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Activation of the Envelope Proteins by a Metalloproteinase Enables Attachment and Entry of the Hepatitis B Virus into T-Lymphocyte

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Previously, we identified an HBV binding factor (HBV-BF), a 50-kDa serum glycoprotein which interacts with HBV envelope proteins and which is also located in the membrane of normal human hepatocyte (A. Budkowska *et al.* (1993) *J. Virol.* 67, 4316). Here we show that HBV-BF is a neutral metalloproteinase which shares substrate specificity and properties with a newly described family of membrane type matrix metalloproteinases. HBV-BF treatment of the HBV resulted in the cleavage of the N-terminal part of the middle HBV envelope protein at the pre-S2(136–141) amino acid sequence VRGLYF/L (containing a single arginine cleavage site). HBV-BF affected the reactivity of the large HBV protein with pre-S1-specific MAbs, probably inducing the conformational change of the pre-S1 domain. The HBV-BF-digested virus remained morphologically intact with unchanged S antigenic determinants. The structural modifications of the viral envelope proteins induced by HBV-BF enabled cell membrane attachment and viral entry into the T-lymphocyte. Both processes were blocked by the metalloproteinase inhibitor 1,10 phenanthroline. Thus, the host-dependent proteolytic activation of the envelope proteins seems to be essential for the HBV entry into the cell. HBV-BF under a membrane bound or a secreted form could be (one of) the molecule(s) responsible for the HBV proteolytic activation.

INTRODUCTION

The entry of enveloped viruses into their target cells requires the binding of the virus to one or more cellular receptor(s) and the fusion of the envelope with the target cell membrane. In some cases the same molecule responsible for virus attachment can also mediate internalization. For other viruses such as Sendai virus, influenza A virus, Newcastle disease virus, murine coronavirus, human immunodefficiency virus type 1, and rotaviruses (*14, 26*) proteolytic cleavage of viral surface glycoproteins by host cell proteases is necessary for viral infection. The cleavage results in the exposure of a hydrophobic stretch of amino acids which acts as the mediator of membrane fusion and determines tissue tropism of these viruses, especially if the virus utilizes ubiquitous molecules as receptors.

Hepatitis B virus (HBV) is a member of the hepadnavirus family. It is composed of a 3.2-kb DNA genome, a nucleocapsid core and a viral envelope, containing three coterminal viral glycoproteins termed large (L), medium (M), and small (S). These HBV surface proteins are encoded by a single open reading frame — gene S — composed of the pre-S1, pre-S2, and S regions with alternative translational initiation sites. The domains of the M and L proteins, encoded by the pre-S1 and pre-S2 fragments of the viral genome, are located at the surface of virions and of subviral particles. They may act as viral attachment proteins in the interaction of HBV with the membrane receptor(s) on the surface of the hepatocyte (*8, 9, 28*). The S protein is probably fusogenic (*37*).

The early stages of the HBV infection remain unknown due to the failure to cultivate HBV *in vitro.* The major target organ of HBV is the liver. Hepatocytes are apparently the only cells susceptible and permissive to infection, although viral antigens and DNA have been detected in many extrahepatic tissues. Cellular components involved in the entry of HBV into its target cell have not been identified and little is known about the biological bases of its restricted host range and tissue tropism. Various molecules have been described as candidates for HBV receptors (*7, 12, 28, 30, 42*); however, there is no evidence that a particular component mediates productive infection. Recent data have shown that peripheral mononuclear cells of HBV-infected patients exhibit adsorbed viral particles but not the replicating virus (*15*), thus raising the question of the presence of HBV receptors on human lymphoid cells and the possibility of the HBV entry into this type of cell.

We have described in human serum a 50-kDa molecule, called "HBV-binding factor" (HBV-BF) which interacts with the pre-S1 and pre-S2 epitopes of the viral envelope (*5*). The immunochemical properties of this molecule, its reactivity with the putative viral attachment pro-

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teins, and the localization of an antigenically related component in the membrane of the human hepatocyte suggested that HBV-BF may be involved in the HBV entry into the cell. Here we show that HBV-BF displays the properties of a member of recently described membrane-type matrix metalloproteinase family (*38*) and induces structural modifications of the viral envelope enabling attachment and internalization of HBV into the T-lymphocyte cell line.

The proteolytic activation of the envelope proteins with an enzyme identical or related to HBV-BF seems to be required for HBV entry into the cell. Proteolysis which triggers a conformational change of the HBV envelope might induce the exposure of the fusogenic sequence necessary for the fusion of the viral envelope with the cell plasma membrane. Thus, the presence of this type of enzyme in the active form in the membrane or in the vicinity of the target cell could be determinant for this process.

MATERIAL AND METHODS

Cell culture

The human T lymphoblastoma cell line CEM was cultured in a RPMI 1640 medium (Gibco BRL) supplemented with 10% of fetal bovine serum, 2 m*M* L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The medium was changed three times a week.

HBV and recombinant HBsAg particles

HBV particles were purified from the plasma of HBV carriers by a sequence of ultracentrifugations as previously described (*5*). Recombinant HBsAg particles (m-HBsAg) were produced in Chinese hamster DHFR$^-$ ovary cells transfected with a plasmid encoding the pre-S region of the S gene of HBV and murine dihydrofolate reductase (*23*). These particles contained three polypeptides of a molecular mass of 22, 26, and 43 kDa corresponding to the small (24 and 26 kDa) and the middle (34 kDa) envelope proteins. Polypeptides of 26 and 34 kDa were glycosylated. Recombinant m-HBsAg particles were purified by the producer and were kindly provided by R. Vinas (Pasteur Mérieux).

Purification of HBV-BF

HBV-BF was purified from human serum as previously described (*5*). Globulins were precipitated from serum with 50% ammonium sulfate, followed by ion exchange chromatography (DE 52, Whatman) and gel permeation with a series of three Sephacryl S200 columns (Pharmacia, Sweden). The final preparation was incubated with insoluble rabbit antibodies to human IgG to remove contaminating IgG or its fragments.

The level of purity of HBV-BF was determined using high-performance liquid chromatography (HPLC) with a Superose 12 column (Pharmacia) and by SDS–PAGE. As previously described (*5*) the purified HBV-BF was eluted from the HPLC column as a single peak of activity of a molecular mass of 50 kDa, corresponding to a single peak of protein (O.D. 0.2×10^{-3}). The trace amounts of protein components of a molecular mass of 10 kDa or less were eluted after HBV-BF peak and did not show any reactivity with HBV envelope. No bands or only a weak 50-kDa protein band could be detected in the purified HBV-BF preparation after SDS–PAGE under nonreducing conditions followed by a silver staining.

Determination of the inhibitory effect of HBV-BF on the binding of MAbs to the HBV envelope proteins

Inhibition assays for detection of HBV-BF were carried out as previously described (*5*). The assays were based on the inhibition by HBV-BF of the binding of horseradish (HRPO)-labeled MAbs to the pre-S1, pre-S2, and S region encoded epitopes on HBV or on recombinant m-HBsAg particles. The wells of microtiter plates (Nunc, Roskilde, Denmark) were coated with HBV or m-HBsAg particles at a concentration of 1 μg of protein per milliliter. The plates were overcoated with 1% bovine serum albumin–0.05% Tween 20 in phosphate-buffered saline. Samples tested for HBV-BF activity were incubated for 2 hr at 37° on HBV or m-HBsAg-coated plates and after washing the wells were incubated with HRPO-labeled MAbs and then with *o*-phenylenediamine as a substrate. MAbs used in the assay and their epitope specificity are detailed in Table 1. MAbs Ma18/7 (*39*) and E21/14 (*22*) were kindly provided by W. H. Gerlich, MAbs F124 (*4*), F376 (*6, 26*) 39-10 (*6*), and 5a-19 (*6*) were produced in our laboratory, MAbs 144 and 112 were kindly provided by J. P. Bourgeois (Sanofi Diagnostics Pasteur) and MAb E6 (*22*) by B. Porstmann.

The absorbance (*A*) values at 492 nm were measured by ELISA in the linear range of the assay and the results were expressed as percentage inhibition calculated as follows:

$$\text{Percentage inhibition} = \frac{\text{A negative control} - \text{A test sample}}{\text{A negative control} - \text{A positive control}} \times 100$$

The negative controls were at the absorbance values 1.6–1.9 and positive controls at 0.05 to 0.2.

