p48 (ISGF-3γ) Is Involved in Interferon-α-induced Suppression of Hepatitis B Virus Enhancer-1 Activity*

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Interferon-α (IFN-α) suppresses hepatitis B virus (HBV) gene expression by reducing its enhancer-1 activity. IFN-α induces transcription factors, interferon-stimulated gene factor 3 (ISGF3), and interferon regulatory factor-1 (IRF-1), which activate interferon-inducible gene expression through binding to the interferon-stimulated regulatory element (ISRE) “AGTTTCNNTT-TCNC” in the gene promoters. We found the ISRE-like sequence “AGGCTTTCACTTTCTC” in the HBV enhancer-1 region and elucidated the role of this sequence. Gel mobility shift assay showed binding of in vitro translated IRF-1 and in vitro translated p48 (ISGF3-γ), which is a component of ISGF3 to this sequence. However, nuclear extracts binding to this sequence from human hepatoma cells (HuH-7) treated with IFN-α contained only the protein consisted of p48. In transfection experiments, IFN-α suppressed the HBV enhancer-1 activity, and overexpression of p48 enhanced this inhibitory effect. Both mutation and deletion of the ISRE-like sequence in the HBV enhancer-1 region reduced the suppressive effect of IFN-α. Our results suggest that the ISRE-like sequence in the HBV enhancer-1 can interact with the protein containing p48 and mediate the IFN-α-induced suppression of the enhancer activity.

Hepatitis B virus (HBV) causes acute hepatitis, chronic hepatitis, and liver cirrhosis and is closely linked to hepatocellular carcinoma (1). Interferon-α (IFN-α) has been used as an anti-viral agent against chronic HBV infection (2), resulting in improvement of clinical outcome in patients with chronic HBV infection (3). IFN-α suppresses expression of HBV gene by reducing the HBV enhancer-1 activity (4). We have reported previously that IFN-α in combination with tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β) synergistically suppress the HBV enhancer-1 activity (5). In addition, recent studies have shown that cytotoxic T lymphocytes, which release various cytokines, play an important role in noncytolytic clearance of HBV from hepatocytes (6–8).

Type I IFN (IFN-α and -β) induces the formation of a heterotrimeric transcription factor complex, interferon-stimulated gene factor 3 (ISGF3) (9), which consists of signal transducers and activators of transcription 1 and 2 (STAT1 and STAT2) (10) and p48 (ISGF3γ) (11), after binding to its receptor. ISGF3 translocates into the nucleus and binds to the interferon-stimulated regulatory element (ISRE) in the promoter of a variety of interferon-inducible genes, and transactivates their expression (10). Interferon regulatory factor-1 (IRF-1) is another important factor induced by type I and II IFNs (12).

Since the sequence of IRF-1 binding site termed IRE-E overlaps with ISRE (13), IRF-1 also binds to ISRE sequence and activates the interferon-inducible gene transcription (14). p48 and IRF-1 show a homology within their amino-terminal regions and are members of the IRF family together with other IRFs (15).

However, analysis in mice deficient for the p48 gene has shown that induction of some interferon-inducible genes such as 2',5'-oligoadenylate synthetase (2',5'-OAS) (16) and double-stranded RNA-dependent protein kinase (PKR) (17) are p48-dependent and that IRF-1 cannot compensate completely for the loss of p48 (13), suggesting that p48 and IRF-1 are, in part, functionally different.

