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Detection of Cellular Receptors Specific for the Hepatitis B Virus preS Surface Protein on Cell Lines of Extrahepatic Origin

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Hepatitis B virus infection is primarily mediated by the interaction of the preS region of the viral envelope protein with its still unknown cellular receptor. Using recombinantly expressed preS proteins, the distribution of preS-binding receptors on cell lines from extrahepatic origins was determined by immunofluorescence and flow cytometry. In contrast to human liver cell lines, most cell lines from extrahepatic origins did not bind preS proteins. Nevertheless, exceptions were found in the bone marrow-derived cell line, KG-1, and the osteogenic sarcoma cell line SaOS-2, as well as in the previously reported EBV-transformed B-cell line, Wa. To determine the biochemical nature of these receptors, Wa-cells were cell surface biotinylated and the preS-binding receptors were isolated by immunoprecipitation. A specific band with a molecular weight of ~30 kDa was identified in a SDS-polyacrylamide gel, which further characterization is expected to provide clues regarding the infection mechanism of HBV in hepatic- and extra-hepatic cells.

Key Words: **hepatitis B virus; preS protein; extrahepatic cells; receptor; flow cytometry.**

In general, viruses are attached and internalized into their host cells by the interaction of viral envelope proteins with some surface proteins on the target cells (47). It is widely accepted that the infection of the hepatitis B virus (HBV) operates in the same manner by which the HBV surface antigen (HBsAg) plays a significant role in the interaction with the host cell (6). The envelope of HBV is composed of three kinds of proteins, which are encoded from a single gene that has three distinct initiation sites. These three translation products are designated each as the large (L), middle (M) and small (S) protein respectively, which mainly differ in their N-terminal region. In particular, the N-terminal overhanging part of the L-protein over the S-protein is known as the preS region, which is further divided into the preS(1–119) and preS2(120–174) region (30). Several groups have reported that this preS region of the HBsAg might contain the major interaction site with their cognate cellular receptors (4, 29). Especially the amino acid sequence 21–47 of the preS region is believed to contain a specific binding site for cellular receptors, which can be blocked by competitive binding of synthetic peptides derived from this region (4, 37, 39). Despite these observations, so far no definite information has been obtained regarding the identity of the HBV receptor on the target cell side. Some contradictory roles for polymerized human serum albumin (34) as well as for interleukin-6 (32) as linker molecules bridging the virus and the host cell have been reported, and also a large variety of putative HBV-receptor proteins have been suggested (2, 4, 40, 43, 48) but still there is no definitive report on the identification of the cellular HBV receptor.

Nevertheless using purified HBsAg proteins or synthetic peptides, putative cellular receptors had been detectable on HBV target cells. Indeed in this way, several studies have reported the binding of HBV surface antigens on hepatocytes and hepatoma derived cell lines (4, 43, 48), and it was in the course of such studies that also some cells of extrahepatic origins were identified as possessing HBV-preS binding activities (31). Such findings are interesting in so far as the primary site of infection is known as the liver (6) while the detection of viral DNA or proteins in extrahepatic locations are not uncommon (5, 39).

To evaluate the distribution of HBV receptors in terms of their preS-binding activity on cells of hepatic and extra-hepatic origin, in the present study, recombinant preS region proteins (*adr* subtype) were ex-

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pressed in *E. coli* in form of a maltose binding protein (MBP) fusion protein. Cellular binding of this fusion protein was performed using anti-MBP antibodies and fluorescence-labeled secondary antibodies followed by flow cytometric analysis. Human cell lines from various tissue origins were analyzed upon their binding capacity to the HBV preS region proteins, and further attempts were made to evaluate correlation between binding of the HBV surface protein and virus transmission ability. Finally, to determine the biochemical characteristics of preS-binding proteins on such extrahepatic cell lines, putative receptor proteins were isolated by immunoprecipitation and further analyzed by SDS–PAGE.

MATERIALS AND METHODS

Cell lines and maintenance. All cell lines used in this study were obtained from the Korean Collection of Type Culture (KCTC, Taejon, Korea) if not else indicated. The EBV-transformed B-cell line "Wa" was a kind gift from Dr. Keisuke Sato, Asahikawa Medical College, Japan. All cell lines were maintained in RPMI-1640 or DMEM medium (Life Technologies Inc., Grand Island, NY) supplemented with 10% FCS (Life Technologies Inc.), 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 20 μM β-mercaptoethanol in a humidified 5.6% CO_2 atmosphere in a 37°C incubator.

