J. Microbiol. Biotechnol. (2000), **10**(6), 844–850

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

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Receptor-Mediated Endocytosis of Hepatitis B Virus PreS1 Protein in EBV-Transformed B-Cell Line

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Received: August 30, 2000
Accepted: November 22, 2000

Abstract The specific binding and internalization of viral particles is an essential step for the successful infection of viral pathogens. In the case of the hepatitis B virus (HBV), virions bind to the host cell via the preS domain of the viral surface antigen and are subsequently internalized by endocytosis. HBV-preS specific receptors are primarily expressed on hepatocytes, however, viral DNA and proteins have also been detected in extrahepatic sites, suggesting that cellular receptors for HBV may also exist on extrahepatic cells. Recently, an EBV-transformed B-cell line was identified onto which the preS region binds in a receptor-ligand specific manner. In this study, this specific interaction was further characterized, and the binding region within the preS protein was localized. Also the internalization after host cell attachment was visualized and analyzed by fluorescence-labeled HBV-preS1 proteins using confocal microscopy. Energy depletion by sodium azide treatment effectively inhibited the internalization of the membrane-bound preS1 ligands, thereby indicating an energy-dependent receptor-mediated endocytotic pathway. Accordingly, the interaction of HBV-preS1 with this specific B-cell line may serve as an effective model for an infection pathway in extrahepatic cells.

Key words: Hepatitis B virus, preS region, infection, Receptor, epstein barr virus, B-lymphocytes, confocal laser scanning microscopy

The viral envelope not only protects the virus nucleocapsid from chemical and physical damage, but also plays an important role in the binding and internalization of the virus into its host cell [27]. In the case of the hepatitis B virus (HBV), the viral envelope consists of three glycoproteins embedded in a lipid bilayer. All of these proteins are encoded from a single gene, *env* which is transcribed from three alternative start points [6, 29]. Depending on the lengths of the transcripts, the protein products are differently termed. The shortest protein is the small (S) protein and the longest the large (L) protein, of which the L protein consists of the S protein with an additional 174 amino acid residues. (the preS region) in its N-terminal. This preS region can be further subdivided into two regions, the 119 amino acid preS1 region and the shorter, 55 amino acid preS2 region. The third surface protein is a product of the S protein with the preS2 region and is called the middle (M) protein.

A substantial number of reports have been written about the role of the preS region in viral pathogenesis (reviewed in [3]). In particular, a function as a specific binding domain to the viral host cell has been shown for preS1 [18] as well as for preS2 [21], independently. Currently, it is widely accepted that HBV is bound by the preS domain, either directly or via some intermediate receptor proteins, to the cellular receptor on the host cell [12]. In contrast to these extensively investigated receptor-ligand interactions, the early events after host cell attachment are still the subject of contradictory reports. Neither an endocytotic mechanism nor an alternative nuclear transport and uncoating of the viral envelope have been definitively assigned. Especially, the uptake of viral particles into hepatocytes has been regarded as a conflict point, since data supporting either a direct fusion mechanism with the outer cell membrane [24] as well as an energy-dependent receptor-mediated endocytotic pathway have both been reported [11].

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The evaluation of the precise infection mechanism is of great interest not only for analyzing the pathogenicity in hepatocytes but also for understanding the impact of HBV infection in extrahepatic tissues [2, 31]. The presence and significance of this aspect in HBV infection has been largely ignored mainly due to the major pathogenic feature of HBV involving the liver as the primary target organ. However, along with the accumulation of data locating HBV in extrahepatic sites, the search for possible candidate receptor protein(s) on extrahepatic cells is also ongoing. Whilst the identity of the HBV receptor is still unclear, the presence of virus receptors can be detected by binding assays of the preS region to the corresponding cells. In this context, Neurath *et al.* [16] showed that the preS region not only binds to hepatocytes but also to some cells of extrahepatic origin. Furthermore, in a recent study [4], an EBV-transformed B-cell line, Wa-cells [7], was identified onto which preS region-derived recombinant proteins bind.