Biomolecular interaction analysis (BIA core) of the HBV-BF activity

HBV or recombinant m-HBsAg particles were immobilized on the sensor chip CM5 directly or by the intermediate of 39-10 MAb anti-HBs using the procedure described by Jonsson *et al.* (*13*). The carboxylated dextran matrix of the sensor chip was activated using *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(3-diethylaminopropyl)

carbodiimide (EDC) (Pharmacia Biosensor, Uppsala, Sweden). The ligand − m-HBsAg particles (30 μl) at a concentration of 100 μg/ml in a buffer, pH 5.0, were injected followed by 30 μl of 1 *M* ethanolamine hydrochloride at pH 8.5, to deactivate the unreacted groups. Subsequently, 25 μl of the purified preparation of HBV-BF in PBS were injected over the surface of the sensor chips. Any change in the surface concentration resulting from the interaction of HBV-BF with HBV or recombinant m-HBsAg particles was detected as an optical phenomenon of the surface plasmon resonance (SPR) signal expressed in resonance units (RU). The continuous display of RU as a function of time was referred to as a sensogram. The effect of the action of HBV-BF on the immobilized particles was investigated by incubation of the sensor chips with a series of MAbs directed to the pre-S and S epitopes.

Analysis of the effect of proteinase inhibitors on HBV-BF activity

Inhibitors of different groups of proteases were mixed with purified HBV-BF prior to incubation with recombinant m-HBsAg or HBV. The protease inhibitors were: pepstatin, leupeptin, E64, chymostatin, phenylmethanesulfonyl fluoride (PMSF), antipain, and soybean trypsin inhibitor (Behringer Mannheim Biochemicals); 1,10 phenanthroline, EDTA, phosphoramidon, and aprotinin (Sigma Chemical Co.) and were used at concentrations listed in Table 2. The effect of the inhibitor was expressed as percentage of the residual HBV-BF activity measured by ELISA as compared to the activity of HBV-BF mixed with PBS instead of inhibitor.

Inoculation of CEM-cells

Cells (1×10^7) were washed three times with the cell culture medium without fetal calf serum. The cells were subsequently incubated for 1 hr (at 4 or 37°) with 40 μl of HBV (corresponding to 44 pg of HBV DNA) or with the same amount of HBV mixed with purified HBV-BF (8 μg protein/ml). HBV-BF-treated and control samples were preincubated for 1 hr at 37° before inoculation of the cells. In some experiments, the HBV-BF inhibitor, 10 m*M* 1,10-phenanthroline, was included in the reaction mixture. The effect of HBV-BF on the envelope proteins was controlled by ELISA using pre-S-specific MAbs as described above. The inoculated cells were washed as above, transferred to a complete culture medium containing 10% fetal calf serum, and grown at 37°. In some experiments the cells were subsequently digested with 0.5% trypsin to remove virus adsorbed onto the cell surface. Aliquots of inoculated cells were harvested for PCR analysis at days 1 to 13.

Detection of HBV-DNA in the inoculated cells

Aliquots of inoculated cells (or cellular fractions) were digested with 500 μg/ml of proteinase K in a lysis buffer (10 m*M* EDTA 10 m*M* Tris–HCl, pH 7.8, 150 m*M* NaCl, 0.2% SDS) for 2 hr at 45°. The samples were extracted with phenol/chloroform, and the DNA was precipitated with ethanol and resuspended in 20 μl of 10 m*M* Tris–HCl, pH 7.4, containing 1 m*M* EDTA. The samples were then analyzed by PCR with oligonucleotide primers MD027 (nt position 1861–1880) and MD 031 (nt position 2286–2270) corresponding to the conserved sequences of the C gene.

The PCR products were separated by electrophoresis in a 1.2% agarose gel containing ethidium bromide and were analyzed by Southern blotting on a Hybond-N membrane (Amersham, England). The plasmid (pCP10) containing two complete head to tail copies of HBV genome (subtype ayw) cloned into pBR322 at the *Eco*RI site was labeled with dUTP-fluorescein using the ECL random priming kit (RPMN3040, Amersham) and used as a probe. The hybridized fluorescein-labeled probe was detected on the blots with a horseradish peroxidase-labeled anti-fluorescein antibody, and revealed by chemoluminescence (ECL, Amersham). Using this method, 0.01–0.001 fg/sample of cloned HBV DNA (pCP10) could be detected corresponding to 15–150 copies of the genome.

Detection of ccc DNA

Covalently closed circular DNA (cccDNA) in extracts of cultured cells was detected as previously described (*19*). Briefly, DNA was extracted and precipitated as above and analyzed by PCR using two pairs of primers. The choice of primers was determined by the position of the minus strand gap between nucleotides 1823 and 1834. The forward primer S^+, 5′ GGG AGT GGG CCT CAG CCC GTT TCT CCT G-3′ (nucleotides 643–669 of S gene) and either reverse primer X^-, 5′-GGA ACG GCA GAC GGA GGG GAC GAG A-3′ (nucleotides 1511–1484 of X gene) or reverse primer C^-, 5′-ATG CCC CAA AGC CAC CCA AGG CAC AGC-3′ (nucleotides 1903–1877 of gene C) were used.

To prevent pairing between newly synthesized DNA strands initiated by primers C− and S+ when RC-DNA was the template, the three primers were designed to hybridize above 72° (average T_m of 80°) and were used in excess. The detection limits of this technique after Southern blot and hybridization for both pairs of primers ranged from 0.1 to 1 fg/sample when the plasmid pCP10 described above was used as a template (*19*).

Isolation of cell surface components

Cell surface proteins were isolated by a modification of the method of Maisner *et al.* (*20*). The cells were biotin labeled, washed, and lysed in a buffer containing 1% Triton X-100, 0.1% SDS, 1% DOC, 0.15% NaCl, and protease inhibitors. The labeled components were isolated by adsorption on insoluble streptavidin-agarose and separated by centrifugation.

Isolation of nuclear and cytoplasmic fractions from CEM cells

The method described by Qiao *et al.* (*36*) was used to separate nuclear fraction from cell membranes and cytoplasm. The cells were washed three times with cold PBS, centrifuged at 6000 rpm for 2 min, and the cell pellets were resuspended in 200 μl of 0.5% NP-40, 1 m*M* EDTA, 150 m*M* NaCl in 10 m*M* Tris–HCl, pH 7.4. The suspension was mixed and incubated for 5 min at room temperature to solubilize cell membranes and then centrifuged as above. The pellet was considered as the "nuclei-enriched" fraction and the supernatant as the "cytoplasmic" fraction. The fractions were morphologically examined under the light microscope after staining with hematoxylin. This analysis revealed the presence of soluble components and no visible nuclei in the cytoplasmic fraction and a large amount of free nuclei and very few intact cells in the nuclei-enriched fraction.

HBV-BF analysis by zymography

Samples (0.1 μl) containing HBV-BF (8 ng of protein) were solubilized in a buffer containing 10% glycerol, 1% SDS, and bromophenol blue. Electrophoretic migration was carried out on 7% SDS–polyacrylamide gels and copolymerized with 1 mg/ml of gelatin or casein as previously described (*11, 41*). SDS was extracted with Triton X-100 and the gel was incubated overnight at 37° in a reaction buffer containing 50 m*M* Tris–HCl, pH 7.4, 5 m*M* $CaCl_2$, and 5 m*M* $ZnCl_2$. To investigate the effect of proteinase inhibitors: 50 m*M* 1,10 phenanthroline, 100 m*M* EDTA, 1 m*M* PMSF, or 2 m*M* *N*-ethylmaleimide (NEM) were added to the reaction buffer. Gels were stained with 0.5% Coomassie brilliant blue G250 in 30% methanol, and 10% acetic acid for 4 hr. This technique detects clear bands, the cleaved (active) forms of enzymes as well as their inactive (uncleaved) precursors (proenzymes) due to the exposition of the active site of the enzyme by conformational changes induced under experimental conditions as demonstrated by Herron *et al.* (*11*). This process is not, however, considered as an activation, where the cleavage of a propeptide results in the exposition of the active, enzymatic site since the real activation process can be evidenced by a decrease in the molecular weight of the analyzed molecule.

Activation of MMP-2 by HBV-BF

Conditioned media from hepatic stellate cells, containing a latent form of the matrix metalloproteinase-2 (*1*) were prepared as previously described (*17, 41*). Aliquots of these preparations (15 μl) were incubated with 0.1, 1, or 5 μl of HBV-BF (corresponding to 8, 80, or 400 ng of protein), with or without proteinase inhibitors (10 m*M* 1,10 phenanthroline, 10 m*M* EDTA, 1 m*M* PMSF, or 2 m*M* NEM) 6 hr at 37° and analyzed by zymography in 7% SDS–polyacrylamide gels copolymerized with 1 mg/ml of gelatin as described above.