Expression and purification of MBP-preS. The construction of the prokaryotic expression vector encoding a fusion protein between the HBV-preS region (serotype *adr*) and the maltose-binding protein has been previously described in details (3). In the present study, this vector was used for transformation of *E. coli* cells (strain JM109), which were then further used for overexpression of this recombinant protein. Induction was achieved by addition of IPTG (final concentration 1 mM) at the log growth phase of a freshly inoculated liquid culture. Cells were harvested after 3 h by centrifugation, and the cell pellet was resuspended in lysis buffer (1 mM EDTA, 20 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM PMSF) for sonication. Purification of the recombinant MBP-preS proteins was achieved by binding of the cell lysate with amylose-conjugated agarose resins (New England Biolabs, Beverly, MA), and specifically bound proteins were eluted by competition with free maltose. The purity of the isolated proteins was determined by SDS-polyacrylamide gel electrophoresis.

ELISA. To determine the structural integrity of the recombinant preS proteins, ELISA was performed using preS-epitope-specific monoclonal antibodies. The mouse monoclonal antibody (mAb) F35.25 binds to the preS1(21–47) region (37) and the mAb H8 (22) recognizes the preS2(120–145) regions, respectively. The day before analysis, 96-well plates (NUNC, Roskilde, Denmark) were coated with recombinant MBP-preS protein in 0.1 M Na-carbonate, pH 9.5 to a concentration of 1 μg/well. Next day, excessive reagents were washed out with TBS/0.05% Tween-20, and non-specific binding was blocked with 3% casein by incubation for 30 min at room temperature (RT). Primary antibodies were then added to saturating concentrations and the reaction was incubated for 1 h at RT. In the case of rabbit anti-MBP antiserum (New England Biolabs), specific binding was detected with biotin-conjugated goat anti-rabbit IgG (DAKO A/S, Glostrup, Denmark) followed by peroxidase-conjugated streptavidin. Where mouse monoclonal antibodies (H8 and F35.25) were used, specific-binding was detected with peroxidase-conjugated rabbit anti-mouse IgG (Sigma, St. Louis, MO). After washing out excess reagents, the assay was developed by addition of the corresponding enzyme substrates and analyzed in an Emax ELISA reader (Molecular Dynamics, Sunnyvale, CA).

FACS analysis. For analysis, 2×10^5 cells from each cell lines were first incubated with saturable amounts of the MBP-preS fusion protein at 4°C for 3 h and afterwards washed in PBS/Na-azide (0.05%) for 2 times. Specific binding of the preS-ligand was detected using rabbit anti-MBP antiserum (New England Biolabs) and biotinylated goat anti-rabbit IgG antibodies (DAKO) followed by FITC-conjugated streptavidin (Becton Dickinson Immunocytometry Systems, San Jose, CA). As negative control ligand was used recombinantly expressed MBP encoded from the same original expression vector pMAL-c2 without any additional fusion sequences. Flow cytometry was performed on a FACScan (Becton Dickinson Inc., Mountain View, CA) and the data were analyzed using the software program LYSYS (Becton Dickinson Inc.).

Cell surface biotinylation and immunoprecipitation. For the identification of preS-binding cell surface proteins, live Wa-cells were labeled with biotin (Sulfo-NHS-SS-biotin, Pierce, Rockford, IL) following the protocol as described by Goetrupp *et al.* (11). In brief, 2×10^7 cells were harvested at the log growth phase of the culture and washed once with Biwa's buffer (PBS pH 8.0, with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$). Cells were then resuspended in 20 ml of the same buffer, and the cell surface proteins were labeled with biotin by addition of 1/20 volume of a freshly prepared biotin solution (10 mg/ml in Biwa's buffer). The reaction was terminated after incubation for 1 h at 25°C by washing off excess free biotin with PBS/0.1% BSA. Binding of preS ligand was performed by incubation of these biotinylated cells in each 80 μg/ml of MBP-preS or as control with 80 μg/ml MBP for 3 h at 4°C. After removal of unbound ligands, cells were lysed in 1 ml of cell lysis buffer (50 mM Tris, pH 8.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 1 mM PMSF). After incubation for 30 min at 4°C, the cell lysate was down centrifuged for 5 min at 15,000 rpm and the supernatant was transferred into a new Eppendorf tube. Nonspecific binding to protein A Sepharose was eliminated by a preclearing step with 50 μl protein-A Sepharose for 4 h at 4°C under continuous rotation. The pre-cleared cell lysate was then incubated with 50 μl of anti-MBP monoclonal antibodies (mAb)-conjugated protein-A Sepharose beads overnight at 4°C under continuous rotation. The MBP-specific mAb HAM-19 has been previously determined to be an effective reagent in immunoprecipitation of proteins (36). Next day beads were extensively washed with TBS/0.05% Tween-20, and they were then resuspended in SDS-gel loading buffer for separation in a 12.5% SDS–PAGE. Detection of biotinylated proteins was performed using peroxidase-conjugated streptavidin and a luminol-based chemiluminescense kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