In the current study, to investigate whether the binding of preS in these cells was mediated via the preS1 or the preS2 region, recombinant viral surface proteins were labeled with ^{125}I and used in a ligand binding assay. Also to determine whether this specific binding may be followed by receptor-mediated endocytosis, the cellular events after the binding of the viral ligands were monitored, and the energy dependency was determined by sodium azide treatment. For these experiments, the entire preS and preS1(1-56) regions were expressed as recombinant proteins (Fig. 1) in fusion to the maltose binding protein (MBP) and isolated to high purity [4, 23]. Visualization of binding and the subsequent internalization of the viral ligand proteins were achieved by fluorescence labeling of the recombinant proteins and analysis by confocal laser scanning microscopy. As a result, it was shown that the preS1(1-56) region was responsible for the preS protein binding to the B-cell line Wa, and that the internalization of the preS(1-56) protein followed an energy-dependent, receptor-mediated endocytotic pathway.

MATERIALS AND METHODS

Cell Culture

The EBV-transformed B-cell line "Wa" was a kind gift from Dr. Keisuke Sato, Asahikawa Medical School, Japan. The cells were maintained in an RPMI-1640 medium (Gibco, Grand Island, U.S.A.) supplemented with 10% FCS (Gibco), 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 20 μM β-mercaptoethanol at 37°C in a humidified CO_2 incubator [1, 8]. During extensive culture of the Wa-cells, a subpopulation of these cells changed their morphological phenotype to fibroblast-like attached growing cells, which is in contrast to the usual phenotype of EBV-transformed cells growing in clusters. It was confirmed that the acquisition of this phenotype was reversible such that attached growing cells released from the flask bottom could generate both normal clustering as well as attached growing Wa-cells. For a separate analysis, Wa-cells growing attached to the culture flasks were harvested by cell-scraping and extensive washing of the flask bottom with phosphate buffered saline (PBS) after the removal of the floating cells.

Expression and Purification of MBP-preS(1-174) and MBP-preS1(1-56)

The expression and isolation of the HBV-preS(1-174) region as a fusion product with the maltose binding protein (MBP) were performed as previously described [4, 9]. The genetically engineered *Escherichia coli* expressing a fusion protein of MBP and the HBV-preS1(1-56) region was a kind gift from Dr. H. J. Hong (KRIBB, Taejon, Korea). The expression and purification of these recombinant proteins were performed as described previously [23]. Briefly, IPTG-induced cells were disrupted by sonication, and the supernatant was dialyzed against a binding buffer (20 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, pH 7.4) and afterward purified by DEAE-Sepharose CL-6B and gel filtration.

Ligand Binding Assay

For each receptor-ligand binding assay, 1×10^5 Wa cells were transferred into Eppendorf tubes and incubated with either iodinated MBP-preS1(1-56), MBP-preS(1-174) or MBP alone at various concentrations for 2 h at 4°C. The corresponding ligand proteins were iodinated using an IODO-BEADS® iodination kit (Pierce, Rockford, U.S.A.) following the protocol as described by the supplier. After incubation, cells were extensively washed with PBS by repeated centrifugation at 1,600 rpm for 5 min until the supernatants showed no radioactivity. The cpm for the bound ligands on the cell surface was determined by a γ-counter (Beckman Gamma 5500, Irvine, U.S.A.). The non-specific binding was defined by the competition of the excess amounts of unlabeled ligands with each of the corresponding fusion proteins or MBP at the same concentration as above for 2 h at 4°C.

FITC Labeling of MBP-preS1(1-56) and Hen Egg Lysozyme (HEL)

For FITC conjugation, the concentration of each protein was adjusted to 1 mg/ml in an FITC-labeling buffer (0.05 M Boric acid, 0.2 M NaCl, pH 9.2) and incubated with 100 μg FITC per mg protein at room temperature. The reaction was stopped after 2 h by the addition of a tenth volume of 0.1 M glycine, and the free FITC was removed by gel filtration using Sephadex-G25 (Pharmacia Biotech, Uppsala, Sweden).

