Human hepatitis B virus (HBV), a hepatotropic DNA virus, is a leading cause of human hepatitis.

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2 The abbreviations used are: HBV, human hepatitis B virus; HBeAg, e antigen of HBV; mIL-1RAcP, membrane form of interleukin-1 receptor accessory protein; IL-1, interleukin-1; IL-1RI, type 1 interleukin-1 receptor; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; iκB, inhibitor of NF-κB; HCC, hepatocellular carcinoma; Th2 cell, T helper 2 cell; Th1 cell, T helper 1 cell; GST, glutathione S-transferase; CMV, cytomegalovirus; HA, hemagglutinin; (His)6, polyhistidine; IL-6, interleukin-6; TNF, tumor necrosis factor; MIP-1α, macrophage inflammatory protein-1α; iNOS, inducible nitric-oxide synthase; GM-CSF, granulocyte-macrophage colony stimulating factor; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; IFN, interferon; IL-10, interleukin-10; siRNA, small interfering RNA; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate; TK, thymidine kinase; RT, reverse transcriptase.
HBeAg and IL-1RAcP

stress, and tissue damage (19). IL-1 also functions as a costimulator to activate Th2 cells (20, 21). Binding of IL-1 to type I IL-1 receptor (IL-1RI) leads to recruitment of membrane form of IL-1RAcP (mIL-1RAcP), which is essential for signal transduction (22). This is followed by recruitment of several intracellular adaptor proteins and kinases, including Toll-interacting protein (Tollip), MyD88, and members of the interleukin-1 receptor-associated kinase family. Tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) is also recruited transiently to the receptor complex and triggers signal events that culminate in the activation of IκB kinase complex and specific mitogen-activated protein kinase kinase kinases. Activated IκB kinases phosphorylate the NF-κB inhibitor IκB, leading to the degradation of IκB and activation of NF-κB, whereas activated mitogen-activated protein kinase kinase kinases phosphorylate and activate c-Jun N-terminal kinase and p38 mitogen-activated protein kinase. IL-1 signal transduction ultimately leads to the activation of IL-1-responsive genes (19, 23).

In this study, we show that HBeAg interacts and colocalizes with mIL-1RAcP on the cell surface. Furthermore, HBeAg is able to induce the interaction of IL-1RI with mIL-1RAcP, trigger the recruitment of adaptor protein MyD88 to the IL-1RI/mIL-1RAcP complex, activate NF-κB through IκB-α degradation, induce NF-κB-dependent reporter gene expression, and induce the expression of IL-1-responsive genes. Silencing IL-1RAcP dramatically abolishes HBeAg-mediated NF-κB activation. Our observations reveal an important function of HBeAg. The physiological significance of this interaction is discussed.

EXPERIMENTAL PROCEDURES

Vectors, Reagents, and Antibodies—Vector pGEX-3X, Ready-to-Go™ kit, nickel-agarose Hitrap chelating column, anion exchanger Hitrap Q column, protein A-agarose slurry, [35S]methionine (1000 Ci/mmole), and [γ-32P]ATP (3000 Ci/mmole) were purchased from Amersham Biosciences. NF-κB-driven firefly luciferase plasmid, pLexA-BD, and MATCHMAKER liver cell oligo(dT)-primed library in pB42AD for yeast two-hybrid screen were from Clontech. Antibody against HCV core protein was from DakoCytomation. IL-1RAcP and control small interfering RNAs (siRNAs) were from Dharmacon. TNF-α and granulocyte macrophage-colony-stimulating factor (GM-CSF) ELISA kits were from Endogen. Anti-mouse IgG-TRITC conjugated and anti-rabbit IgG-FITC conjugated antibodies were from Jackson ImmunoResearch. Vector pRSETB was a gift by Dr. Kin-Fu Chak, Institute of Biochemistry, School of Life Science, National Yang-Ming University (24).

Expression Vectors—For bacterial expression, the full-length HBeAg was fused downstream of a polyhistidine tag of a pRSETB vector. The full-length HBeAg and different segments of core protein, respectively, were fused downstream of a GST tag of a pGEX-3X vector. For in vitro transcription and translation reaction, the #46 cDNA was inserted downstream of an influenza viral HA epitope in a pGEM-HA vector. For cytoplasmic expression in mammalian cells, the full-length HBeAg and different segments of core protein, respectively, were fused downstream of a GST tag of a pCMV-GST vector. The #46 protein, sIL-1RAcP356, and MyD88, respectively, were fused downstream of an HA epitope of a pCMV-HA vector. For membrane expression, the mIL-1RAcP and IL-1RI, respectively, were fused downstream of the FLAG tag of a pFLAGCMV vector or the HA tag of a pHACMV vector.