RESULTS

Evaluation of the Presence and Intactness of Antigenic Determinants on Purified MBP-preS Fusion Proteins by ELISA

To show that the detection of preS-binding receptors using a protocol employing MBP-preS proteins, rabbit anti-MBP antisera, biotinylated anti-rabbit IgG antibodies, and finally using FITC-conjugated streptavidin, is indeed antigen-specific, and that the immune complex remains stable during the various incubation and washing steps, ELISA was performed mimicking the formation of this antigen/antibody complex. Figure 1 shows the result from this assay as the signals of the enzyme reactions were quantified using an ELISA reader. Instead of using FITC-conjugated streptavidin as in the actual staining reaction, peroxidase-conjugated streptavidin or peroxidase-conjugated anti-mouse IgG antibodies were used for development of the

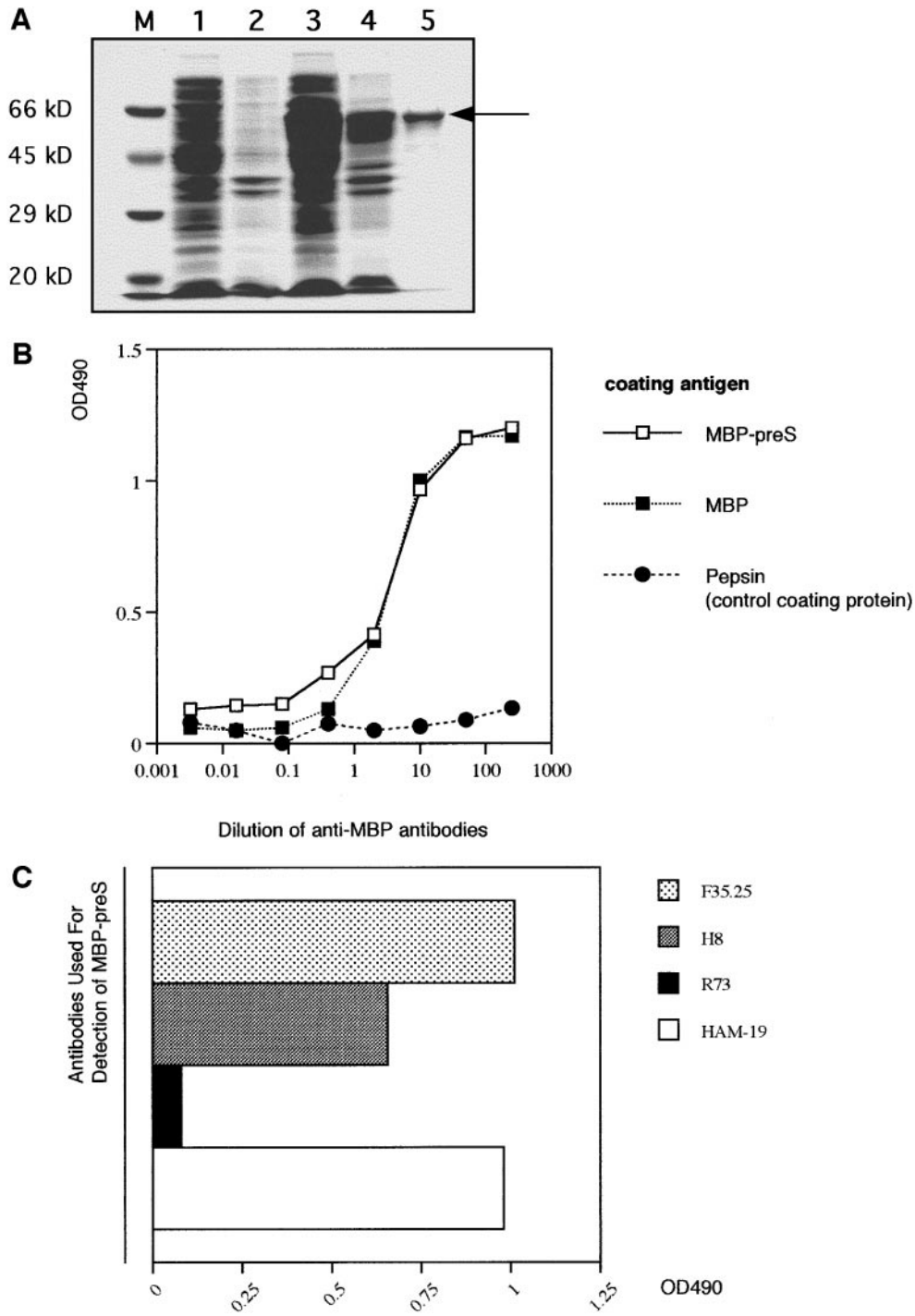

FIG. 1. Expression, purification, and identification of the MBP-preS fusion protein. (A) The recombinantly expressed and purified MBP-preS protein is shown by Coomassie blue staining of a 12.5% SDS–PAGE. M, molecular weight marker; 1, soluble fraction of the uninduced cell lysate of MBP-preS expressing *E. coli;* 2, insoluble fraction of the uninduced cell lysate; 3, soluble fraction of IPTG-induced MBP-preS expressing *E. coli;* 4, insoluble fraction of IPTG-induced cells. 