Confocal Microscopy and FACS Analysis

The binding and internalization of the FITC-labeled ligands were visualized by confocal laser scanning microscopy (CLSM) [13]. Each 5×10^5 Wa-cells was transferred into a round-bottomed 5 ml centrifuge tube (Falcon, Lincoln Park, U.S.A.) and incubated with 40 μg/ml of either MBP-preS1(1-56)-FITC or FITC-labeled hen egg lysozyme (HEL) for different time intervals. To inhibit any receptor-mediated internalization, the cell suspension was treated with 0.05% sodium azide during ligand binding. After incubation, the excess ligands were removed by extensive washing with PBS, and the cells were mounted on slide glasses for microscopic analysis. The CLSM was performed using a Leica TCS 4D connected to a Leica DAS upright microscope (Leica Lasertech GmbH, Heidelberg, Germany). For the FACS analysis, the cells were treated in the same manner as for the CLSM. After removal of any excess ligands, the cell pellet was resuspended in 500 μl PBS, and the cells were analyzed by a FACScan (Becton Dickinson, San Jose, U.S.A.).

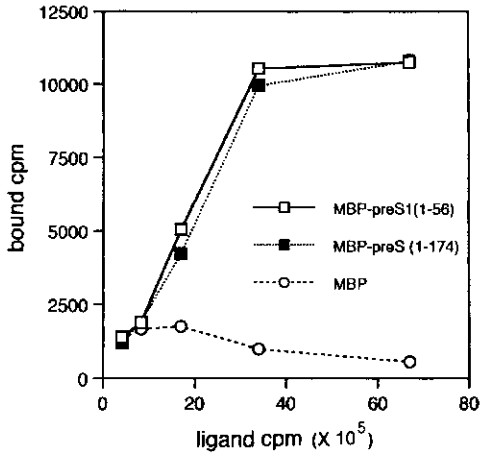

Fig. 2. Ligand binding assay with Wa-cells.
Each 1×10^5 of Wa-cells was transferred into Eppendorf tubes and incubated with ^{125}I-MBP-preS1(1-56), ^{125}I-MBP-preS and ^{125}I-MBP at various concentrations for 2 h at 4°C. After extensive washing, the bound cpm was detected using a gamma counter. The results are expressed as the specific bound cpm by subtracting the background signals.

RESULTS

Specific Binding of preS1(1-56) to EBV-Transformed B-Cell Line, Wa

As regards the receptor-binding epitope within the preS domain, the preS1(21-47) as well as the entire preS2 region have been independently described as putative ligands for the receptor on the host cell [17, 21, 30]. Accordingly, to define which part of the preS is actually involved in binding to Wa-cells, ligand binding assays were performed using iodinated MBP-preS, that is the preS(1-174) region, and MBP-preS1(1-56) as well as with MBP alone as the negative control (Fig. 1). Figure 2 shows the measured cpm of each ligand after incubation with Wa-cells. While iodinated MBP alone only showed a background level, MBP-preS as well as MBP-preS1(1-56) specifically were bound to Wa-cells, reaching a plateau of binding activity when included in excess amounts (>3.4×10^6 cpm, corresponding to 35 μg/ml of the corresponding ligand). Since this signal plateau indicates the receptor saturation on these cells, it would appear to confirm might the presence of a specific receptor-ligand interaction between the preS region and the Wa-cells [22]. Regarding the specificity of this receptor, the results as shown in Fig. 2 suggest a rather restricted binding to the preS1(1-56) part of the preS region. As indicated in the saturation curve, the bound cpm in the case of incubation with preS1(1-56) was nearly identical to that of the entire preS region, and the additional preS1 and preS2 parts apparently had a minimal effect in ligand binding.

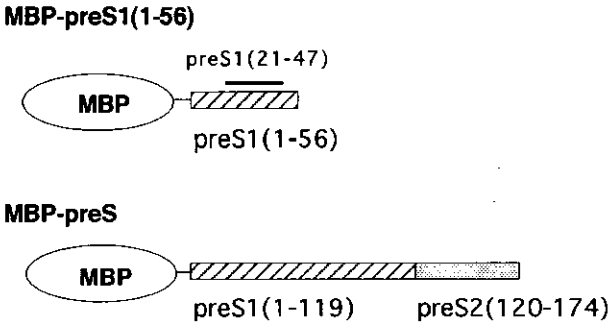

Fig. 1. Schematic representation of the recombinantly expressed preS region proteins.
The preS1 and the entire preS regions were recombinantly expressed in C-terminal fusion to the maltose-binding protein.