Recent studies have demonstrated the presence of ISRE-like sequence in several viral genomes, such as Qp promoter region of the Epstein-Barr virus (EBV) nuclear antigen 1 gene (18, 19), U5 region of the bovine leukemia virus long terminal repeat (20), and downstream region of the human immunodeficiency virus type 1 long terminal repeat (21) and that IRF-1, but not ISGF3, interacts with these ISRE-like sequences and regulates viral gene expression (19–21). We also found the ISRE-like sequence in the 5′ region of the HBV enhancer-1, which is located just before the X gene promoter and enhances the transcription of not only the X gene but also the Core, Pre-S, and S gene of the HBV specifically in hepatocytes (22). In the present study, we determined whether the ISRE-like sequence in the HBV enhancer-1 could interact with p48 or IRF-1 and act as a cis-element mediating the IFN-α action. We showed that IFN-α induced a complex formation of this sequence with the protein containing p48 and that the complex formation was relevant to the IFN-α-induced suppression of the enhancer activity. This is the first report suggesting that p48, together with its partners, directly binds to HBV DNA sequence and participates in the regulation of HBV gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture—The HuH-7 human hepatoma cell line was maintained in a chemically defined medium, IS-RPMI (23) with 2% fetal bovine serum. In each experiment, the medium was replaced with serum-free IS-RPMI containing recombinant human IFN-α 2a (Nippon Roche Co., Tokyo, Japan), recombinant human IFN-γ 1a (Shionogi Co., Osaka, Japan), and tumor necrosis factor-α (25 ng/ml) or interleukin-1β (10 ng/ml) to induce the transcription of ISRE-like sequences. After the induction of ISRE-like sequences, nuclear extracts were prepared from HuH-7 cells (14).

The abbreviations used are: IFN, interferon; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; ISGF3, interferon-stimulated gene factor 3; STAT, signal transducers and activators of transcription; ISRE, interferon-stimulated regulatory element; IRF, interferon regulatory factor; 2',5'-OAS, 2',5'-oligoadenylate synthetase; EBV, Epstein-Barr virus; PKR, double-stranded RNA-dependent protein kinase; CAT, chloramphenicol acetyltransferase; ISG54, interferon-stimulated gene encoding protein of 54 kDa; GBP, guanylate-binding protein; bp, base pair(s).

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Japan, or IL-1β (Osuka Pharmaceutical Co., Tokushima, Japan), then incubated for the indicated periods.

**Gel Mobility Shift Assay**—The full-length human p48 (ISGF3γ) cDNA cloned into pBluescript SK+ (11), which was kindly provided by Dr. D. E. Levy (Department of Pathology, New York University School of Medicine), was subjected to in vitro transcription and translation with TNT T7 quick-coupled transcription/translation system (Promega Corp., Madison, WI). pUC IRF-1 and pHRFAS-51 plasmids containing the full-length human IRF-1 and IRF-2 cDNA, respectively, were kindly provided by Dr. T. Taniguchi (Department of Immunology, Tokyo University, Tokyo, Japan). The IRF cDNA plasmids were subcloned to PGEM 7 (+) (Promega Corp.) and subjected to in vitro transcription and translation. The HuH-7 cells were treated with 1,000 IU/ml IFN-α (2 h) or 100 IU/ml IFN-γ (2 h and 24 h), and the nuclear extract was prepared as described previously (24). Anti-human p48 rabbit serum was also provided by Dr. D. E. Levy, and anti-human IRF-1 rabbit serum was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The following double-stranded oligonucleotides were used as a probe or competitor in the assays (only the sense strand is shown): HBV ISRE-like sequence (25), GAGGCTTCTACTTTCTGG; mutated HBV ISRE-like sequence (mutations are underlined), GAGGCGCTACCTTCTGG; 2′-OAS ISRE (26), CGTTGGTTGGTTCTTCAGA; C13 oligonucleotide (IRF-E) (27), TCTACATCTATCTCCTATTCT. The probe was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. The proteins prepared from the in vitro transcription and translation or nuclear extract were incubated with 10 fmol of the labeled probe for 30 min at 24°C in the presence of 40 mM KCl, 20 mM HEPES (pH 7.9), 1 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 2 μg of poly(dI-dC), 1 μg of salmon testes DNA, and 5% glycerol. The reaction mixture was electrophoresed on a 4% polyacrylamide gel containing 25 mM Tris borate and 0.25 mM EDTA.