5, purified MBP-preS proteins. Arrow indicates the position of MBP-preS. (B) The successful formation of the immune complex consisting MBP/rabbit anti-MBP antibodies/biotinylated anti-rabbit IgG antibodies and peroxidase-conjugated streptavidin was shown by an ELISA. (C) Intactness and accessibility of the preS region within the MBP is shown in an ELISA using preS1 and preS2 subregion-specific monoclonal antibodies F35.25 and H8, respectively. MBP was detected using the monoclonal antibody HAM-19, and as negative control was used an isotype matched control antibody (R73; 14).

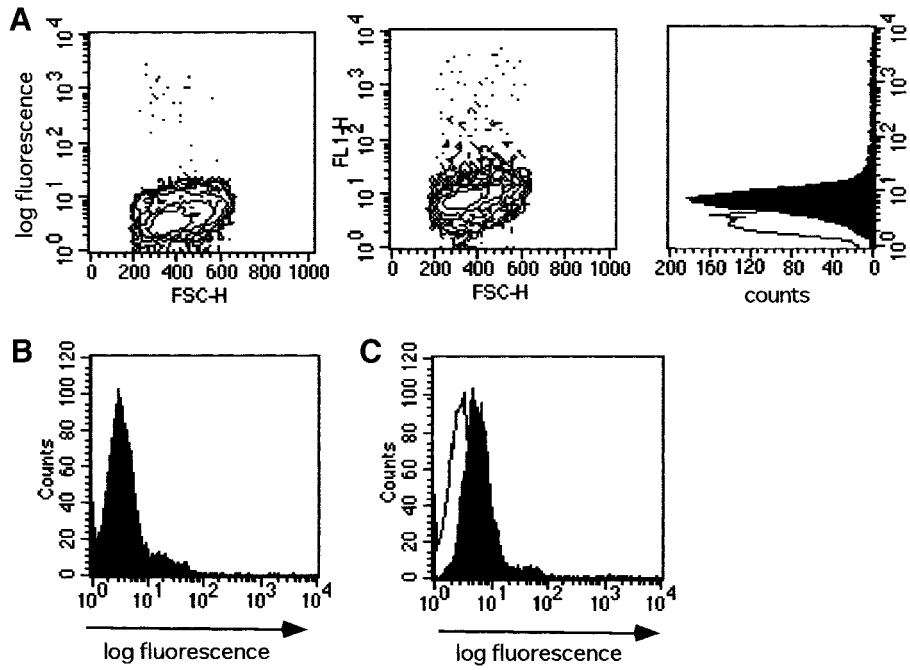

FIG. 2. Determination of MBP-preS binding onto cell lines of hepatic origin. Specific binding of recombinant MBP-preS proteins to cells from hepatic origin was shown by FACS analysis using HepG2 hepatoma cells or as negative control with HT-29 human colon cancer cells. Contour plot shows the relative cell size in forward scatter (horizontal axis) versus the log immunofluorescence (vertical axis) of cell surface bound MBP (left) or MBP-preS ligands (right) in HepG2 cells. The histograms show the quantitative analysis of the bound MBP and MBP-preS fluorescence signals as compared to each other. A., HepG2 cells; B., HT-29 cells. C., Wa cells. Total 10,000 cells were analyzed. Filled histogram shows staining of MBP-preS while unfilled histogram indicates the negative control staining with MBP.

assay. Here is to see that, for one, the immune complex can specifically detect MBP-preS proteins (Fig. 1B), and to the other that the preS region of the recombinantly expressed fusion protein remains structurally intact as shown by the specific staining with preS-subregion specific monoclonal antibodies (Fig. 1C).