Receptor-Mediated Endocytosis of preS1(1-56)

To examine whether and how these receptor-ligand complexes are internalized, the events after the binding of preS were monitored by confocal microscopy. To synchronize the binding and internalization, the Wa-cells were first incubated with the FITC-labeled MBP-preS1(1-56) protein for 3 h min at 4°C. Thereafter the cells were either left under these conditions or transferred into a 37°C environment and incubated for a further hour. As shown in Fig. 3, in the former case, the preS ligands were only detected on the cell surface (A), however, after the temperature shift to 37°C, when active endocytosis can occur, the surface-bound ligands were transported into the cytoplasm (B). In the latter case, a strong signal was detected throughout the cytoplasm which proves that, instead of a non-specific diffusion, an active internalization of the receptor bound preS1(1-56) was responsible for this observation. For a further evaluation of the internalization mechanism, the energy-dependent activities were inhibited by treatment with sodium

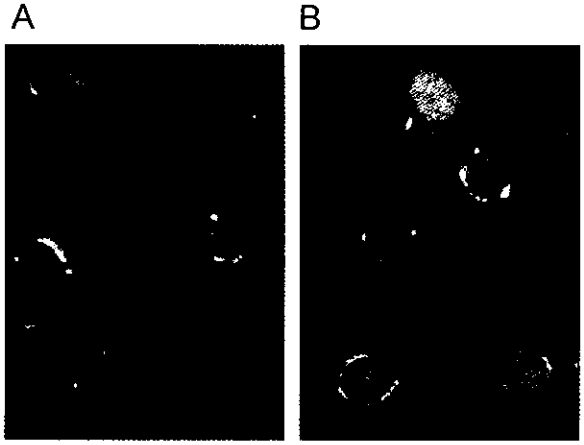

Fig. 3. Receptor-mediated endocytosis of preS1(1-56) ligands in Wa-cells as analyzed by confocal microscopy.
Each 5×10^5 of Wa-cells was incubated with 40 µg/ml of FITC-labeled MBP-preS1(1-56) at 4°C for 3 h. Then the incubation temperature was either shifted to 37°C to enable active endocytosis of the receptor/ligand complexes or left at 4°C. The cells were harvested after 60 min, and the images were analyzed by confocal laser scanning microscopy. The cell image is shown at a 1,000 × magnification.