**Cell Transfection and Chloramphenicol Acetyltransferase (CAT) Assay**—To construct the pXEn-αCAT plasmid containing the HBV enhancer-1 and X promoter region, NeiI (937) to NcoI (1244) fragment from pBRHBAdv125 (25) was inserted into the pCAT-basic plasmid (Promega Corp.). To construct the pXEnαISRE/M-CAT plasmid, which contains T to C substitutions in the ISRE-like sequence, the NeiI fragment containing the ISRE-like sequence in the pXEn-αCAT plasmid was replaced with double-stranded oligonucleotide with the sequence 5′-ATAACATCTAAAGCAGGCCCTCACCTTCTCGCCAACTT-ATAAAGG-3′ (mutations are underlined). To construct the pXEnαISRE-CAT plasmid, the NeiI (937) to StuI (985) fragment containing the ISRE-like sequence was deleted from pXEn-αCAT plasmid. Similarly, to construct the pXEn-αCAT plasmid, which has only the X promoter region, the NeiI (937) to StuI (985) fragment containing the full HBV enhancer-1 region was deleted from pXEn-αCAT plasmid. The full-length IRF-1 and IRF-2 cDNAs from pUC IRF-1 and pHRFAS-51 plasmids, respectively, were inserted into the expression vector, pcDNA3 (Invitrogen Corp., Carlsbad, CA), to yield the IRF-1 and IRF-2 expression plasmids, pcIRF-1 and pcIRF-2, respectively. p48 expression plasmid, pcP48, was also constructed by inserting the full-length p48 cDNA into the pCDNA2. Transfection was performed using 3 μg of plasmid DNA per flask (25 cm2) by the lipofection method (28). Two days later, cells were harvested and lysed by five cycles of freezing and thawing. The lysate was heated at 60°C for 15 min to inactivate deacetylase, centrifuged at 15,000 rpm for 5 min, and the supernatant was used for determination of CAT activity as described previously (29). pSV-β-galactosidase control vector (Promega Corp.) was cotransfected with CAT plasmids to normalize the protein levels.

**Northern Blotting**—The HuH-7 cells were treated with 1,000 IU/ml IFN-α or 100 IU/ml IFN-γ. Total RNA was isolated from the cells before and at 1, 2, 4, 8, and 24 h after treatment by the guanidium isothiocyanate method for analyzing the levels of p48 and IRF-1 mRNA using p48 and IRF-1 cDNA probe, respectively.

**RESULTS AND DISCUSSION**

We found the ISRE-like sequence “AGCCATTACCTTTCCT” in the HBV enhancer-1 region at positions 958–973 (25), which is similar to the ISRE sequence in interferon-inducible gene promoters (26, 30–33) (Table 1). Moreover, the HBV ISRE-like sequence is precisely conserved among the different subtypes of HBV (25, 34–36). Therefore, we examined whether p48 or IRF-1 could bind to this sequence by the gel mobility shift assay using a oligonucleotide probe that contained the HBV ISRE-like sequence. The in vitro translated p48 formed a complex band with the probe (Fig. 1A, lane 1). Addition of 100 times molar excess of nonlabeled 2′,5′-OAS ISRE and HBV ISRE-like oligonucleotide competitors reduced the density of the complex band (Fig. 1A, lanes 2 and 4), but addition of nonlabeled mutated HBV ISRE-like oligonucleotide did not (Fig. 1A, lane 5), in which T to C substitutions at key positions known to impair the ISRE function were introduced (20, 24). The in vitro translated IRF-1 also formed a complex band with the probe (Fig. 1A, lane 6). This interaction was abolished by addition of 100 times molar excess of the nonlabeled C13 oligonucleotide that contains the IRF-1 binding consensus sequence (IRF-E) (13, 27) and HBV ISRE-like oligonucleotide competitors (Fig. 1A, lanes 7 and 9), but addition of nonlabeled mutated HBV ISRE-like oligonucleotide did not (Fig. 1A, lane 10). These results indicate that p48 and IRF-1 can recognize the ISRE-like sequence in the HBV enhancer-1 region.