Detection of preS-Specific Binding Proteins on HepG2 Cells by Flow Cytometry

HepG2 human hepatoma cells were incubated with different amounts of MBP-preS proteins, and their specific binding to the cell surface was analyzed by fluorescence-activated flow cytometry (FACS). The saturating concentration of MBP-preS ligand proteins for staining 2×10^5 cells was determined to be 80 μg/ml (data not shown). HT-29 cells are known from the authors own previous studies to have no reactivity to the HBV-preS region, and using this property, these cells were used as negative controls in the present assay. Figure 2A shows the result from MBP-preS protein binding to HepG2 cells both in contour plot and histogram. Here is to see that the difference in the relative fluorescence of MBP and MBP-preS binding as displayed in the two contour plots can be more easily quantified and compared to each other when using an overlay histogram. In this regard, further analyses of preS binding were all displayed in histograms as seen in Figs. 2B and 2C. While Fig. 2B shows that HT-29 cells indeed have no binding activity to preS proteins, the EBV-transformed B-cell line Wa (17), which is a previously described extrahepatic cell line with HBV-preS protein binding activity (3), was specifically bound with the recombinant MBP-preS proteins (Fig. 2C) so that the fidelity of this assay system was assured.

FACS Analysis of MBP-preS Fusion Protein Binding on Cells of Extrahepatic Origin

Expression of preS-specific binding proteins was detected by the incubation of MBP-preS fusion proteins followed by fluorescence-labeled secondary reagents as described under Materials and Methods. Cells from extrahepatic origins used in this study were as following: HT-29 (human colon cancer cell line; 9), Wa (EBV-transformed B-cell line; 17), Capan-2 (pancreatic carcinoma; 51), A-498 (renal carcinoma; 19), HL-60 (promyelocytic cells; 35), U-87 MG (human glioma cells; 18), MCF-7 (human breast cancer cell line; 49), WISH (amnion cells; 25), primary lung cells (a kind gift of Dr. K. D. Kim, KRIBB, Taejon, South Korea), Tera-1 (testis; 45), Hut-78 (T-cells; 44), SaOS-2 (osteosarcoma cells; 46), and KG-1 (bone marrow cells; 42). The results from staining of these cell lines with MBP-preS are shown in Figs. 3

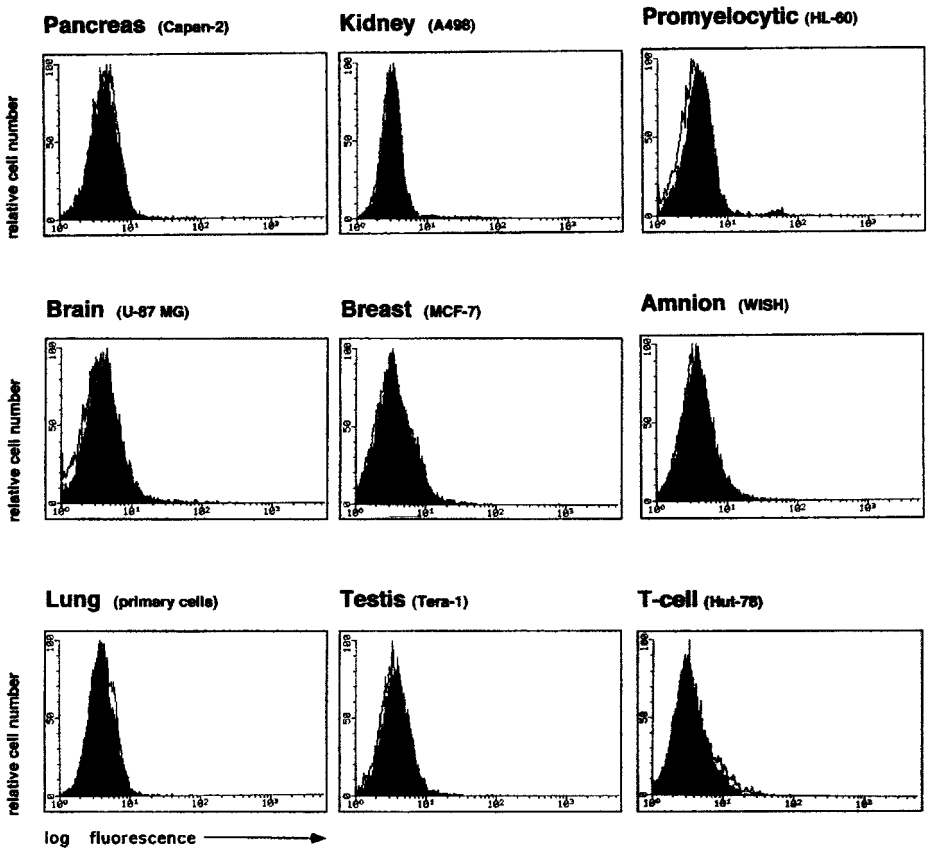

FIG. 3. FACS analysis of MBP-preS binding onto cell lines of extrahepatic origin. The following human cell lines were analyzed upon their expression of HBV-preS-binding activities by incubation with recombinant MBP-preS proteins. Analysis followed the same scheme as used in Fig. 2, but only histograms are shown as results. Capan-2, A-498, HL-60, U87-MG, MCF-7, WISH, primary lung cells, Tera-1, and Hut-78 cells. The tissue origin of each cell lines is indicated in front of the name of the cell line. Filled histogram shows staining of MBP-preS, while unfilled histogram indicates the negative control staining using recombinant MBP.