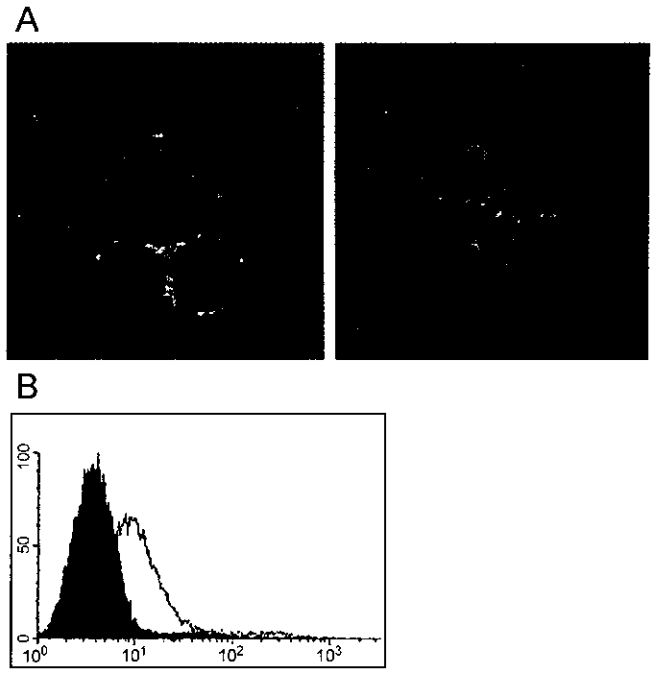

Fig. 4. Inhibition of receptor-mediated endocytosis by sodium azide.
5×10^5 Wa-cells were incubated in 40 µg/ml of FITC-labeled MBP-preS1(1-56) at 37°C with or without sodium azide for 2 h. After removal of the excess ligands, the distribution of the fluorescence labeled preS1(1-56) proteins on the Wa-cells was analyzed by confocal laser scanning microscopy. A. On the left is the Wa-cells incubated with MBP-preS1(1-56)-FITC under normal conditions, whereas on the right is the Wa-cells incubated with MBP-preS1(1-56) in the presence of 0.05% sodium azide. B. The quantitative analysis of the fluorescence signal intensity was performed on the same cells as used for the CLSM by FACS. The histograms show the signal intensity of the specifically bound preS proteins (filled histogram) compared to the binding and internalization of the negative control protein, hen egg lysozyme (unfilled histogram).

azide. Since receptor-mediated endocytosis is an ATP-driven, energy-dependent process, the blockage of oxidative phosphorylation by sodium azide was expected to result in a failure of active internalization [10]. The left panel in Fig. 4A shows the normal situation of the binding and internalization of preS1(1-56) in Wa-cells after incubation at 37°C for 2 h. In contrast, the addition of 0.05% sodium azide prior to ligand incubation effectively inhibited the internalization of the receptor ligand complexes (Fig. 4A right).

To confirm the specificity of this assay as well as compare the receptor binding-mediated signal to non-specific internalization by endocytosis, Wa-cells were either incubated with FITC-labeled preS(1-56) proteins or with FITC-labeled hen egg lysozyme for 2 h at 37°C. After the removal of the excess ligands, the cells were analyzed by fluorescence-activated flow cytometry (FACS) and their relative signal intensities compared to each other. Fig. 4B shows the result of this experiment, where a much higher signal was found to be generated when the cells were incubated with the receptor-specific ligand preS1(1-56).

Expression of preS1(1-56) Specific Receptors on Normal and Attached-Growing Wa-cells

After establishing the specific binding and receptor-mediated endocytosis of preS1(1-56), the next question concerned the identity of the cellular receptor. While there have been several previous reports regarding the identification of possible candidates for the HBV receptor, no definite receptor protein has been isolated so far. To examine the nature of the HBV preS receptor on this specific EBV-transformed B-cell line, it first needs to be questioned why this cell line expresses this receptor, which is not detectable in other B-cell lines [4]. Therefore, the regulatory mechanism behind the expression of this receptor was the primary subject of analysis. For this, a special feature of Wa-cells was used for further investigation. As described in Materials and Methods, Wa-cells grow in two different, interchangeable phenotypes in a tissue culture. One is the usual cluster-forming phenotype, typical for EBV-transformed B-cells, and the other is a rather epithelial-cell-like morphology growing attached to the bottom of culture flasks (Fig. 5). Besides these morphological changes, a drastic differentiation in the cell surface markers was observed between these two phenotypes. As shown in Fig. 6, the FACS analysis revealed that the attached growing Wa-cells down-regulated their expression of MHC-class I as well as class II molecules when compared to the normal suspension culture. Based upon this observation, it was then investigated whether the expression of the preS1 receptors on the Wa-cells also was dependent on these control mechanisms. However, when using the same incubation protocol as for the confocal

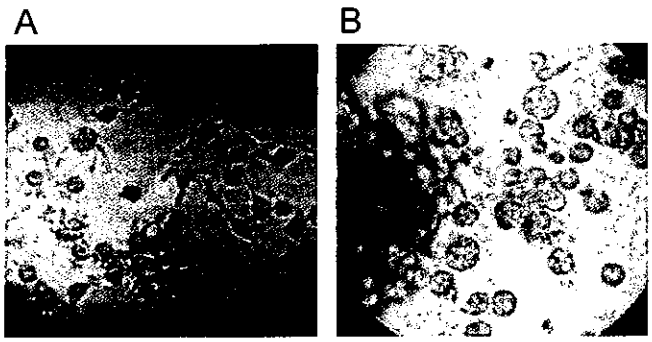

Fig. 5. Microscopic analysis of attached-growing versus normal Wa-cells.
The extensive culture of Wa-cells resulted in the appearance of a morphologically changed subpopulation which grew attached to the culture dishes and showed a phenotypic similarity to fibroblasts. A. attached growing Wa-cells, B. normal Wa-cells growing in suspension as small clusters. Each cell population was grown in separate culture flasks and the cells were analyzed by a phase contrast light microscope with a 200 × amplification.