TABLE 1

Influence of HBV-BF on the Binding of MAbs to the HBV Envelope Proteins

Solid phase antigen	MAb	Epitope recognized (aminoacids)	Percentage inhibition by HBV-BF
m-HBsAg (ay)	F124	pre-S2 (120-126)	90–100
	Q19-10	pre-S2 (120-126)	90–100
	F376	pre-S2 (133-145)	40–60
	E6	pre-S2 (136-141)	40–60
	39-10	*a* determinant of S protein	0–5
	144	*a* determinant of S protein	0–5
	112	*a* determinant of S protein	0–5
m-HBsAg (ad)	E21/14	pre-S2 (162-168) ad	0–2
HBV (ay)	F124	pre-S2 (120-126)	90–100
	E6	pre-S2 (136-141)	40–60
	Ma 18/7	pre-S1 (28-36)	80–100
	5a-19	pre-S1 (36-43)	80–100
	39-10	*a* determinant of S protein	0–5
	114	*a* determinant of S protein	0–5
	122	*a* determinant of S protein	0–5
HBV (ad)	E21/14	pre-S2 (162-168) ad	0

Western immunoblotting

Specimens were solubilized in a buffer containing 2% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue, and 20% glycerol for 2 min at 100°. Proteins were separated on 5–15% polyacrylamide gradient gels and electroblotted onto nitrocellulose. The membrane strips were blocked overnight at 4° with 5% skim milk, washed, and let to react for 1 hr at room temperature with MAbs diluted in 1% skim milk. The MAbs used for immunoblotting included anti-pre-S1 MAb 5a-19 (*6*), anti-pre-S2 MAbs F124 (*4*), and E21/14 (*22*) and anti-HBs MAb H166 (*25*) reactive with all denatured HBV envelope proteins, kindly provided by L. T. Mimms.

Electron microscopy

Two-step staining procedure was used: 400-mesh grids coated with plastic-carbon films were placed onto a drop of virus suspension, dried with a filter paper, and finally floated on a drop of negative stain (1% phosphotungstic acid). The specimens were observed with a JEOL JEM-1010 electron microscope.

RESULTS

Modification of the L and M envelope proteins by HBV-BF

To define the mode of action of HBV-BF on the envelope proteins we analyzed the inhibitory activity of this molecule on the binding of MAbs directed to different epitopes on the recombinant m-HBsAg and serum HBV (Table 1). The first series of experiments with recombi-

nant m-HBsAg as a solid phase antigen demonstrated that the binding of MAbs F124 and Q 19-10 specific to the N-terminal, pre-S2 epitopes (aa 120–126), was strongly inhibited (90–100%) by HBV-BF. Binding of MAbs F376 and E6 reactive with the epitopes localized in the pre-S2 (aa 133–145) sequence was only partially inhibited (40–60%), whereas binding of MAb E21/14 specific to the C-terminal pre-S2 sequence (aa 162–168) was not blocked by HBV-BF. The reactivity of the S determinants of HBsAg particles remained unchanged as evidenced by the binding of three different anti-S-specific MAbs. These results suggested that the interaction site of HBV-BF with the pre-S2 domain is located in the central part of the pre-S2 region (aa 133–145) corresponding to the epitopes recognized by MAbs F376 and E6.

Another series of experiments with complete virions as the solid phase antigen showed that HBV-BF blocked the binding of the pre-S2-specific MAbs F124 and E6 to the viral particles in the same way as to the m-HBsAg (Table 1). Interestingly, the binding of the MAb E21/14 specific to the C-terminal epitope of the pre-S2 domain of HBV was not inhibited but significantly enhanced (150–230%) following incubation with HBV-BF. This observation suggested a conformational change and exposition of the C-terminal part of the pre-S2 domain. HBV-BF blocked the binding of the two pre-S1 domain-specific MAbs Ma18/7 and 5a-19 (80–100% inhibition), recognizing pre-S1 (*28–36*) and pre-S1 (*36–43*) aa sequence, respectively. The reactivity of the three different S region-specific MAbs remained unchanged.

The interaction of HBV-BF with the surface HBV antigens was further analyzed by SDS–PAGE followed by immunoblotting with anti-pre-S antibodies (Fig. 1). The M HBV protein (gp34/gp36) was undetectable or only weakly stained with the pre-S2-specific MAbs F124 after treatment of both m-HBsAg particles and HBV with HBV-BF (Figs. 1A and 1B), suggesting that this epitope was cleaved from the M protein. It should be mentioned that the L protein is not reactive with MAb F124 since this MAb selectively binds to the pre-S2 (120–126) epitope glycosylated at the Asp 123 in the M protein, whereas there is no glycan linked to the corresponding epitope in the L protein (*8, 10*). In further experiments a faster migrating 28-kDa band, corresponding to a cleaved form of the M protein, was detected in the HBV-BF-treated m-HBsAg using MAb E24/14 specific to the C-terminal pre-S2 (162–168) epitope (Fig. 1C). A protein band of the same molecular weight was detected in HBV-BF-treated virions when MAb H166, reactive with a sequential epitope of the S domain was used for immunoblotting (Fig. 1D).

In contrast, the L HBV protein (p39/gp41) of unchanged molecular weight was evidenced by immunoblotting in virions preincubated with HBV-BF using anti-pre-S1 MAb (5a-19). (Fig. 1B). Immunoblotting analysis of the native and HBV-BF-treated virions with anti-S MAb H166 confirmed that the molecular mass of the M protein decreased of about 6–8 kDa after incubation with HBV-BF, whereas the S and L HBV proteins remained unchanged (Fig. 1D).

All these data suggested that HBV-BF can be a proteinase cleaving the M protein within the pre-S2 sequence in recombinant as well as in the viral particles. HBV-BF did not cleave the L protein, since the potential cleavage sites in the pre-S2 domain are not accessible in the L protein (*8*). However, HBV-BF hampered the reactivity of the pre-S-specific MAbs with the corresponding epitopes in the L protein, suggesting that either a conformational modification or blocking of the pre-S domains occurred. Indeed, the large HBV protein of unchanged molecular weight, reactive with pre-S1- and pre-S2-specific MAbs was evidenced by Western blotting in HBV-BF-treated HBV particles. The observed modifications did not concern the S protein domain since the conformational *a* determinant of the HBV envelope remained immunologically unchanged and the electron-microscopic analysis of the HBV-BF-digested virions showed their well-conserved structure (Fig. 2B) in comparison with the native HBV (Fig. 2A).

The proteinase activity of HBV-BF was further evidenced by the real-time biospecific interaction analysis (BIA core). HBV or m-HBsAg particles were immobilized on the sensor chips (Fig. 3A) and incubated with purified HBV-BF (Fig. 3B). Identical results were obtained for both systems involving m-HBsAg or HBV on the sensor chips: cleavage of the immobilized components could be directly observed as a release of the material from the sensor chip surface evidenced by a lowering sensogram baseline after injection of HBV-BF (Fig. 3B). HBV-BF-treated HBV and m-HBsAg did not bind MAb F124, suggesting that the corresponding epitopes were removed from the surface. MAb E6 bound weakly, whereas MAb 39-10 conserved its binding capacity (data not shown). These observations confirmed the partial removal and stability of the corresponding epitopes, respectively. Therefore, the results obtained with BIA core were consistent with those obtained with ELISA and confirmed HBV-BF to be a proteolytic enzyme, which cleaved the M protein.

Determination of the cleavage site in the M HBV protein

To determine the cleavage site of HBV-BF in the pre-S2 domain, different anti-pre-S MAbs were reacted in ELISA with m-HBsAg or HBV particles prior to their incubation with HBV-BF to determine which of the pre-S-specific MAbs could prevent the effect of HBV-BF. Most of the pre-S2-specific MAbs (F124, Q-19/10, and E 21/14) did not affect the reactivity of HBV-BF with the pre-S2 domain; MAb F376, partially inhibited HBV-BF (about 50%), whereas MAb E6 completely (100%) blocked the action of HBV-BF on the M protein. These results suggested that HBV-BF cleaves the pre-S2 domain at or