and 4, whereby most of the tested cells showed no binding reactivity to preS (Fig. 3). Nevertheless, exceptions were found in the osteosarcoma cell line SaOS-2 and the bone marrow-derived cell line KG-1 (Fig. 4), where the specific binding of MBP-preS but not MBP alone was observed.

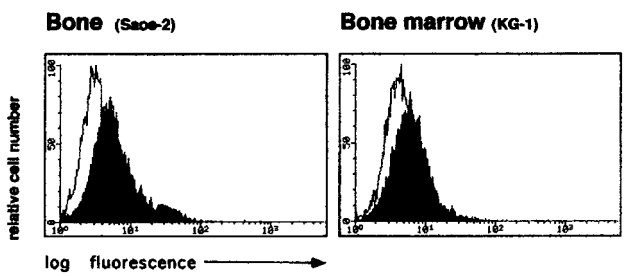

FIG. 4. Detection of preS-binding on SaOS-2 and KG-1 cells. After screening of various cell lines from extrahepatic origin, two cell lines were identified where MBP-preS binding was observed. Filled histogram shows staining of MBP-preS, while unfilled histogram indicates the negative control staining using recombinant MBP.

Immunoprecipitation of preS-Specific Binding Proteins

Cell surface biotinylation provides a powerful mean for the isolation of membrane proteins, which are then detected by enzyme- or fluorescence-conjugated streptavidin. Furthermore, the use of water-soluble biotins such as sulfo-NHS-biotin enables the biotinylation under physiological conditions so that these biotinylated cells can be further used in ligand-binding assays or for other manipulations before performing immunoprecipitations of proteins in interest.

In order to isolate preS-binding receptors from extrahepatic cells, the same strategy was employed to label membrane proteins of an extrahepatic cell line. For this, the EBV-transformed human B-cell line Wa, which has been previously described to possess preS-binding activity (3), was harvested from a stationary culture and resuspended in biotin-labeling buffer. The biotinylated cells were then further used for incubation with MBP-preS proteins, and immunoprecipitation itself was performed by building an immunecomplex

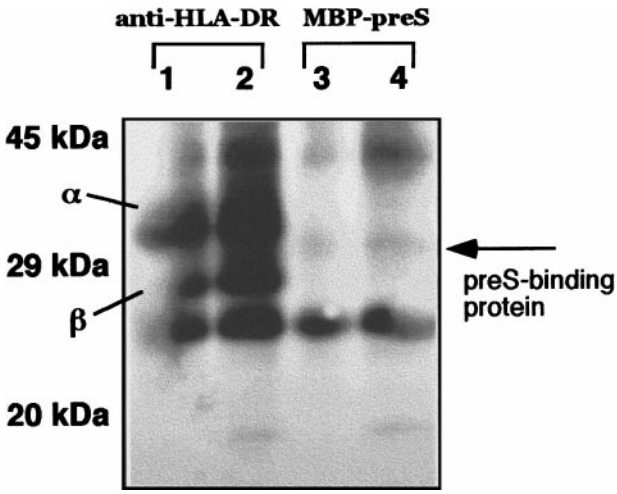

FIG. 5. Immunoprecipitation of preS-binding proteins from Wa cells. The preS-binding proteins on Wa cells were immunoprecipitated using HAM-19-conjugated anti-MBP immunoaffinity resins after cell surface biotinylation and incubation of Wa cells with MBP-preS proteins. Immunoprecipitated proteins were then released from the beads by boiling and these were then separated in a 12.5% SDS–polyacrylamide gel. Protein bands derived from Wa-cell lysate was identified by peroxidase-conjugated streptavidin and chemiluminescence (ECL, Amersham Pharmacia Biotech.). As positive control for showing the fidelity of this assay, HLA-DR molecules were coimmunoprecipitated using L243, anti-HLA-DR-specific monoclonal antibody-conjugated resins.

with the MBP-preS proteins using anti-MBP antibodies prior conjugated to Sepharose beads. For this, the anti-MBP mouse IgG1 mAb (HAM-19, 36) was bound to protein A Sepharose beads to get optimal orientation of the primary antibody for binding of MBP-preS proteins. Figure 4 shows the result of this assay as performed with HAM-19-conjugated protein A Sepharose beads or with the anti-HLA-DR monoclonal antibody L243-conjugated protein A Sepharose as control. While the L243-conjugated beads successfully precipitated two protein bands corresponding to the α- and β-chain of the HLA-DR complex, HAM-19-conjugated beads immunoprecipitated a single molecule of $\sim$30 kDa, which is regarded as the putative HBV-preS binding protein on Wa cells (Fig. 5).