microscopy, MBP-preS1(1-56) was shown to bind to the attached-growing Wa-cells with the same fluorescence intensity as to Wa-cells in the suspension culture (Fig. 6C).

DISCUSSION

The major target organ of the hepatitis B virus is the liver. Nevertheless, there have also been reports about the presence of HBV in extrahepatic cells. Since the first report of HBV DNA in extrahepatic sites by Lie-Injo *et al.* [14], viral DNA as well as viral proteins have been detected in various cells such as mononuclear blood cells, bone marrow cells, the kidney, and pancreas *etc.* [20, 25]. In particular, the detection of HBV in blood leukocytes had been regarded as a possible explanation for some immunological abnormalities as well as functional and morphological alterations of lymphocytes in HBV infected patients. While there has been speculation about the identity of the cellular receptor for HBV, cells expressing putative HBV receptor proteins can be identified based on their binding property to the preS region of the viral surface antigen. Using this epitope, either in the form of synthetic peptides or recombinant proteins, a large array of extrahepatic cells have been identified on which HBV receptors are expressed [16]. Recently, an EBV-transformed B-cell line was identified, which also exhibited a binding specificity to the preS region of HBV [4]. However, in this case, in all the other extrahepatic cell lines described so far, no further investigations have been performed on the events following the binding of the viral ligands. To pursue this question in the case of the EBV-transformed B-cell line "Wa", first, the specificity of the HBV preS receptors on these cells was determined. Recombinant MBP-preS, MBP-preS1(1-

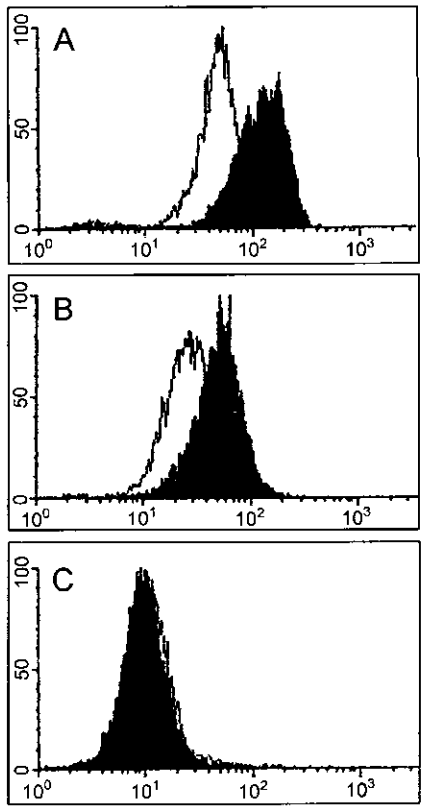

Fig. 6. FACS analysis of attached growing and normal Wa-cells.
Each 2×10^5 Wa-cells of the attached growing or normal phenotype was harvested and analyzed by FACS. The expression of MHC class I and class II was detected by the mouse monoclonal antibodies, W6/32 (A) and L243 (B), respectively, followed by FITC-labeled anti-mouse Ig secondary reagents. The expression of the preS1 binding receptors were detected by FITC-labeled MBP-preS1(1-56) proteins (C). The open histograms represent the distribution of the fluorescence intensity of the attached growing Wa-cells, whereas the closed histograms show that of the normal Wa-cells.