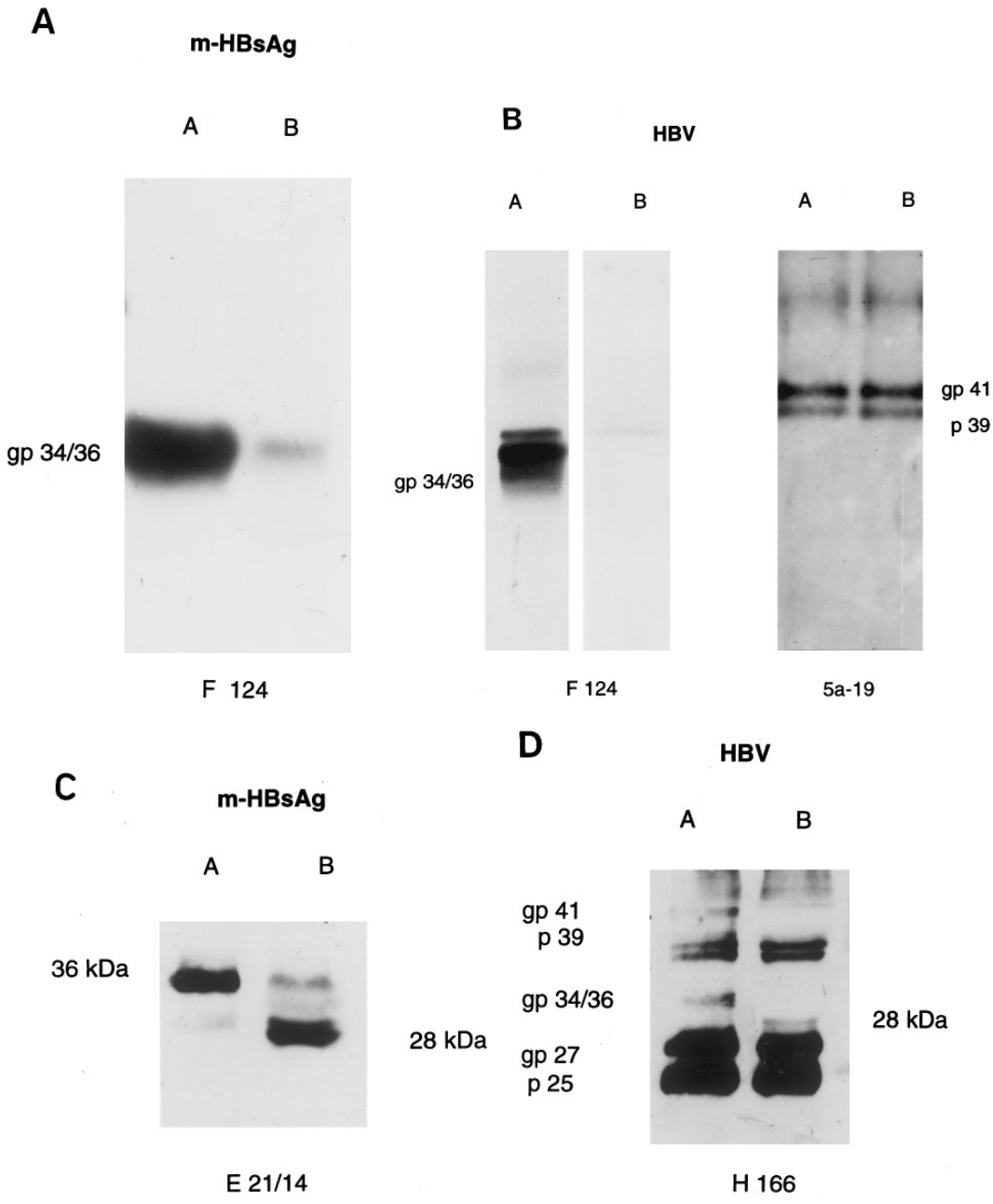

FIG. 1. Cleavage of the middle HBV-envelope protein by HBV-BF evidenced by Western blot. Recombinant m-HBsAg or HBV particles were incubated with HBV-BF and subsequently subjected to electrophoresis and immunoblotting with MAbs anti-pre-S2 F124 (A and B), E21/14 (C), anti-pre-S1 MAb 5a-19 (B), and anti-S MAb H166 (D). Lanes A, native, untreated HBV or recombinant m-HBsAg; lanes B, HBV or m-HBsAg treated with HBV-BF. The cleaved form of the M protein of a molecular mass of 28 kDa is shown on C and D.

spatially close to the aa sequence pre-S2 (136–141) (VRGLYF/L) corresponding to the epitope recognized by MAb E6 and containing arginine residue (Arg 137) (Fig. 4). This cleavage (i) abolished the reactivity of MAb F124 with the N-terminal pre-S2 epitope (120–126), (ii) partially blocked the reactivity of the antibodies to the central part of the pre-S2 domain (133–145), (iii) increased the reactivity of the MAb E21/14 with a corresponding C-terminal epitope pre-S2 (162–168), which probably became exposed on the surface of viral particles and better accessible for this MAb. The cleavage of the pre-S2 protein at the Arg 137 as a potential cleavage site and removal of 17 amino acids (including glycosylated Asp 123 residue) is in accordance with the decrease of molecular mass of the M-HBV protein of about 6–8 kDa observed by Western Blotting.

HBV-BF is a neutral metalloproteinase

To investigate what type of proteinase is HBV-BF we studied the effect of various proteinase inhibitors on the

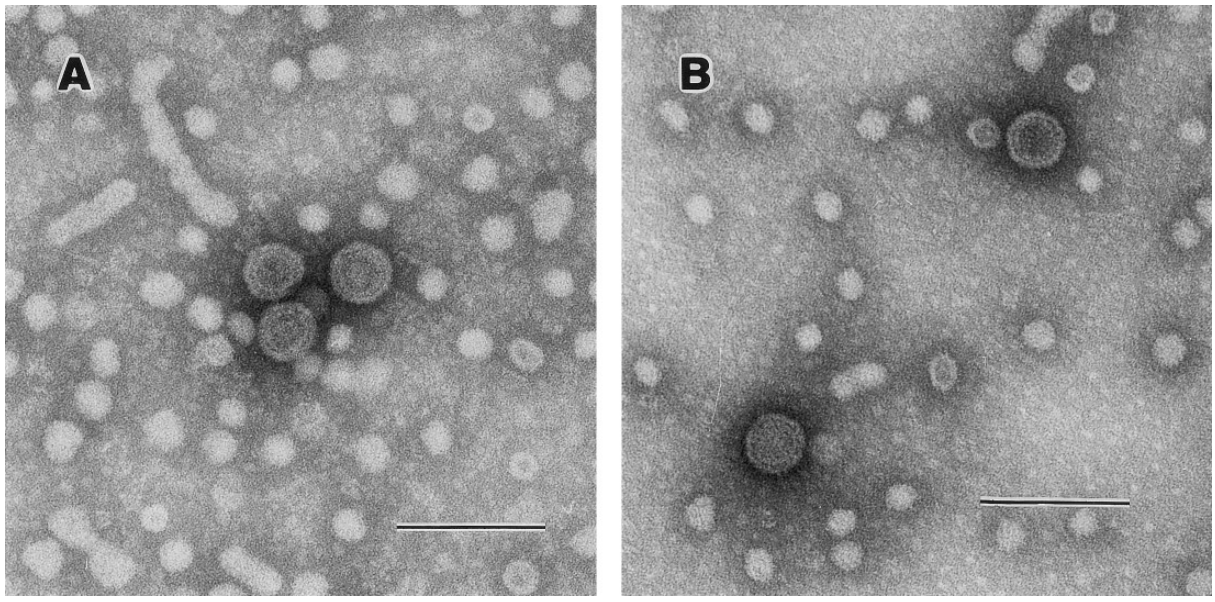

FIG. 2. Morphology of HBV-BF-digested virions used for inoculation of the CEM cells. Negatively stained preparation of the native virus (A) and virus digested with HBV-BF (B). Bars correspond to 100 nm.

reactivity of HBV-BF. As shown in Table 2, the interaction of HBV-BF with the two pre-S domains of HBV was unaffected by inhibitors of aspartic proteinases, serine proteinases, and cysteine proteinases. In contrast, the effect of HBV-BF on both M and L proteins was abolished by two metalloproteinase inhibitors: EDTA and 1,10 phenanthroline, but was unaffected by phosphoramidon, which is a specific inhibitor of bacterial metalloproteinases and does not inhibit matrix metalloproteinases.

The optimum pH for the reactivity of HBV-BF was in the neutral range and rapidly decreased at the low as well as at basic pH values (showing 30% of activity at pH 5.0 until complete inactivation at pH 3.5 and 50% activity at pH 9.5). This feature, as well as the sensitivity to the action of chelating agents, suggested HBV-BF to be a member of the neutral metalloproteinase family.

HBV-BF shows the properties of a matrix metalloproteinase

Zymography analysis showed that HBV-BF migrated in SDS–PAGE as a 50-kDa band capable of degrading gelatin and casein, two typical substrates of the matrix metalloproteinases (Fig. 5). Both activities were inhibited by EDTA and 1,10 phenanthroline (partially for gelatin and completely for casein), whereas other proteinase inhibitors such as PMSF or NEM did not influence HBV-BF activity.