DISCUSSION

The major target organ for the hepatitis B virus is the liver. Nevertheless viral DNA as well as viral proteins are also frequently detected in various extrahepatic sites, including lymphocytes, pancreatic islet cells, renal tubule epithelial cells, testicular spermatogenic cells and so on (5, 33, 50). Such reports implicate the possibility of viral replications also in extrahepatic cells, which is in fact also observed in clinical cases even though in a limited fashion (28). What consequences such extrahepatic infections might have are well described in studies, where extrahepatic carcinoma were observed by chromosomal integration of HBV DNA in lung cells (1) or where these extrahepatic sites served as sanctuary in evading the immune surveillance resulting in recurrent HBV infections (21, 15).

Since tropism of the virus mainly determines the site of infection, analysis of the distribution of putative HBV receptor expression will facilitate the understanding of HBV pathogenicity and transmission pathways. To clarify this issue, in this study, recombinant HBV envelope proteins (MBP-preS, Fig. 1) were used to simulate the attachment of virions to putative target cells. The preS region is known to contain the major binding site for attachment and internalization into the host cell, and native or recombinant preS proteins as well as synthetic peptides derived from the preS region have been successfully employed in determining HBV susceptibility (41, 43).

The validity of this assay system was confirmed in the present study by flow cytometric analysis, which is shown in Fig. 2. Here, preS protein binding was specifically detected on HepG2 cells, which is a representative hepatic cell line and on which the presence of preS-specific cellular receptors has been already proved on prior occasions (3, 26, 43). Based on these results, further experiments were performed with other cell lines, and here it is to see that nearly all of the extrahepatic cell lines as used in this study had no preS-binding activity. Neither Tera-1, which is a testicular cancer cell line, nor the pancreatic carcinoma cell lines Capan-2 showed interaction with the preS protein, which was also the case with cells from other tissue origins such as kidney, lung and brain etc. Since the tissue origins of the analyzed cells also comprised cells and organs where in other studies HBV DNA and proteins have been observed, it was questioned why there were disagreements between preS receptor expression and HBV infectivity. That is, while tissues such as testis, pancreas, and lung cells are known to be susceptible to HBV infection, the present study shows the lack of preS receptor expression in the corresponding cell lines. A possible explanation for this discrepancy might be that while the preS-mediated internalization pathway would be the major infection route, that also alternative infection pathways would exist, which are working independent of preS-binding. The interaction of the S-region with annexin V (7) as well as receptor-independent membrane-fusion mechanisms (13) might be some possibilities which would explain the absence of preS-binding receptor on HBV-susceptible extrahepatic cells. Albeit such reasoning, the lack of preS binding activity on most of the analyzed extrahepatic cells was still controversy. For example, the failure of preS-binding to the T-cell line Hut-78 was also rather embarrassing (Fig. 3) since in a previous study the transferrin receptor on T-cells had

been supposed to be the putative cellular receptor for HBV. This interaction was mapped to the preS2 and S-region of the HBV envelope region (8) and hence Hut-78 cells do express transferrin receptors on their cell surface (27), a specific binding of preS proteins was expectable. Nevertheless, most of the current results on the distribution of preS-specific receptors on extrahepatic cell lines confirms the classical study performed a decade ago by Neurath *et al.* (1990), where serum-purified HBsAg proteins were used for screening of HBV receptors on extrahepatic cells. These authors reported in that study that most extrahepatic cell lines including the T-cell lines CEM, MOLT-3 and HUT-102B as well as the cell lines MCF-7 and Capan-1 etc. were lacking preS binding activity. Thus in agreement with such prior studies, it can be concluded that the expression of preS-specific receptors seems to be rather restricted to hepatic tissues.

The interaction of preS to the bone marrow-derived cell line, KG-1 (Fig. 4) was in this context an exception where preS-binding activity was present. On the other hand, HBV infection in lymphoid cells, in particular in the bone marrow, has been also reported previously by other groups so that this observation for itself is not novel (39). Indeed the bone marrow seems to serve as an effective site for the HBV to hide itself from immunsurveillance during viral clearance, and the virus might then induce recurrent infections from these tissues (15). The detection of HBV DNA in leukocytes and bone marrow aspirates as observed by others (12, 23) as well as in the bone marrow-derived cell line by ourselves (Fig. 4) urge for the re-examination of lymphoid cells as potential targets for HBV.