56), and MBP were iodinated and used in a ligand binding assay. The results (Fig. 2) show that the binding kinetics of MBP-preS1(1-56) were identical to those of the MBP-preS protein, which covers the entire preS region. It is therefore obvious that the HBV preS receptors on Wa-cells are specific for the preS1 region. This is also in agreement with previous reports describing potential HBV receptors on liver-derived cells, like the hepatoma cell line HepG2 or primary hepatocytes, on which the preS1 region alone was sufficient for virus binding and internalization [5, 15, 19, 28]. To examine whether this binding structure on Wa-cells can also function as a receptor for the active endocytosis of viral particles, the following events after ligand binding were monitored by confocal microscopy. Using this device, living cells can be optically sectioned and the intracellular events monitored without disrupting the cell membrane or other cellular structures. As shown in Fig. 3, after incubation for 3 h in the cold, fluorescence

labeled ligands were only detectable on the cell surface, thereby indicating the necessity for an active mechanism in the internalization of the receptor-ligand complexes. With a subsequent temperature shift to 37°C, where physiological activities are reactivated, the plasma membrane-bound ligands were rapidly internalized, resulting in a broad intracellular distribution outside of the nucleus (Fig. 3). The specificity of this assay was further confirmed using an FITC-labeled hen egg lysozyme, a 14 kDa small protein, which has no specific receptor expressed on mammalian-cells, and which is receptor-independently internalized into B-cells [26]. Here it was shown that under the same conditions, only a trace amount of non-specifically internalized proteins were detectable (Fig. 4B). These observations prove that the preS1 binding receptors on Wa-cells not only serve as binding structures, but also act as putative mediators for the entry of virus particles into the host cells, since *in vivo*, not only preS1 protein fragments but the whole virus particle with protruding preS1 regions would interact with these receptors.

To further establish that this internalization was not an artifact due to the higher permeability of the plasma membrane when raising the incubation temperature, the endocytosis was chemically inhibited at 37°C by incubation with sodium azide. As expected, sodium azide (0.05% final conc.) effectively suppressed the endocytosis of the preS1(1-56) ligands, which showed that this was an energy-dependent cellular process. Accordingly, the present data documents the first description of a cell line of extrahepatic origin, where the HBV surface antigen not only specifically binds but is also actively internalized by receptor-mediated endocytosis. This observation also supports the hypothesis of an uptake of HBV particles by endocytosis instead of the direct fusion of the viral envelope with the outer cell membrane. The specificity of the HBV receptors on Wa-cells implicates that this receptor protein may be similar, if not identical, to those expressed on hepatic cells. Therefore, the identification of this extrahepatic receptor may also have consequences for the analysis of HBV receptors on hepatic cells. While experiments are currently under way for the identification of this preS-binding protein using immunoprecipitation and other molecular biological methods, an initial attempt to evaluate the regulatory mechanism of this receptor protein was presented in this study. Besides the characteristic phenotype of expressing preS1-binding proteins, Wa-cells exhibit another interesting phenotypic feature by growing in two alternative states (Fig. 5), that is either as adherent cells or in a clustering suspension. To investigate whether the expression of the preS receptor was also affected by this morphological and phenotypic change, attached-growing or normal clustering Wa-cells were isolated, and the expression of the preS receptor was analyzed. As shown in Fig. 6C, no difference was detected regarding the level of HBV-preS1(1-56) receptor expression, which was in contrast to the down regulation of the MHC class I and class II molecules (Fig. 6A, B). Without further knowledge about the identity of this putative HBV receptor, the results reported so far lead to the conclusion that this receptor protein is rather constitutively expressed on Wa-cells, and is independent of the cell morphology of these cells. As such, the preS-binding property would appear to be an acquired ability by transformation with EBV, plus the induction and regulation of preS-receptor expression would seem to be rather independent of cell conditions.

Whether this receptor protein is indeed characteristic of EBV-transformed B-cells or singularly expressed on Wa-cells will be revealed by further investigation with a larger array of EBV-transformed cell lines. Nevertheless, the identification of a functional HBV receptor candidate in an extrahepatic cell line as well as the similar binding specificity to receptors on hepatic cells provides a more precise picture of the pathogenicity in extrahepatic cells as well as the infection and transition mechanism of HBV into tissues and organs other than the liver.

Acknowledgments

This study was supported in part by a grant (HS2470) from the Ministry of Science and Technology, Republic of Korea.

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