HBV-BF was further incubated with conditioned medium from hepatic stellate cells which secrete a latent form of a matrix metalloproteinase 2 (MMP-2, gelatinase A, EC 3.4.24.24) (*17, 41*) and the mixtures were analyzed by zymography. The latent form of MMP-2 (proenzyme)

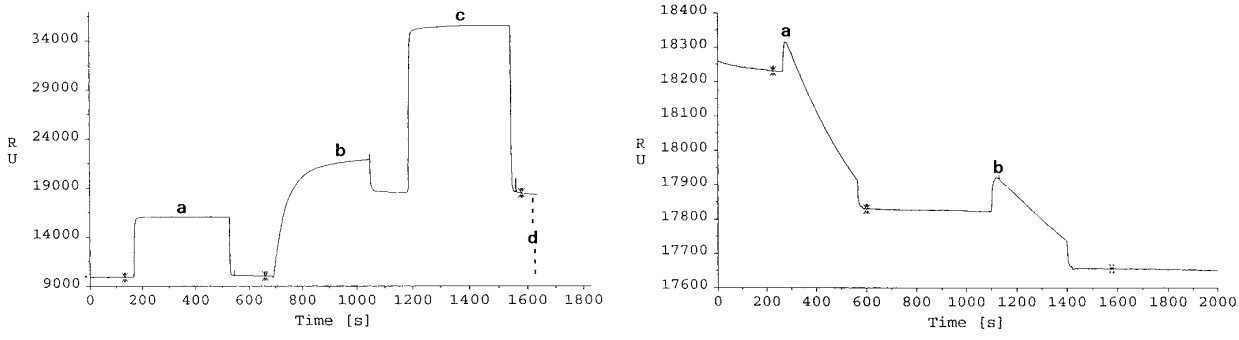

FIG. 3. Proteolytic activity of HBV-BF evidenced by a real time Biospefic Interaction Analysis (BIA core). (A) Sensogram showing immobilization of recombinant m-HBsAg particles on a sensor chip. (a) The pulse of NHS and EDC gives an increase in the SPR signal due to the change in the bulk refractive index. (b) Corresponds to the injection of 30 μl of m-HBsAg and (c) to the injection of ethanolamine to deactivate the sensor chip surface. The amount of immobilized particles corresponds to 8264 RU (d). (B) HBV-BF (25 μl) was subsequently injected twice (a and b) over the sensor chip surface. The baseline difference between the beginning and the end of the sensogram after the first injection indicates cleavage of the portion of the surface-bound m-HBsAg, corresponding to 400 RU.

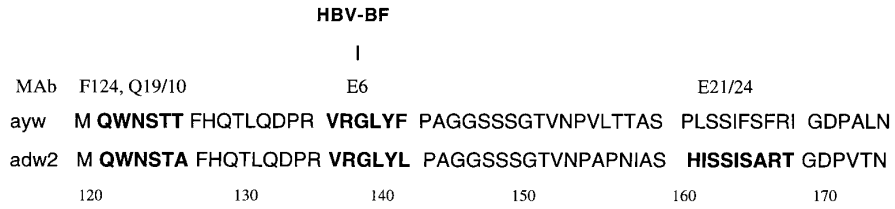

FIG. 4. Localization of the cleavage site of HBV-BF in the pre-S2 domain of the M HBV protein. Linear maps of the pre-S2 sequences of ayw and adw2 subtypes are presented. Numbers indicate aminoacids along the M protein. Epitopes recognized by pre-S2 specific MAbs are shown in boldface types. The cleavage site of HBV-BF corresponds to the VRGLYF/L epitope recognized by MAb E6.

appeared as a clear band of a molecular mass of 66 kDa, as previously described (*41*), HBV-BF as a 50-kDa band and an additional band of a molecular mass of 62 kDa corresponded to a cleaved (activated) form of MMP-2. The 62-kDa band was detected in a dose-dependent manner following treatment with increasing concentrations of HBV-BF (Fig. 6A). The activation of MMP-2 by HBV-BF was inhibited by EDTA, 1,10 phenanthroline, and partially by PMSF (Fig. 6B). Serine proteinase inhibitor NEM failed to inhibit the processing of MMP-2 by HBV-BF. All these results showed that HBV-BF has the enzymatic properties of a typical matrix metalloproteinase.

Activation of the envelope proteins by HBV-BF enables adsorption and entry of HBV into the cell

Human peripheral blood mononuclear cells can adsorb viral particles (*15, 33, 34*), but seem to be refractory to HBV infection (*15*). Therefore we addressed the question whether the structural modifications induced by metalloproteinase (HBV-BF) treatment can facilitate internalization and/or replication of HBV in T-lymphocytes. CEM-cells were inoculated with either the native or HBV-BF-digested virus. The adsorption was carried out at 4° to prevent endocytosis, which usually occurs above 10°, and to ensure that the virions remained bound and intact during the incubation and washing procedure. Control experiments were performed at 37°. After the binding and washing steps the cells were transferred to a complete medium and grown at 37° up to 13 days after inoculation. DNA was extracted from the cells and assayed for HBV sequences by PCR. In studies of HBV internalization the washing steps were followed by trypsin digestion to eliminate any bound virus.

The extracts tested up to 13 days after inoculation (either at 4 or 37°) with HBV-BF-digested virus showed intense HBV-DNA bands corresponding to the amplified product of 430 bp (Fig. 7A). In contrast no HBV-DNA was detected in the extracts prepared from CEM-cells inoculated with the native, untreated HBV when tested from days 1 to 13 postinoculation.

To investigate whether modifications of the viral envelope proteins by HBV-BF influence either viral attachment or internalization, subcellular components were isolated and obtained fractions assayed for the presence of HBV-DNA by PCR. The cell surface fraction isolated from the cells inoculated with metalloproteinase (HBV-BF)-digested virus and harvested on day 2 postinoculation showed a

TABLE 2

Effect of Protease Inhibitors on the Interaction of HBV-BF with Pre-S Epitopes

Proteinase group	Inhibitor	Percentage HBV-BF activity (a)	(b)
Aspartate proteinases	Pepstatin (1 m*M*)	95	96
Cysteine proteinases	Leupeptin (100 m*M*)	92	94
	E 64 (50 m*M*)	93	96
	Chymostatin (100 m*M*)	96	96
Serine proteinases	PMSF (1 m*M*)	98	96
	Soybean trypsin inhibitor (100 μg/ml)	95	98
	Aprotinin (0.6 m*M*)	94	95
Metalloproteinases	EDTA (10 m*M*)	0	0
	1,10 phenantroline (5 m*M*)	0	0
	Phosphoramidon (600 m*M*)	94	97
Trypsin, papain, plasmin	Antipain (120 m*M*)	96	98

Note. Purified HBV-BF was incubated with HBV particles coated on the solid phase without (control sample) or with the indicated proteinase inhibitors. The results are expressed as percentage of residual activity of HBV-BF measured by inhibition ELISA with peroxidase-labeled (a) anti-pre S1 Ma 18/7 and (b) anti-pre-S2 F124 MAbs as described under Material and Methods.

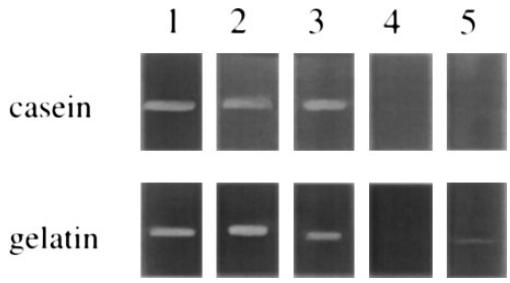

FIG. 5. Analysis of HBV-BF activity by zymography on gelatin and casein gels. The gels were incubated overnight at 37° with reaction buffer containing or not inhibitors as described under Materials and Methods. Proteolytic activity was detected as a 50-kDa band. Lane 1, no inhibitor added; lane 2, NEM; lane 3, PMSF; lanes 4 and 5, EDTA and 1,10 phenanthroline, respectively, added to the reaction buffer.

strong 430-bp band of HBV-DNA (Fig. 7B). No bands could be observed in the membrane fraction from the cells inoculated with the native HBV, and only weak bands were observed in the membrane fraction from the cells inoculated with the virus treated with HBV-BF in the presence of 1,10 phenanthroline. These results showed that the action of HBV-BF enhances viral binding to the cell surface and that this process is susceptible to a specific metalloproteinase inhibitor, 1,10 phenanthroline. Furthermore, HBV-DNA bands were detected up to day 13 in both cytoplasmic and nuclear fractions from cells inoculated with HBV-BF-digested virus, whereas no, or only trace amounts of HBV-DNA, were found in fractions isolated from the cells inoculated with the native virus (Fig. 7C). Therefore, HBV-BF treatment apparently facilitated not only attachment to the cell surface, but also viral internalization. This process was also inhibited by 1,10 phenanthroline, confirming the role of the metalloproteinase digestion in this event.

The cell extracts were also tested for the presence of covalently closed, circular HBV-DNA (ccc-DNA) by the strand-specific PCR. No amplification product was obtained with the ccc-DNA-specific primers, although the HBV-DNA signal did not decline with the time in the cell culture inoculated with HBV-BF-digested virus. Thus, the HBV DNA was exclusively in the RC form and there was no evidence for HBV replication in this type of cell.