Yeast Two-hybrid Screen—The HBV core protein (adw subtype) from amino acids 119–185, designated as 2/5C (Fig. 1A), was fused downstream of the LexA DNA-binding domain of a pLexA-BD vector and used as a bait to screen a human liver cDNA library. A total of 5 x 10^5 cfu was screened.

Preparation of Recombinant Proteins and Bead-bound Proteins—Expression of the 19-kDa (His)_6-HBeAg was induced by 1 mM isopropyl β-d-galactopyranoside in bacterial strain Escherichia coli BL21(DE3). Recombinant (His)_6-HBeAg protein was purified under native condition on a nickel-agarose column and an anion exchanger column. The purification process was performed by fast protein liquid chromatography. The protein was >95% pure as judged by SDS-PAGE followed by silver staining. Fifty μg of purified (His)_6-HBeAg in 500 μl of binding buffer (5 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl (pH 7.9)) was incubated with 40 μl of nickel-charged His.Bind beads. The beads were washed three times with washing buffer (60 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl (pH 7.9)). Recombinant GST fusion proteins, GST-2/5C, GST-Cd2/5C, and GST-HBeAg, were induced by 1 mM isopropyl-β-d-galactopyranoside in bacterial strain RII. Approximately 0.3 mg of GST fusion proteins in 1 ml of phosphate-buffered saline (PBS) was incubated with 40 μl of glutathione-conjugated agarose beads (50% slurry). The beads were washed three times with PBS containing 0.5% Triton X-100.

In Vitro Interaction—In vitro transcription and translation reactions were performed with TNT quick-coupled transcription/translation system in the presence of [35S]methionine according to the manufacturer’s recommendation. In vitro transcription/translation products were incubated with glutathione bead-bound GST, GST-2/5C, and nickel bead-bound (His)_6-HBeAg, respectively, in binding buffer (25 mM HEPES (pH 7.9), 12.5 mM MgCl₂, 100 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 0.1% Nonidet P-40). The beads were then washed four times in 0.5% Triton X-100 in PBS. Bead-bound
proteins were eluted with SDS-PAGE sample buffer, resolved on a 12% SDS-PAGE, and detected by autoradiography.

Cell Culture and Transfection—Human embryonic kidney 293T (HEK293T) cells were grown in Dulbecco's modified eagle's medium supplemented with 10% bovine calf serum, 102 units/ml penicillin, and 10 \( \mu \)g/ml streptomycin at 37 °C at 8% CO2. Human hepatoma HA22T/VGH cells were grown in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM non-essential amino acid, 102 units/ml penicillin, and 10 \( \mu \)g/ml streptomycin at 37 °C at 5% CO2. Plasmid DNAs were transfected into HEK293T cells or HA22T/VGH cells in a 10-cm dish by the calcium phosphate method. Each set of experiments was performed with two different preparations of plasmids and repeated two to three times for each preparation.

Pulldown Assay—Cells in a 10-cm dish were lysed in 500 \( \mu \)l of 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5)) on ice for 20 min. Whole-cell extracts were incubated with 30 \( \mu \)l of glutathione-conjugated agarose beads for 1 h at 4 °C with rocking. Beads were washed three times with 1% Nonidet P-40 lysis buffer and once with wash buffer (150 mM NaCl, 50 mM Tris-Cl (pH 7.5)).

Immunoprecipitation—Cells in a 10-cm dish were lysed in 400 \( \mu \)l of 0.1% Nonidet P-40 lysis buffer (0.1% Nonidet P-40, 250 mM NaCl, 5 mM dithiothreitol, 1 mM EDTA, 10% glycerol) containing Complete protein inhibitor mixture on ice for 2–3 h. Twenty \( \mu \)l of M2 affinity beads was added to the whole-cell extracts and incubated for 3 h at 4 °C with rocking. Alternatively, 40 \( \mu \)l of anti-core antibody was added to the whole-cell extracts and incubated overnight at 4 °C with rocking. After adding 20 \( \mu \)l of protein A-agarose slurry, the mixtures were incubated for 3 h at 4 °C with rocking. The M2 beads and anti-core protein A beads, respectively, were washed three times with 0.1% Nonidet P-40 lysis buffer.

Western Blot—Proteins were transferred to Hybond ECL or Hybond-P filter and detected by using primary antibody and secondary antibody. The immunoreactive proteins were visualized with Western Lightning Chemiluminescence Reagent Plus.

Indirect Immunofluorescence and Confocal Microscopy—Transfected HEK293T cells were precooled for 20 min at 4 °C and then incubated with HBeAg or IL-1β for 1 h at 4 °C. After washing three times with PBS, cells were incubated with PBS containing 1% paraformaldehyde and 1% sucrose for 10 min at room temperature. Fixed cells were washed twice with PBS, blocked by incubation with 10% bovine serum albumin (BSA) in PBS for 30 min at 37 °C, and incubated with primary antibody in 3% BSA/PBS for 1 h at 37 °C. After extensive washing with PBS, cells were incubated with anti-mouse IgG-TRITC conjugated and anti-rabbit IgG-FITC conjugated secondary antibodies and H33342 in 3% BSA/PBS for 45 min at 37 °C. After extensive washing with PBS, cells were examined by laser confocal microscopy.