To determine whether IFN-α could induce the binding of nuclear proteins to the HBV ISRE-like sequence in the cells, nuclear extracts were prepared from HuH-7 cells treated with 1,000 IU/ml IFN-α for 2 h. The nuclear extract from the IFN-α-treated cells formed a complex band with the probe (Fig. 1B, lane 3). Addition of 100 times molar excess of the nonlabeled 2′,5′-OAS ISRE and HBV ISRE-like oligonucleotide competitors reduced the density of the complex band (Fig. 1B, lanes 4 and 7), but addition of nonlabeled mutated HBV ISRE-like
Fig. 2. Effects of IFN-α, IL-1β, or both on the HBV enhancer-1 and X promoter activities. A, schema of the HBV enhancer-1 to X promoter region (25) and constructs of chimeric CAT plasmids. Shaded ellipse indicates the ISRE-like sequence. B, the HuH-7 cells were transfected with 3 μg of the indicated plasmids and incubated with IFN-α (1,000 IU/ml), IL-1β (100 IU/ml), or both. Two days later, CAT activity was analyzed. The amount of extracts and incubation time were 10 μg of protein and 30 min, respectively. Data represent CAT activity relative to the control (pXEn-CAT without treatment; lane 1) and are expressed as mean ± S.D. of three separate experiments.

oligonucleotide did not (Fig. 1B, lane 8). Furthermore, addition of anti-p48 serum led to a supershift of the complex band (Fig. 1B, lane 5), but addition of anti-IRF-1 did not (Fig. 1B, lane 6), suggesting that the binding protein induced in the cells by IFN-α contained p48 but not IRF-1 in its molecule. In addition, the position of the p48-associated complex band using in vitro translated one (Fig. 1B, lane 1) was different from that the nuclear extract from IFN-α-treated cells (Fig. 1B, lane 3). This would account for ISGF3 formation in IFN-α-treated cells, although our work did not address whether STAT1 and STAT2 are actually involved in its complex formation. We could not detect any shifted bands corresponding to IRF-1 in this condition. The nuclear extract from HuH-7 cells treated with IFN-γ for 24 h, but not that treated with IFN-γ for 2 h, formed a complex band with the HBV ISRE-like oligonucleotide probe (Fig. 1B, lanes 9 and 10) at the similar position of the p48-associated complex band (Fig. 1B, lane 1). Addition of anti-p48 serum led to a supershift of this complex band (Fig. 1B, lane 11), suggesting that 24-h IFN-γ treatment induces p48 in HuH-7 cells as described previously (11, 15).

By the functional analysis of the HBV ISRE-like sequence, varying lengths of the HBV enhancer-1 and X promoter region were linked to the CAT gene (Fig. 2A). pXEn-CAT contains the full-length of the HBV enhancer-1 and X promoter region, while pXEnISRE/M-CAT contains three nucleotide mutations in the ISRE-like sequence as described under “Experimental Procedures.” pXEn.ISRE-CAT lacks the 48-bp region containing the ISRE-like sequence in pXEn-CAT, and pXCAT contains only the X promoter region. These CAT plasmids were transfected into HuH-7 cells. Afterward, the cells were treated with 1,000 IU/ml IFN-α, 100 IU/ml IL-1β, or both, since at least 1,000 IU/ml IFN-α was required for an apparent suppression of the HBV enhancer-1 activity (Fig. 3A, lanes 1–5), and since we have shown previously that IFN-α, in combination with IL-1β or TNF-α produced a more profound suppression of the HBV enhancer-1 activity than IFN-α alone (5). CAT expression from pXEn-CAT was suppressed by approximately 45 and 80% by IFN-α alone and IFN-α in combination with IL-1β, respectively (Fig. 2B, lanes 2 and 4), but its expression was not suppressed by IL-1β alone as reported previously (5) (Fig. 2B, lane 3). CAT expression from pXEnISRE/M-CAT and pXEn.ISRE-CAT, which showed a base-line CAT activity almost similar to that from pXEn-CAT, showed only a slight suppression by IFN-α alone and IFN-α in combination with IL-1β, respectively (Fig. 2B, lanes 6 and 8 and lanes 10 and 12). IFN-α alone or IFN-α in combination with IL-1β did not affect CAT expression from pX-CAT (Fig. 2B, lanes 14 and 16). These results suggest that the ISRE-like sequence in the HBV enhancer-1 region is likely to mediate the IFN-α-induced suppression of the enhancer activity. This is a surprising result, because ISRE existing in the promoters of interferon-inducible genes behaves as a positive cis-element (26, 30–33). It is not clear, at present, why the HBV ISRE-like sequence works negatively. However, our results are supported by the following findings. 1) IFN-α suppresses the activity of EBV nuclear antigen 1 gene promoter containing ISRE-like sequence (19). 2) IRF-2, which counteracts with IRF-1 by competitive binding to ISRE (37), can directly activate the expression of human histone H4 gene through binding to ISRE in its promoter (38).

