV binding; ○, HBsAg binding.](image-url)
THBV-associated component, p35, is a core-related polypeptide.

**Tryptic Peptide Mapping.** Purified THBV was dialyzed into borate buffer containing NaDodSO₄, lyophilized, and radio-labeled with the Bolton–Hunter reagent (4, 5, 10). After NaDodSO₄/PAGE, peptide maps were successfully generated from THBV p14.5, p15.5, and p17. Each of these polypeptides yielded peptide maps sharing at least 80% homology, suggesting that each of these components was closely related to each other (unpublished data). Since p15.5 was the dominant band by silver staining as well as 125I labeling on every gel, its peptide map was used for comparison with all others, as shown in Fig. 4 and summarized in Table 1. When the peptide map of HBsAg p25 (Fig. 4A) was compared to that of THBV p15.5 (Fig. 4B) by resolving digests of each on a single thin-layer plate (Fig. 4C), many shared spots were observed. These are diagrammed in Fig. 4D. Twenty-six percent of the spots on the tryptic map of THBV p15.5 from animal 3008957 were shared with HBsAg p25 isolated from a human chronic carrier. In the reciprocal comparison, 39% of the spots on the tryptic map of HBsAg p25 were also present on the map of THBV p15.5. The results of other peptide mapping comparisons are summarized in Table 1 and demonstrate that THBV p15.5 from two different livers shares some homology with the corresponding major surface antigen polypeptides of the HBV-like family of viruses. Comparison of THBV p15.5 from two different livers revealed homology characteristic of HBsAg subtypes (5). Together these results suggest that THBV p15.5 and related polypeptides (p14.5 and p17) are surface antigen related.

**DISCUSSION**

The results of this study show that purified THBV particles share a number of antigenic and structural characteristics with HBV and related viruses. For example, the detection of THBV in the Auszyme II and Auszyme assays shows that THBV, like GSHsAg and WHsAg determinants, cross-reacts with reagents that test for the presence of HBsAg (refs. 1, 4, 12, and 13; Fig. 1). Since HBsAg, WHsAg, and GSHsAg each contain a major polypeptide pair between 20 and 30 kDa by NaDodSO₄/PAGE analysis (4, 5, 14, 15), it seemed likely that THBV-associated particles, also derived from a mammalian host, would share this characteristic. Surprisingly, NaDodSO₄/PAGE analysis of THBV-associated components resulted in the appearance of major bands at 14.5, 15.5, and 17 kDa instead (Fig. 2). Subsequent peptide mapping showed these major components to be closely related to the major surface antigen components of HBV and related viruses, strongly suggesting these are surface antigen-related polypeptides. Although the size range of these major THBV-associated components was similar to that of the major DHBsAg polypeptide (12, 14), comparative peptide mapping did not demonstrate a closer relationship between THBV p15.5 and DHBsAg p18.5 than between THBV p15.5 and the major surface antigen polypeptide of HBsAg or other related viruses (Table 1). Together these results are also consistent with the presence of a THBV surface antigen gene which is truncated compared to the corresponding gene in human, ground squirrel, and woodchuck hepatitis viruses. Although the S gene of duck hepatitis B virus (DHBV) is also truncated, any deletion in the THBV S gene relative to HBV must be different from that in DHBV, since there are shared antigenic determinant(s) among THBV and HBsAg but none thus far reported among DHBsAg and HBsAg.
The binding of THBV-associated p35 to two different antibodies capable of signaling the presence of HBcAg-associated polypeptides in a protein blot assay suggests that this component is core related (Fig. 3). These results are consistent with those recently obtained with similarly assayed core particles from HBV and related viruses, namely, that core-associated polypeptides specifically bind antibodies raised against synthetic peptides derived from the X region of HBV DNA (9). The phosphorylation of the major HBcAg-, GSHcAg-, and DHBCAg-associated polypeptides in the in vitro protein kinase reaction (16–18) is also found with the major core-associated polypeptide, p35, of THBV (unpublished data). The fact that surface antigen polypeptides are not phosphorylated in Dane particles (16) and that THBV surface antigen components p14.5, p15.5, and p17 do not become phosphorylated in this reaction suggests that the protein kinase activity in THBV is endogenous, just as with HBV (17). Although THBV is a HBV-like virus naturally infecting a mammalian host, the detection of a single core-associated polypeptide at approximately 35 kDa is similar to what is observed with DHBV (19). Consequently, the genome organization of THBV resulting in core gene expression may be similar to that of the DHBV genome. THBV DNA cloning and nucleotide sequencing should allow us to determine the number and size of open reading frames in the genome of this HBV-like virus.

Table 1. Tryptic peptide mapping comparisons among THBV isolates and surface antigen particles

<table>
<thead>
<tr>
<th>Comparison</th>
<th>% homology*</th>
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<tbody>
<tr>
<td>308960 p15.5 vs HBsAg ( adr) p25 isolates</td>
<td>37</td>
</tr>
<tr>
<td>308957 p15.5 vs HBsAg (adr) p25 isolates</td>
<td>39</td>
</tr>
<tr>
<td>308960 p15.5 vs GSHsAg p22 isolates</td>
<td>41</td>
</tr>
<tr>
<td>308957 p15.5 vs GSHsAg p22 isolates</td>
<td>42</td>
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<tr>
<td>308960 p15.5 vs WHsAg p22 isolates</td>
<td>43</td>
</tr>
<tr>
<td>308957 p15.5 vs WHsAg p22 isolates</td>
<td>34</td>
</tr>
<tr>
<td>308960 p15.5 vs DHBSAg p18.5 isolates</td>
<td>47</td>
</tr>
<tr>
<td>308957 p15.5 vs DHBSAg p18.5 isolates</td>
<td>50</td>
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<td>308960 p15.5 vs 308957 p15.5</td>
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</tr>
<tr>
<td>308960 p15.5 vs 308957 p15.5</td>
<td>36</td>
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The THBV major polypeptides, p15.5, from animals 308960 and 308957 are compared with each other and with the major surface antigen component from HBV, ground squirrel hepatitis virus, woodchuck hepatitis virus, and duck hepatitis B virus. Comparisons are made based upon at least two independent maps of each polypeptide digest and upon mixing experiments in most cases. The variability in percent homology in a given comparison due to iodination or two-dimensional gel separation is ±4% (5). ND, not determined.

*The left and right columns show homology values based upon comparison of the appropriate THBV p15.5 with related surface antigen particles from two different sources. For example, the percent homology between 308960 p15.5 and HBsAg (adr) p25 from one source is 37% of the HBsAg spots, while comparison of the same squirrel antigen map with HBsAg (adr) p25 from another source revealed 56% homology.

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