Electrophoretic Mobility Shift Assay—Nuclear extracts of HA22T/VGH cells were prepared as described previously (25). Electrophoretic mobility shift assays were performed by incubating 10 \( \mu \)g of nuclear extracts with 1 \( \times \) 10^5 cpm of [\( \gamma \)-32P]ATP end-labeled double-stranded wild type NF-κB oligonucleotides (5’-AGTTGAGGCGACTTCCCTT-3’) in 20 \( \mu \)l of binding buffer at 30 °C for 30 min as described previously (25). For competition experiments, unlabeled wild type or mutant NF-κB oligonucleotides (5’-AGTTGAGGGCACTTCCCTGAG-3’) were added. After washing three times with PBS, cells were incubated with PBS containing 1% paraformaldehyde and 1% sucrose for 10 min at room temperature. Fixed cells were washed twice with PBS, blocked by incubation with 10% bovine serum albumin (BSA) in PBS for 30 min at 37 °C, and incubated with primary antibody in 3% BSA/PBS for 1 h at 37 °C. After extensive washing with PBS, cells were incubated with anti-mouse IgG-TRITC conjugated and anti-rabbit IgG-FITC conjugated secondary antibodies and H33342 in 3% BSA/PBS for 45 min at 37 °C. After extensive washing with PBS, cells were examined by laser confocal microscopy.
FIGURE 2. Interaction of HBeAg with IL-1RαCp. A, in vivo interaction of HBeAg with #46 protein and sIL-1RαCp356. Pulldown experiments of lysates from HEK293T cells coexpressing GST, GST-2/5C, GST-1/5C, GST-2/5Cd1/5C, or GST-HBeAg with HA-46 or HA-sIL-1RαCp were performed with glutathione beads. Proteins in GST pulldown and in whole-cell extracts were analyzed by Western blot with anti-HA and anti-GST antibody. B, in vitro interaction of HBeAg with HA-46 protein. In vitro transcribed/translated [35S]methionine-labeled HA or HA-46 was incubated with glutathione bead-bound GST and GST-2/5C and nickel bead-bound (His)_6-HBeAg, respectively. After extensive wash, bead-bound proteins were analyzed by autoradiography. C, in vitro interaction of HBeAg with endogenous mIL-1RαCp. Pulldown experiments of precleared lysates of HEK293T cells were performed with glutathione bead-bound GST-2/5C and GST-HBeAg and nickel bead-bound (His)_6-HBeAg, respectively. The endogenous mIL-1RαCp in pulldown products was detected by anti-IL-1RαCp antibody. The bead-bound GST-HBeAg and (His)_6-HBeAg were detected with anti-core antibody. D, interaction of exogenous (His)_6-HBeAg added into culture medium with the overexpressed mIL-1RαCp. HEK293T cells were transfected with FLAG or f:mIL-1RαCp. The purified (His)_6-HBeAg was added into the culture medium at 4 °C for 20 min. HBeAg in lysates was immunoprecipitated down by anti-core protein A beads. The f:mIL-1RαCp protein in the immunoprecipitates and in whole-cell extracts was detected by anti-FLAG antibody. (His)_6-im, a His-tagged bacterial immunity protein of irrelevant function, served as a negative control. E, colocalization of HBeAg with mIL-1RαCp on cell surface. HEK293T cells were transiently transfected with f:mIL-1RαCp or f:IL-1R. After incubation with HBeAg or IL-1β for 1 h at 4 °C, cells were fixed with 1% paraformaldehyde for 10 min at room temperature. After blocking, cells were incubated with primary antibody for 1 h at 37 °C followed by anti-mouse IgG-TRITC-conjugated and anti-rabbit IgG-FITC-conjugated secondary antibodies and H33342 for 45 min at 37 °C and analyzed by laser confocal microscopy.
TCCAGGC-3') were added to the binding reaction on ice 5 min before addition of the radiolabeled probe.

RNA Interference Technique—Fifty nm concentration of a 21-nucleotide siRNA duplex of IL-1RαCp gene (accession number NM_002182) at nt 254–272 (sense: 5'-GCAAGUGAUAGCAGGAGGAAGCUAGUU-3'; antisense: 5'-UUUCUGAGGCAUCUUCGCUU-3') or control siRNA (siCONTROL non-targeting siRNA #2) in 12 μl of TransIT-TKO was transfected into HA22T/VGH cells in 6-cm dish for 72 h.