It has been shown that pretreatment of HeLa cells with IFN-γ before stimulation with IFN-α results in increased levels of p48 and enhances the expression of IFN-α-inducible genes.
(39) and that the stable transfection of p48 expression vehicle effectively enhances the response to IFN-α treatment in melanoma cells (40). As suspected, the treatment of HuH-7 cells with 100 IU/ml IFN-α followed by 1,000 IU/ml IFN-α enhanced the suppressive effect of IFN-α on pXEn-CAT activity by approximately 45–70% (Fig. 3A, lanes 4 and 8), although the IFN-γ treatment alone showed a little effect on its activity (Fig. 3A, lanes 6 and 7). In addition, we cotransfected the p48 expression plasmid together with pXEn-CAT into HuH-7 cells, followed by incubation with or without 1,000 IU/ml IFN-α. p48 transfection without IFN-α treatment resulted in approximately 20% decrease in pXEn-CAT activity (Fig. 3B, lane 3), compared with vehicle transfection (Fig. 3B, lane 1). In contrast, p48 transfection followed by IFN-α treatment markedly reduced pXEn-CAT activity by 80% (Fig. 3B, lane 4). However, cotransfection experiments by IRF-1 or its counterpart IRF-2 expression plasmid together with pXEn-CAT showed no significant changes (Fig. 3B, lanes 5 and 6), even though the in vitro transfected IRF-1 interacted with the HBV ISRE-like sequence (Fig. 1A, lane 6). These results suggest that abundance of p48 enhances the IFN-α-mediated suppression of the HBV enhancer-1 activity in HuH-7 cells. Kimura et al. (13) have reported that p48 is essential to the induction of anti-viral genes such as 2′,5′-OAS and PKR by IFN-α, and IRF-1 cannot compensate for the loss of p48. The authors also showed that expression of guanylate-binding protein (GBP) was largely dependent on IRF-1, indicating the diversity of p48 and IRF-1 function. In addition, p48 is a key factor involved in IFN-α and IFN-γ-induced anti-viral responses against encephalomyocarditis virus, vesicular stomatitis virus, and herpes simplex virus (13). Taken together, it is possible that suppression of HBV enhancer activity by IFN-α is largely dependent on p48 rather than IRF-1.

In an attempt to investigate whether IFN-α induced p48 or IRF-1 mRNAs in HuH-7 cells, Northern blot analysis was performed. IRF-1 mRNA was induced at 1 h after 1,000 IU/ml IFN-α treatment (Fig. 4A, lane 2), but its induction was transient as described previously (37), while induction of p48 mRNA was still detected 24 h after treatment with IFN-α (Fig. 4A, lane 4). Similar results were obtained with 100 IU/ml IFN-γ treatment (Fig. 4B). It is known that p48 is induced by IFN-γ and by viral infection (11, 15). However, recent reports have also revealed that p48 mRNA is induced by IFN-α in human peripheral blood mononuclear cells (41) and that IFN-α/β can stimulate the promoter activity of p48 gene in murine macrophage cell line (42).

The suppression of HBV enhancer-1 activity by IFN-α in combination with IL-1β was apparently greater than that induced by IFN-α alone. Such synergistic effect was also observed with TNF-α (5) or IFN-γ (Fig. 3A, lane 8). These cytokines are associated with the noncytolytic clearance of HBV from hepatocyte by cytotoxic T lymphocytes (6–8). Accordingly, it is likely that IFN-α interacts with other cytokines to eradicate the infected virus more efficiently, although further studies are necessary to clarify how IFN-α and other cytokines act cooperatively in viral clearance.

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