DISCUSSION

Several reports have suggested that the pre-S region encoded protein domains of HBV envelope are responsible for attachment of HBV to cellular receptors (*8, 9, 28, 33, 34*). The major S protein might have a potential fusogenic activity, since the 23 N-terminal amino acids show a high degree of homology with fusogenic peptides of other viruses and are conserved among different HBV antigenic subtypes and within the hepadnavirus family (*37*). The attachment of the HBV to various cells of human origin, which are not susceptible to infection has been demonstrated (*29*). This observation and the impossibility to cultivate HBV *in vitro* suggest that the mechanisms of natural HBV infection may require several factors for viral binding, penetration, and transport into an adequate cellular compartment. The receptors present on nonsusceptible cells may function in focusing the virus onto the cell membrane. Other factors present only in, or in the vicinity of the target cell, would be required for subsequent steps of infection, such as internalization and intracellular transport of the virus to the cell nucleus where replication starts.

In this study we characterized HBV-binding factor, the molecule previously found in hepatocyte membranes and under a soluble 50-kDa form in normal human serum. HBV-BF is a member of the neutral metalloproteinase family and displays the substrate specificity and the properties of a matrix metalloproteinase. HBV-BF modified the structure of the envelope proteins and enhanced the capacity of HBV to bind and enter into a T-lymphocyte cell line. First, HBV-BF cleaved the M envelope protein in the pre-S2 sequence. Cleavage resulted in (i) the decrease of a molecular mass of the M protein of about 6 kDa, (ii) disappearance of the binding of the N-terminal pre-S2 antibodies, (iii) reduction of the binding of the MAbs directed to the central part of the pre-S2 domain, and (iiii) increase of the binding of C-terminal MAbs due to the exposition of the corresponding epitopes. Cleavage of the M protein by HBV-BF was completely blocked by metalloproteinase inhibitors, but also by MAb E6 spe-

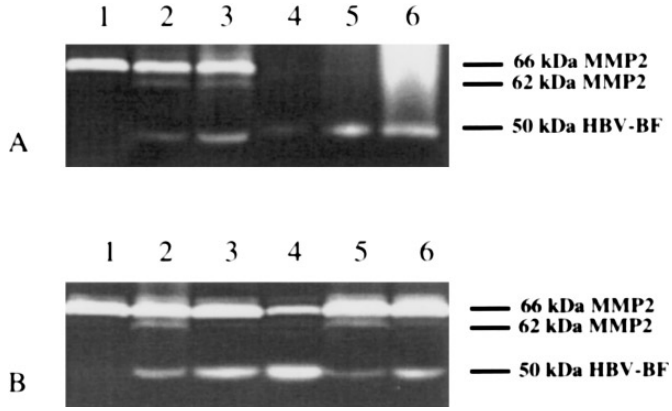

FIG. 6. (A) Activation of MMP-2 by HBV-BF. Conditioned medium of hepatic stellate cells containing a latent form of MMP-2 of a mol. mass of 66 kDa was incubated with 8, 80, and 400 ng of the HBV-BF preparation for 6 hr at 37° (lane 1, 2, and 3, respectively). The mixtures were subsequently submitted to gelatin zymography analysis. The 62-kDa band corresponds to the cleaved form of MMP-2 and 50-kDa band to HBV-BF. Lanes 4, 5, and 6 represent control samples containing 8, 80, and 400 ng of HBV-BF, respectively, incubated in the same conditions in the absence of conditioned medium. (B) Inhibition of MMP-2 activation. Conditioned medium of hepatic stellate cells containing a latent form of MMP-2 (66 kDa, lane 1) was incubated with 40 ng of the HBV-BF preparation for 6 hr at 37° without (lane 2) or in the presence of proteinase inhibitors (EDTA, lane 3; 1,10 phenanthroline, lane 4; NEM, lane 5; and PMSF, lane 6). Subsequently, the mixtures were submitted to gelatin zymography analysis.

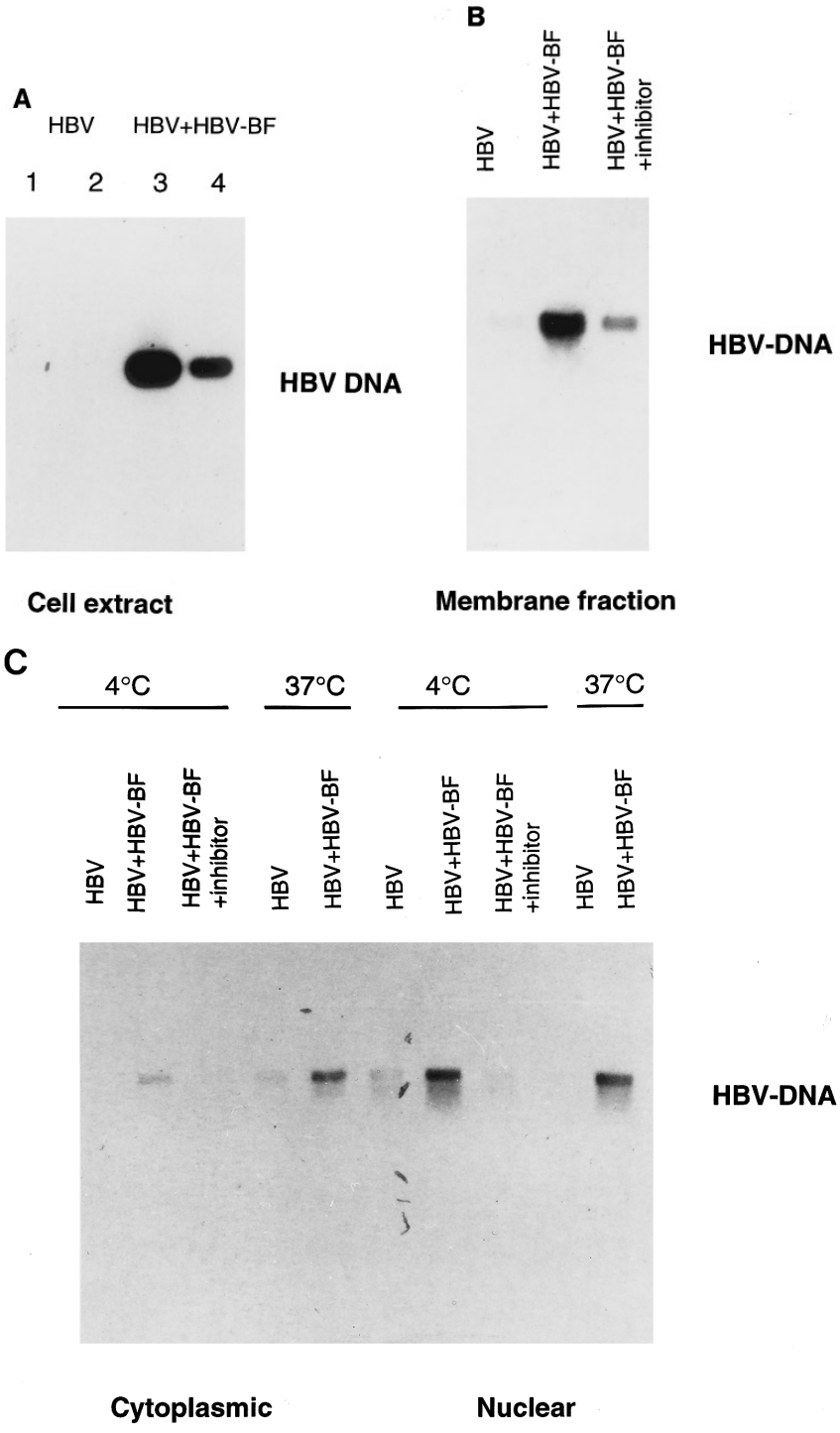

FIG. 7. (A) Detection of amplified HBV-DNA on day 8 in DNA extracts from cells inoculated with the virus digested with HBV-BF. CEM-cells (10^7) were incubated either with 40 μl of HBV (40 pg of HBV DNA) or with the same amount of the virus preincubated with HBV-BF (8 μg of protein) as described under Materials and Methods. The cells were subsequently trypsin digested, transferred to complete medium, and grown at 37°. Aliquots of cells were harvested for PCR analysis. Lanes 1 and 2 represent extracts of the cells inoculated with native HBV, lanes 3 and 4 extracts from cells inoculated with HBV-BF-digested virus (lanes 2 and 4 represent 10-fold dilutions of the extracts analyzed in lanes 1 and 3, respectively). (B) Detection of amplified HBV-DNA on day 2 in the membrane fraction isolated from cells inoculated with the metalloproteinase-digested virus. Biotinylated surface proteins of CEM-cells inoculated with HBV, HBV digested with HBV-BF, or HBV pretreated with HBV-BF in the presence of inhibitor (10 m*M* 1,10 phenanthroline) were isolated on streptavidin-agarose and the presence of HBV-DNA determined by PCR. (C) Detection of amplified HBV-DNA on day 13 in the "cytoplasmic" and "nuclear" fractions isolated from CEM-cells inoculated with the native or with metalloproteinase-digested HBV. The inoculation was carried out at either 4 or 37°. HBV-BF inhibitor, 10 m*M* 1,10 phenanthroline.

cific to the pre-S2 (136–141) epitope, suggesting that the VRGLYF/L sequence is the cleavage site of HBV-BF in the pre-S2 domain. This aa sequence contains the arginine residue (Arg 127) and corresponds to a monobasic cleavage site present in other viral glycoproteins activated by enzymes secreted from epithelial cells (*14*).