Luciferase Reporter Assay—HA22T/VGH cells were cotransfected with plasmids containing NF-κB-driven firefly luciferase and TK-driven Renilla luciferase. Twenty-four h posttransfection, cells were treated with different reagents. To assay firefly and Renilla luciferase activities, cells were lysed using passive lysis buffer, and luciferase activities were determined by the manufacturer's standard protocols. Firefly luciferase activity values were normalized to Renilla luciferase activity values to control for transfection efficiency. Luciferase activity was measured in duplicate wells, and experiments were repeated at least four times.

Reverse Transcriptase-PCR—The Ready-to-Go™ kit was used to measure the expression of IL-1 responsive genes and mIL-1RαCp mRNA in HA22T/VGH cells according to the manufacturer's recommendation. RT-PCR reactions containing total cellular RNA and primers were done in a single-tube format and a 50-μl reaction volume. PCR primers used for analyses were the following: IL-1β, ATGATGGCTTATATTACAGTGCCGAA (forward primer, 24-mer) and GGAAGAACAAATTTGCATGTTGAA (reverse primer, 24-mer); RT-PCR product, 777 bp; IL-6, AGTTGCCTTCTCCCTGG (forward primer, 17-mer) and ATTTGGCAAGAGACCTCA (reverse primer, 19-mer); RT-PCR product, 603 bp; TNF-α, GCTTCCTAGCCTTCTCTCTCT (forward primer, 20-mer) and AAGACCCCTCCCCAGTAGAT (reverse primer, 20-mer); RT-PCR product, 507 bp; macrophage inhibitory protein-1α (MIP-1α), CCGCTGTCTGCTCAGCTACACCTCCCCGGCA (forward primer, 30-mer) and TGGACCCTCAGGCACCTAGCTCAGCTGGTCG (reverse primer, 30-mer); RT-PCR product, 195 bp; inducible nitric-oxide synthase (iNOS), AGATTCCAGTTGTCACCATCCACC (forward primer, 19-mer) and GCCCACTTCCCTCAGGATG (reverse primer, 19-mer); RT-PCR product, 582 bp; GM-CSF, AGGCCACGACGAGCCCTGG (forward primer, 21-mer) and CTCTGGACTGCGCTCCCCAGCAGTCAAGGG (reverse primer, 30-mer); RT-PCR product, 363 bp; mIL-1RαCp, TAGGCGGCTAGTGTGCTGCTAGTG (forward primer, 23-mer) and GAGCTCGAGCACCTACCTGTTCTTT (reverse primer, 24-mer); RT-PCR product, 798 bp; β-actin, TGAAGGGGTTACCCCAACTGTGGCCCATCA (forward primer, 31-mer) and CTGAAGGACATTGGTCGGGTCGATGG (reverse primer, 30-mer); RT-PCR product, 661 bp. RT-PCR results were analyzed with a Personal Densitometer using ImageQuant (GE Healthcare).

ELISA—HA22T/VGH cells were seeded in 24-well flat-bottom plates at 2 × 10⁵ cells/ml and cultured for 2 days before stimulation. Cells were washed three times with PBS and stimulated with different reagents. Culture media were collected at 15 h for TNF-α and 24 h for GM-CSF, respectively, after stimulation. Levels of TNF-α and GM-CSF in the culture media were measured by ELISA. ELISA was performed by adding 50 μl of each sample to a 96-well plate of TNF-α and GM-CSF ELISA kits according to the manufacturer's recommendation. Each experiment was performed in triplicate wells and final results were the average of three independent experiments.

RESULTS

In Vivo and in Vitro Interaction between HBeAg and IL-1RαCp—HBeAg shares a large stretch of sequence in common with HBV core protein, which assembles to form viral capsid. They only differ at the N and C termini (Fig. 1A). To identify the cellular protein(s) that interact with HBeAg and/or core protein, the C-terminal 67 amino acid region from amino acid 119 to 185 of core protein (2/5C) was used as a bait to screen the human liver cDNA library in a yeast two-hybrid system. As shown in Fig. 1B, a ~0.6-kb #46 cDNA was identified and confirmed to interact with the bait in yeast two-hybrid system. Sequence analysis of #46 shows it corresponds to amino acids 247–356 segment of the soluble form of human IL-1RαCp, sIL-1RαCpP356. For human IL-1RαCp, one 570-amino acid membrane form (mIL-1RαCp) and two soluble forms, 356-amino acid form (sIL-1RαCpP356) and 346-amino acid form (sIL-1RαCpPβ) that are generated by alternative splicing have been identified (22, 26, 27). Both soluble forms lack the intracellular and transmembrane domains of the membrane form. The sequence of mIL-1RαCp and sIL-1RαCpP356 is identical from amino acid 1 to 350. The N-terminal segment of
HBeAg and IL-1RAcP