The observation of preS binding to SaOS-2 osteosarcoma cells, on the contrary (Fig. 3), raised questions about the biological meaning of this result. Studies on the association between HBV infection and the bone are extremely rare so that in contrast to bone marrow cells neither osteoblasts nor osteoclasts as well as osteosarcoma and other cells from the bone have been brought in connection with HBV. The mineralizing osteosarcoma SaOS-2 that has been used in this study expresses several phenotypic characteristics of primary human osteoblasts (46), and in this regard literature search upon osteoblasts infections by HBV was performed, which however gave no results. Indeed HBV infection seems to have no relation with bone cells, except for some reports where oesteopenia were observed during chronic hepatitis (30), which however is to be addressed to a rather indirect effect of HBV infection. Therefore, the preS-binding activity in SaOS-2 cells must be analyzed in another context. Rather than representing the situation *in vivo,* the binding of preS proteins to SaOS-2 cells might be due to the expression of some cellular receptors that are merely expressed on this particular cell line without further involvement in HBV infection. This might be possible when regarding the fact that osteoblasts are origined from the same pluripotent stem cells as hematopoietic cells and stroma cells in the bone marrow (16). In this regard, some shared cell surface molecules with these cells could be expressed in SaOS-2, which would then serve as the preS binding proteins in this case. For example, c-kit expression on SaOS-2 cells is a well documented fact (10), whereby c-kit is also detected on a wide range of lymphoid cells including T- and B-cells as well as hematopoietic precursor cells (20). Furthermore, cytokine receptors that are mostly expressed on lymphatic cells are also detected on SaOS-2 cells, that include the IL-2 receptor γ-chain, the interleukin-7 receptor, GM-CSF receptor and 4-1BB (24). Thus the common binding property of bone marrow cells and bone cells as well as that of the EBV-transformed B-cell line Wa (3 and Fig. 2) might have been resulted from the expression of a receptor molecule, that is commonly expressed on cells of hematopoietic origin.

While this might explain the current observation, on the other hand, it is also contradictory in itself in that liver cells, which are not derived from hematopoietic stem cells, nevertheless are infected by HBV. As well as that also other cell lines from hematopoietic origin such as the T-cell line HuT-7 and other B-cell lines (3) are negative for preS-binding. To clarify this issue, the best approach would be the direct isolation of preS-binding receptor proteins from these cells and to identify and characterize the preS-binding protein. In an attempt to isolate such preS-binding receptors from extrahepatic cells, in the present study, recombinant MBP-preS proteins were used to immunoprecipitate such proteins from the EBV-transformed B-cell line Wa. These cells are positive in their preS-binding property (Fig. 2C) and are also easily to maintain and to culture for mass production, so that these cells were regarded as good starting materials in the identification of extrahepatic preS receptors. By cell surface biotinylation and immunoprecipitation of preS binding proteins, a ~30 kDa protein that selectively binds to the preS protein (Fig. 4) was identified. This protein was not observable when immunoprecipitation was performed with the same cell lysate but with different immunoaffinity beads for protein-binding. As shown in the control immunoprecipitation with anti-HLA-DR antibodies-conjugated protein A agarose beads, only the α- and β-chain of the HLA-DR complex was visible.

The present observation is not the sole report on the isolation of potential HBV receptors (4, 43, 48). However it is remarkable insofar that it shows the physical presence of preS-binding proteins also on cells from extrahepatic origin, which was so far largely neglected. Whether this molecule might be identical to the 30 kDa receptor protein isolated from human liver cells as reported in the study of Dash *et al.* (4) is not evident. Also the difference to other putative HBV receptors as

have been reported for the asialoglycoprotein (48) or other unidentified molecules of higher molecular weight (43) is not clear. For the examination of this matter, further studies regarding the biochemical characterization, such as the analysis of the glycosylation content and amino acid analysis of this immunoprecipitated protein, are required. Also the isolation of and comparison to preS-binding proteins from other cell lines, both of hepatic and extrahepatic origin, will be the focus of further studies.

It is clear that the current study using various established cell lines can not project the accurate situation *in vivo*, since many cells are transformed and might have been further differentiated *in vitro* so that they could have lost some essential characteristics of the original representing tissues. In this regard, the mere binding of preS proteins to certain cells might have little relevance to their transmissibility of HBV *in vivo*. Nevertheless, the screening for preS binding protein expression on various cell lines from extrahepatic origin will provide a draft figure on the mechanism of the mainly hepatotropic feature of HBV in its pathogenic mechanism.

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