Moreover, HBV-BF hampered the reactivity of the pre-S-specific MAbs with the corresponding epitopes on the L HBV protein. Since (i) this effect was prevented by metalloproteinase inhibitors and (ii) the intact L protein could be evidenced by Western blot, it is conceivable that cleavage of the M protein by HBV-BF affected the conformation of the large HBV protein. The HBV-BF-digested virus remained morphologically unchanged and conserved its intact conformation-dependent S domain reactivity. Whether all these structural changes induced by HBV-BF exposed potential N-terminal fusogenic sequence of HBV-S protein is at present under investigation.

The human $CD4^+$ T-lymphocyte cell line, CEM only weakly bound native HBV and much more efficiently recombinant S,L* HBsAg particles composed of the major S protein and of a truncated L* protein containing pre-S1 (12–52) and pre-S2 (133–145) aa sequences (*32*) as previously demonstrated by our group by immunofluorescent techniques (*35*). In this study the CEM cells did not (or very weakly) adsorb and internalize the native virus, in contrast with the metalloproteinase (HBV-BF)-digested virions. Therefore, the proteolytic activation of the envelope proteins by HBV-BF enabled HBV binding to the cell surface and viral entry into the cell.

The substrate specificity and other enzymatic properties of the HBV-BF suggested that this molecule is a member of the matrix metalloproteinase (MMP) family. MMPs are zinc requiring matrix-degrading enzymes which include collagenases, gelatinases, and stromelysins, all of which are involved in the physiological as well as pathological remodeling of the extracellular matrix (*3*). MMPs are secreted as catalytically latent forms (zymogens). Their activity is tightly controlled by mechanisms of proteolytic activation and modulated by tissue inhibitors of matrix metalloproteinases in the pericellular and extracellular environments. (*3*). Two collagen degrading enzymes MMP-1 and MMP-2 were detected in normal and fibrosis livers (*24*). Recently, new members of this family, the membrane-associated ectoenzymes were discovered and named "the membrane-type matrix metalloproteinases" (MT-MMP-1, -2, and -3) due to an additional transmembrane domain (*2, 31, 38*). Particular attention has been focused on the ability of some MT-MMPs to induce processing of the MMP-2 zymogen to its activated form (gelatinase) (*38*). The substrate specificity of the HBV-BF and its capacity to activate MMP-2 suggest that this molecule can also be a membrane-located enzyme. Indeed, it has been recently shown that hepatocytes induce the activation of MMP-2 produced by hepatic stellate cells, via plasma membrane-dependent mechanism (*41*). This process is inhibited by EDTA and can therefore be due to the metalloproteinase activity related to HBV-BF, in accordance with our previous report showing the localization of a component related to the soluble serum HBV-BF in the membranes of normal human hepatocytes (*5*). The membrane-type matrix metalloproteinases exist also as HBV-BF in both membrane bound and soluble, truncated forms of a molecular mass of about 50 kDa (*2, 31*).

Our findings are consistent with recently published data by Lu *et al.* (*18*), who showed that HBV digested with streptococcal V8 protease can efficiently infect the human hepatoblastoma cell line HepG2, refractive to infection with native HBV. The authors suggested that the lack of the suitable fusion-activation protease could be responsible for the lack of susceptibility of this cell line to HBV infection. We therefore believe that the host-dependent proteolytic activation of the virus contributes to the entry of HBV into the cell. This mechanism would implicate a fusogenic process presumably through a cell surface interaction with the newly exposed sites of the viral envelope. A neutral metalloproteinase identical or related to HBV-BF is a good candidate for the enzyme involved in the proteolytic activation which can be of importance for the spread of HBV infection. The availability of the activating proteinase in a membrane-bound or secreted form might be determining for HBV host range and/or tissue tropism. Metalloproteinases are particularly abundant at the apical surface of the epithelial cells in the digestive and respiratory tracts and have been reported to be involved in the entry of other mammalian viruses into cells (*43*).

The conformational changes induced by exposure to low pH provide an efficient mechanism of entry of many pH-dependent viruses (*21*). A number of other enveloped viruses do not require exposure to acid pH for entry and a protease might function as a fusion trigger for these pH-independent viruses (*21*). The activity of HBV-BF was highest at neutral pH, and sharply decreased with acid pH. Moreover, the metalloproteinase-digested virus could enter the cell without exposure to low pH. Therefore HBV may also *in vivo* enter the cell by a pH-independent mechanisms; e.g., by direct fusion between the viral envelope and the plasma membrane.

The detection of viral antigens and HBV DNA in peripheral blood mononuclear cells during the natural course of infection raises the question whether the viral replication can take place in this type of cell (*15, 16*). In our studies we did not find in the inoculated cells the covalently closed, circular DNA (ccc-DNA), the form of the HBV genome considered as the evidence of viral replication, although the same viral preparation under the same experimental conditions was infectious in a primary culture of normal human hepatocytes (P. Gripon, personal

communication). These findings suggest that despite viral binding and protease-induced internalization, the *in vitro* infection of lymphoid cells by HBV is abortive. The question, whether or not peripheral mononuclear blood cells can be infected with HBV *in vivo* remains unanswered. Different experimental approaches express doubt (*16*) or support (*40*) of the possibility of the productive infection of PBMC in persistently infected patients.