A. IL-1β  HBeAg  LPS  WT  Mut

B. IL-1β  HBeAg  PMB  LPS

C. IL-1β  HBeAg  LPS

D. IL-1β  HBeAg  PMB

NF-κB

IKB-α

β-actin

mIL-1RAcP

NF-κB

NF-κB
sIL-1RacPβ, from amino acid 1 to 301, is identical to that of sIL-1RacP356. A unique second half of the Ig3 domain makes up the rest of sIL-1RacPβ. mIL-1RacP is essential for IL-1-mediated response such as activation of interleukin-1 receptor-associated kinase, NF-κB, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase (19, 22, 23). sIL-1RacP356 has been shown to act as an inhibitor of IL-1 signaling (26, 28). The function of sIL-1RacPβ is still unknown (27).

To confirm the interaction between 2/5C and #46 protein in mammalian cells, GST or GST-tagged 2/5C (GST-2/5C) were coexpressed with HA-tagged #46 (HA-46) in HEK293T cells. Interaction between HA-46 and GST-2/5C, but not GST, was observed by glutathione-beads pull down followed by Western blot using anti-HA antibody (Fig. 2A). In vitro 35S-labeled HA or HA-46 was incubated with bead-bound GST or GST-2/5C. HA-46 was pulled down by GST-2/5C, but not GST, demonstrating in vitro interaction between #46 and 2/5C proteins (Fig. 2B). To identify the minimal sequence that is required for the interaction with #46, GST-1/5C and GST-2/5Cd1/5C containing amino acids 144–185 and 116–149 of core, respectively, were coexpressed with HA-46 in HEK293T cells, only the latter could interact with HA-46 (Fig. 2A). Because this amino acids 116–149 segment is present in HBeAg, interaction between HBeAg and #46 protein was tested. Interaction of GST-HBeAg with HA-46 in HEK293T cells was observed (Fig. 2A). In vitro 35S-labeled HA-46, but not HA, was pulled down by (His)6-HBeAg (Fig. 2B). Therefore, HBeAg can interact with #46 protein in vivo and in vitro. Since the protein sequence encoded by #46 is present in sIL-1RacP356, the interaction of sIL-1RacP356 with different core mutants and HBeAg in HEK293T cells was investigated. Identical to the results in HA-46, the cytoplasmic HA-sIL-1RacP protein was able to interact with GST-2/5C, GST-2/5Cd1/5C, and GST-HBeAg but not GST and GST-1/5C (Fig. 2A).

Binding of HBeAg to mIL-1RacP on the Cell Surface—HBeAg is a secreted protein produced during HBV replication, and present in the sera of the patients in natural infection. The above results raise the intriguing possibility that HBeAg in the sera of the patients may interact with the extracellular domain of a membrane-bound IL-1RacP protein. To determine whether there is an interaction between endogenous mLIL-1RacP with HBeAg in vitro, crude HEK293T lysates were precleared with glutathione bead-bound GST-Cd2/5C protein to remove proteins that could bind to GST or the portion of core protein not implicated in the interaction with IL-1RacP. The precleared lysates were then incubated with glutathione bead-bound GST, GST-2/5C and GST-HBeAg, and nickel bead-bound (His)6-HBeAg, respectively. The endogenous mLIL-1RacP was pulled down by GST-2/5C, GST-HBeAg, and (His)6-HBeAg but not by GST (Fig. 2C). To further confirm whether the HBeAg can interact with mLIL-1RacP in vivo, purified (His)6-HBeAg was added into the culture medium of HEK293T cells, which was transiently transfected to overexpress the membrane FLAG:mIL-1RacP protein, for 30 min. (His)6-HBeAg in lysates was immunoprecipitated with anti-core antibody and protein A-agarose beads. FLAG:mIL-1RacP was communoprecipitated with (His)6-HBeAg, suggesting the interaction between exogenous (His)6-HBeAg added to the culture medium and mLIL-1RacP on the cell surface (Fig. 2D). To further demonstrate that the binding of HBeAg to mLIL-1RacP indeed takes place on the cell surface, indirect immunofluorescence followed by confocal microscopy was performed on HEK293T cells transiently transfected to overexpress the membrane FLAG:mIL-1RacP protein. As shown in Fig. 2E, binding of fluorescence labeled HBeAg to the cell surface of HEK293T cells was observed. The decoration of cell surface by HBeAg was only noted in HEK293T cells that also overexpressed FLAG: mIL-1RacP. HBeAg binding intensity paralleled the level of expression of FLAG:mIL-1RacP in the transfectants. Moreover, colocalization of HBeAg and FLAG:mIL-1RacP in transfected HEK293T cells was clearly demonstrated by confocal microscopy. Binding of IL-1β to HEK293T cells overexpressing the membrane FLAG:IL-1RI protein displayed the same pat-

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**FIGURE 4.** NF-κB activation induced by HBeAg. A and B, induction of NF-κB DNA binding activity by HBeAg. HA22T/VGH cells were treated with 3 μg/ml HBeAg or boiled HBeAg, 10 ng/ml IL-1β, or 1 μg/ml LPS or boiled LPS for 1 h. Pretreatment was performed with 30, 50, or 100 μg/ml polymyxin B for 30 min. To detect NF-κB DNA binding activity by electrophoretic mobility shift assay, an end-labeled wild type NF-κB binding site was incubated with nuclear extracts in the absence of competitor and in the presence of unlabeled wild type (WT) or mutant (Mut) NF-κB binding site competitor at the indicated molar excesses. Proteins in cytoplasmic lysates were analyzed by Western blot using anti-IκB-α antibody to measure the IκB-α degradation or anti-β-actin antibody to detect β-actin as protein loading control. B indicates boiled HBeAg or LPS. C, abolishment of HBeAg-induced NF-κB activation by silencing IL-1RacP expression. HA22T/VGH cells were transfected with siRNA targeting the N-terminal region of IL-1RacP or non-targeting control siRNA for 72 h followed by treatment with 3 μg/ml HBeAg, 10 ng/ml IL-1β, or 1 μg/ml LPS for 1 h. Expression of endogenous mLIL-1RacP or β-actin mRNA was determined by RT-PCR, while NF-κB activation was determined by electrophoretic mobility shift assay. D, enhancement of IL-1β-mediated NF-κB activation by HBeAg. HA22T/VGH cells were treated with 1 μg/ml HBeAg, 3 ng/ml IL-1β, or in combination for 1 h. Pretreatment was performed with 10 μg/ml polymyxin B for 30 min.
tern, which served as a positive control. These observation establishes specific interaction between HBeAg and FLAG: mIL-1RACp on the cell surface.

Association of IL-1RI and mIL-1RACp and Recruitment of Adaptor Protein MyD88 Induced by HBeAg Binding—The demonstration that HBeAg binds to mIL-1RACp on the cell surface raises the intriguing possibility that HBeAg can trigger the IL-1-mediated signal transduction. We first tested whether HBeAg could induce the association of IL-1RI and mIL-1RACp. Human hepatoma HA22T/VGH cells overexpressing the membrane HA:IL-1RI and FLAG:mIL-1RACp proteins were treated with HBeAg. These cells were also stimulated with IL-1β as a positive control. As shown in Fig. 3A, the association of IL-1RI and mIL-1RACp was strongly induced by HBeAg, the association of which was also induced by IL-1β. To ensure that the association between IL-1RI and mIL-1RACp was triggered by HBeAg itself rather than contaminants from the HBeAg preparation, we did the following two control experiments: 1) this association was not affected by pretreatment with polymyxin B, a LPS-specific binding antibiotic. This demonstrates that this effect does not result from LPS, which is a possible contaminant carried with the HBeAg preparation; 2) this association could be abolished by boiling of HBeAg. Boiling can denature HBeAg but not LPS. Together, these results demonstrate that HBeAg is able to induce the association of IL-1RI and mIL-1RACp. Adaptor protein MyD88 is recruited into the IL-1RI and mIL-1RACp complex when association of IL-1RI and mIL-1RACp is induced by IL-1β. To test whether MyD88 is recruited into the IL-1RI and mIL-1RACp complex by treatment with HBeAg, HA22T/VGH cells overexpressing overexpressing IL-1RI, FLAG:mIL-1RACp, and HA:MyD88 were treated with either HBeAg or IL-1β with the latter serving as a positive control. As shown in Fig. 3B, recruitment of MyD88 to the IL-1RI and mIL-1RACp complex could be induced by HBeAg. This observation was not affected by pretreatment with polymyxin B, demonstrating that HBeAg is able to induce the recruitment of MyD88 to the IL-1RI and mIL-1RACp complex. These results strongly indicate that HBeAg can trigger the IL-1 signaling pathway.

Induction of IkB-α Degradation and Increase of NF-κB DNA Binding by HBeAg—IL-1β induces the phosphorylation and degradation of IkBα leading to release and activation of NF-κB. HBeAg-induced NF-κB activation was assessed by electrophoretic mobility shift assay. As shown in Fig. 4, A and B, HBeAg increased the NF-κB DNA binding activity, which was specifically competed by wild type NF-κB but not mutant NF-κB oligonucleotides. This correlated with an increased degradation of IkBα. NF-κB DNA binding activity and IkBα degradation induced by HBeAg was not affected by polymyxin B pretreatment but abolished by boiling of HBeAg. In contrast, the LPS-induced IkBα degradation and NF-κB DNA binding activity were abolished by polymyxin B pretreatment but not affected by boiling. These results demonstrate that HBeAg can induce degradation of IkBα and NF-κB activation.