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REFERENCES

1. Arthur, M. J. P., Friedmann, S., and Bissel, D. M. (1989). Lipocytes from normal rat liver release a neutral metalloproteinase that degrades basement membrane (type IV) collagen. *J. Clin. Invest.* 84, 1076–1085.
2. Cao, J., Sato, H., Takino, T., and Seiki, M. (1995). The C-terminal region of membrane-type type matrix metalloproteinase is a functional transmembrane domain required for pro-gelatinase A activation. *J. Biol. Chem.* 270, 801–805.
3. Birkedal-Hansen, H. (1995). Proteolytic remodeling of extracellular matrix. *Curr. Opin. Cell Biol.* 7, 728–735.
4. Budkowska, A., Riottot, M. M., Dubreuil, P., Lazizi, Y., Bréchot, C., Sobczak, E., Petit, M. A., and Pillot, J. (1986). Monoclonal antibody recognizing pre-S2 epitope of hepatitis B virus. Characterization of pre-S2 epitope and anti-pre-S2 antibody. *J. Med. Virol.* 20, 111–125.
5. Budkowska, A., Quan, C., Groh, F., Bedossa, P., Dubreuil, P., Bouvet, J. P., and Pillot, J. (1993). Hepatitis B virus (HBV) binding factor in human serum: Candidate for a soluble form of hepatocyte HBV receptor. *J. Virol.* 67, 4316–4322.
6. Budkowska, A., Bedossa, P., Groh, F., Louise, A., and Pillot, J. (1995). Fibronectin of human liver sinusoids binds hepatitis B virus: Identification by an anti-idiotypic antibody bearing the internal image of the pre-S2 domain. *J. Virol.* 69, 840–848.
7. Franco, A., Paroli, M., Testa, U., Benvenuto, R., Peschle, C., Balsano, F., and Barnaba, Y. (1992). Transferrin receptor mediates uptake and presentation of hepatitis B envelope antigen by T lymphocytes. *J. Exp. Med.* 175, 1195–1205.
8. Gerlich, W. H., and Heermann, K. H. (1991). Functions of Hepatitis B virus proteins. *In* "Viral Hepatitis and Liver Disease" (B. F. Hollinger, S. M. Lemon, and H. Margolis, Eds.), pp. 121–136. Williams & Wilkins, Baltimore.
9. Gerlich, W., Lu, X., and Heermann, K. H. (1993). Studies on the attachment and penetration of hepatitis B virus. *J. Hepatol.* 17(suppl. 3), S10–S14.
10. Heermann, K. H., Waldeck, F., and Gerlich, W. H. (1988). Interaction between native human serum albumin and the pre-S2 domain of the hepatitis B virus surface antigen. *In* "Viral Hepatitis and Liver Disease" (A. J. Zuckerman, Ed.), pp. 697–700. A. R. Liss, NY.
11. Herron, G. S., Werb, Z., Dwyer, K., and Banda, M. J. (1986). Secretion of metalloproteinases by stimulated capillary cells. *J. Biol. Chem.* 261, 2810–2813.
12. Hertogs, K., Leenders, E., Depla, W. P. J., De Brun, W. C. C., Meheus, L., Raymackers, J., Moshage, H., and Yap, S. H. (1993). Endonexin II, present on human liver plasma membranes, is a specific binding protein of small hepatitis B virus (HBV) envelope protein. *Virology* 197, 549–557.
13. Jonsson, U., Fagerstam, L., Ivarsson, B., Karlsson, R., Lundh, K., Lofas, S., Persson, B., Roos, H., and Ronnberg, I. (1991). Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *Biotechniques* 11, 620–627.
14. Klenk, H-D., and Garten, W. (1994). Activation cleavage of viral spike proteins by host proteases. *In* "Cellular Receptors for Animal Viruses" (E. Wimmer, Ed.), pp. 241–280. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
15. Kock, J., Theilemann, L., Galle, P., and Schlicht, H.-J. (1996). Hepatitis B virus nucleic acid associated with human peripheral blood mononuclear cells do not originate from replicating virus. *Hepatology* 23, 405–413.
16. Lamelin, J-P., Zoulim, F., and Trepo, C. (1995). Lymhotropism of hepatitis B and hepatitis C viruses: An update and a newcamer. *Int. J. Clin. Lab. Res.* 25, 1–6.
17. Loreal, O., Lavasseur, F., Fromaget, C., Gros, D., Guillouzo, A., and Clement, B. (1993). Cooperation of Ito cells and hepatocytes in the deposition of an extracallular matrix in vitro. *Am. J. Pathol.* 143, 538–544.
18. Lu, X., Block, T. M., and Gerlich, W. H. (1986). Protease induced infectivity of hepatitis B virus for a human hepatoblastoma cell line. *J. Virol.* 70, 2277–2285.
19. Maillard, P., and Pillot, J. (1996). Polymerase chain reaction to monitor repair of the HBV genome, the first step in viral replication. *Res. Virol.* 147, 5–16.
20. Maisner, A., Schneider-Schaulies, J., Liszewski, M. K., Atkinson, J. P., and Herrler, G. (1994). Binding of measles virus to membrane cofactor protein (CD46): Importance of disulfide bonds and N-glycans for the receptor function. *J. Virol.* 68, 6229–6303.
21. Marsh, M., and Pelchen-Mattews, A. (1994). The endocytic pathway and virus entry. *In* "Cellular Receptors for Animal Viruses" (E. Wimmer, Ed.), pp. 215–240. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
22. Meisel, H., Slominskaya, I., Pumpens, P., Pushko, P., Borisowa, G., Deepen, R., Lu, X., Spiller, G. H., Krûger, D. H., Grens, E., and Gerlich, W. H. (1996). Fine mapping and functional characterization of two immunodominant regions from the pre-S2 sequence of hepatitis B virus. *Intervirology* 37, 330–339.
23. Michel, M-L., Pontisso, P., Sobczak, E., Malpiece, Y., Streeck, R. H., and Tiollais, P. (1984). Synthesis in animal cells of hepatitis B surface antigen particles carrying a receptor for polymerized human serum albumin. *Proc. Natl. Acad. Sci. USA* 81, 7708–7712.
24. Milani, S., Herbst, H., Schuppan, D., Grappone, C., Pellegrini, G., Pinzani, M., Cassini, A., Calabro, A., Ciancio, G., Stefanni, F., and Bourroghs, A. K. (1994). Differential expression of matrix metalloproteinase-1 and -2 genes in normal and fibrotic human liver. *Am. J. Pathol.* 144, 528–537.
25. Mimms, L. T., Floreani, M., Tyner, J., Whitters, E., Rosellof, R., Wray, L., Goetze, A., Sarin, V., and Eble, K. (1990). Discrimination of hepatitis B virus (HBV) subtypes using monoclonal antibodies to the Pre-S1 and Pre-S2 domains of the viral envelope. *Virology* 176, 604–619.
26. Nagai, Y. (1993). Protease-dependent virus tropism and pathogenicity. *Trends Microbiol.* 1, 81–87.
27. Neurath, A. R., Adamowicz, P., Kent, S. P. H., Riottot, M. M., Strick, N., Parker, K., Offensperger, W., Petit, M. A., Wahl, S., Budkowska, A., Girard, M., and Pillot, J. (1986). Characterization of monoclonal antibodies specific for the preS2 region of the hepatitis B virus envelope protein. *Mol. Immunol.* 23, 991–997.
28. Neurath, A. R., Kent, S. B. H., Strick, N., and Parker, K. (1986). Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. *Cell* 46, 429–436.
29. Neurath, A. R., Strick, N., Sproul, P., Ralph, H. E., and Valinsky, J. (1990). Detection of receptors for hepatitis B virus on cells of extrahepatic origin. *Virology* 173, 448–457.
30. Neurath, A. R., Strick, N., and Li, Y. (1992). Cells transfected with

human interleukin 6 cDNA acquire binding sites for the hepatitis B virus envelope protein. *J. Exp. Med.* 176, 1561–1569.

31. Pei, D., and Weiss, S. (1996). Transmembrane deletion mutants of the membrane-type matrix metalloproteinase-1 process progelatinase A and express intrinsic matrix-degradding activity. *J. Biol. Chem.* 271, 9135–9140.
32. Petre, J., Rutgers, T., and Hauser, P. (1992). Properties of a recombinant yeast-derived hepatitis B surface antigen containing S, pre2-and preS1 antigenic domains. *Arch. Virol. (Suppl)* 4, 137–141.
33. Pontisso, P., Petit, M. A., Bankowski, M. I., and Peeples, M. E. (1989). Human liver plasma membranes contain receptors for the hepatitis B virus preS1 region and *via* polymerized human serum albumin for the preS2 region. *J. Virol.* 63, 1981–1988.
34. Pontisso, P., Morsica, G., Ruvoletto, M. G., Zambello, R., Colleta, C., Chemello, L., and Alberti, A. (1991). Hepatitis B virus binds to peripheral blood mononuclear cells via the pre S1 protein. *J. Hepatol.* 12, 203–206.
35. Possehl, C., Maillard, P., Groh, F., Pillot, J., and Budkowska, A. (1997). Analysis of protein components madiating attachment and entry of the hepatitis B virus into human T-lymphocytes. *In* "Viral Hepatitis and Liver Disease" (M. Rizetto, Ed.). (in press).
36. Qiao, M., MacNaughton, T. B., and Gowans, E. J. (1994). Adsorption and penetration of hepatitis B virus in a nonpermissive cell line. *Virology* 201, 356–363.
37. Rodriguez-Crespo, I., Gomez-Gutiérrez, J., Nieto, M., Peterson, D. L., and Gavilanes, F. (1994). Prediction of a putative fusion peptide in the S protein of hepatitis B virus. *J. Gen. Virol.* 75, 637–639.
38. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994). A matrix metalloproteinase expressed on the surface of univasive tumor cells. *Nature* 370, 61–65.
39. Sominskaya, I., Pushko, P., Drelina, D., Kozlovskaya, T., and Pumpen, P. (1992). Determination of the minimal length of the preS1 epitope recognized by a monoclonal antibody which inhibits attachment of hepatitis B virus to hepatocytes. *Med. Microbiol. Immunol.* 181, 215–226.
40. Stoll-Becker, S., Repp, R., Glebe, D., Schaffer, S., Kreuder, J., Kann, M., Lampert, F., and Gerlich, W. H. (1997). Transcription of HBV in peripheral blood mononuclear cells from persistently infected patients. *J. Virol.* 71, 5399–5407.
41. Theret, N., Musso, O., L'Helgoualc'h, A., and Clément, B. (1997). Activation of matrix metalloproteinase-2 from hepatic stellate cells requires interactions with hepatocytes. *Am. J. Pathol.* 150, 51–58.
42. Treichel, U., Meyer zum Buschenfelde, K. H., Stockert, R. J., Poralla, T., and Gerken, G. (1994). The asialoglycoprotein receptor mediates hepatic binding and uptake of natural hepatitis B virus particles derived from viraemic carriers. *J. Gen. Virol.* 75, 3021–3029.
43. Yeager, C. L., Ashmun, R. A., Williams, R. K., Cardellichio, C. B., Shapiro, L. H., Look, A. T., and Holmes, K. V. (1992). Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature* 357, 420–422.