Abolishment of HBeAg-induced NF-κB Activation by Silencing of Endogenous IL-1RACp—To further determine the role of mIL-1RACp on HBeAg-induced NF-κB activation, specific siRNA was designed and used to silence the expression of endogenous IL-1RACp in HA22T/VGH cells. As shown in Fig. 4C, expression of endogenous mIL-1RACp mRNA was dramatically reduced by IL-1RACp specific siRNA, compared with control siRNA. When endogenous mIL-1RACp mRNA expression was greatly diminished, the effect of HBeAg on NF-κB activation was almost completely abolished. These results demonstrate that endogenous mIL-1RACp plays an important role in HBeAg-induced NF-κB activation. The IL-1β-induced NF-κB activation was also abolished by silencing of mIL-1RACp mRNA expression, which was consistent with the essential role of mIL-1RACp in IL-1-mediated response. In contrast, the LPS-induced NF-κB activation was not affected by silencing of mIL-1RACp mRNA expression, which was consistent with the previous finding that mIL-1RACp plays no role in LPS-induced NF-κB activation.

Enhancement of IL-1β-mediated NF-κB Activation by HBeAg—The signaling cascade initiated by IL-1β binding to IL-1RI involves the assembly of a trimeric protein complex consisting of IL-1, IL-1RI, and mIL-1RACp. Our results shown above demonstrate that HBeAg binds to mIL-1RACp and activates the IL-1 signal transduction pathway. It is therefore important to know whether HBeAg can cooperate with IL-1β in the induction of IL-1 signal transduction pathway. As shown in Fig. 4D, higher NF-κB DNA binding activity was noted with the combination of HBeAg and IL-1β than either stimulus alone. This was not abolished by polymyxin B pretreatment. The results demonstrate that HBeAg enhances IL-1β-mediated NF-κB activation.

Expression of NF-κB-dependent Reporter Gene Induced by HBeAg—We then examined whether NF-κB-dependent reporter gene expression could be induced by HBeAg. As shown in Fig. 5, HBeAg treatment increased the levels of the NF-κB-dependent luciferase activity values by 2.40-fold, compared with that of 2.39-fold induced by IL-1β. This HBeAg effect was not affected by polymyxin B pretreatment but abolished by boiling of HBeAg.

Expression of IL-1β-responsive Genes Induced by HBeAg—IL-1 can induce the expression of multiple genes, including IL-1β, IL-6, TNF-α, iNOS, MIP-1α, and GM-CSF. We examined whether the expression of these genes in HA22T/VGH cells was induced by HBeAg by RT-PCR first. As shown in Fig. 6, HBeAg induced the expression of all of these IL-1-responsive genes. This HBeAg effect was not affected by polymyxin B pretreatment but abolished by boiling of HBeAg.

**FIGURE 6.** Expression of IL-1-responsive genes induced by HBeAg. HA22T/VGH cells were treated with 2 µg/ml HBeAg or boiled HBeAg, 1 µg/ml LPS or boiled LPS, or serum-free medium alone for 1 h. Pretreatment was performed with polymyxin B (10 µg/ml) for 30 min. The expression of IL-1-responsive genes including IL-1β, IL-6, TNF-α, iNOS, MIP-1α, and GM-CSF in total RNA was quantified by RT-PCR analysis. The expression of β-actin was used as a control. A indicates boiled HBeAg or LPS. P indicates polymyxin B pretreatment. A, representative RT-PCR analysis of three experiments. B, fold induction of IL-1-responsive genes is shown as means ± S.D. of three independent experiments.
HBeAg and IL-1R<sub>Acp</sub>

**A.**

![Graph A](image)

**B.**

![Graph B](image)

FIGURE 7. Production of TNF-α and GM-CSF induced by HBeAg. Levels of TNF-α (A) and GM-CSF (B) in culture media after treatment of HA22T/VGH cells with IL-1β (10 ng/ml), HBeAg (2 μg/ml), or serum-free medium alone were measured with ELISA. Results presented are means ± S.D. and represent the average of three independent experiments performed in triplicate wells (p < 0.001 in both TNF-α and GM-CSF induced either by HBeAg or IL-1β compared with medium alone).

In HBV infection, HBeAg-negative variants are not only associated with acute fulminant hepatitis in both neonatal and adult infection but also correlates with acute exacerbation of liver inflammation and viral clearance in chronic infection. These clinical observations indicate that HBeAg may attenuate the cell-mediated inflammatory response (12–15). In animal studies, HBeAg has also been shown to preferentially elicit Th2-like response and deplete HBeAg- and core-specific Th1 cells in mice (16, 17). HBeAg, therefore, has been proposed to play an immunoregulatory role and promote viral persistence. However, the molecular mechanism of the immunoregulatory function of HBeAg is still largely unknown. We show here that HBeAg can bind to mIL-1R<sub>Acp</sub> and induce the IL-1 signaling. IL-1 has been shown to function as a costimulator to activate Th2 cells (20, 21). Other IL-1-responsive proteins such as IL-6 also exert anti-inflammatory effect and/or promote Th2 differentiation (30). Our results therefore indicate that HBeAg secreted from the HBV-infected hepatocytes during HBV replication may tip the immune response to a Th2-like response, leading to suppression of the host immune response against HBV and thus preventing viral clearance and promoting viral persistence.

HBeAg positivity, an indicator of active HBV replication, is associated with an increased risk of HCC (18). In the past, the role of HBeAg in the development of HCC has been explained by its indication of active HBV replication. Active replication of HBV may directly initiate malignant transformation by increasing the expression of X protein of HBV or by increasing the probability of insertion of viral DNA in or near tumor-suppressor genes or proto-oncogenes (31, 32). Indirectly, active replication of HBV causes chronic necroinflammatory disease, which accelerates the hepatocyte turnover and generates mutagenic reactive oxygen species during the inflammatory process. The latter leads to the accumulation of DNA damage or insertion of viral DNA into chromosomal DNA. Our finding that HBeAg can activate IL-1 signaling shows that HBeAg may itself contribute to the development of HCC. First, activation of NF-κB-dependent proinflammatory cytokines may enhance the inflammatory process, which induces the hepatocyte proliferation (33). Second, NF-κB activation can induce anti-apoptotic genes, which may protect the transformed hepatocytes against apoptosis and promote the carcinogenesis of hepatocytes (34). The essential role of NF-κB in promoting the development of HCC from inflammatory hepatitis to a mouse model has recently been demonstrated (35). Moreover, constitutive activation of NF-κB has often been observed in cancer tissues of HBV-positive HCC (36). In addition, recent evidence also shows that IL-1 prevents apoptosis in many cell types including keratinocytes, chondrocytes, osteoclasts, neutrophils, monocytes, and lymphocytes (37–41).

Many viral genes encode homologues of cytokine/chemokine and their receptors to modulate the immune response (42). For example, BCRF-1 of Epstein-Barr virus, an IL-10 homologue, blocks the IFN-γ production and suppresses the Th1 cell response (43). K2 of human herpesvirus 8 is a homologue of IL-10, which is an angiogenic factor and B-cell growth factor (44). The vMIP-I/K6, -II/K4, and -III/K4.1 of human herpesvirus 8, homologues of MIP-1, -II, and -III, respectively, are chemotaxtractants for Th2-polarized T cells (45–47). B8R and B18R of vaccinia virus are IFN-γ receptor homologue and IFN-α/β-binding protein, which inactivate IFN-γ and IFN-α/β and thus inhibit both the antiviral and immune functions of IFN-γ and IFN-α/β, respectively (48, 49). Uniquely, HBeAg of HBV induces IL-1 response not as a homologue of cytokine IL-1 or...
cytokine receptor IL-1R but through interaction with an essential component of IL-1 receptor complex: mIL-1RACp.

IL-1 has a broad range of biological effects on different types of cell in response to infection, tissue damage, and stress. It also acts as a costimulator to activate Th2 cells (20, 21). Different viral proteins have been shown to regulate the IL-1 response through different mechanisms. The CrmA protein of cowpox virus inhibits the production of caspase-1, which prevents the proteolytic cleavage of pro-interleukin-1β (pro-IL-1β) to mature IL-1β (50). B15R of vaccinia virus is a soluble IL-1β receptor and inactivates the IL-1 (51). The A46R and A52R proteins of vaccinia virus have been shown to inhibit IL-1 response by possessing putative TIR domain to suppress TIR domain-dependent signaling (52). N1L protein of vaccinia virus has been shown to regulate the IL-1 response by interacting with the mIL-1RACp component of IL-1RI/mIL-1RACp complex to induce the IL-1 response.

HBV appears to employ several strategies to promote its persistence during infection. Viral X protein has been shown to inhibit cellular proteasome activity and may inhibit antigen processing and presentation (54). During HBV replication, 22-nm surface antigen particles, which are secreted in a large amount from infected hepatocytes into blood stream, can consume neutralizing antibody against HBV. These surface antigen particles, in addition, may also function as a high dose tolerogen to suppress immune elimination of infected hepatocytes (55, 56). Here we describe that HBeAg interacts with mIL-1RACp to have a novel immunoregulatory function. The immunomodulation by HBeAg may represent a major function of HBeAg in the HBV life cycle and valuable target for potential therapeutic intervention.

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