View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS Letters 580 (2006) 755–762

Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-γ-induced upregulation of B7-H1 (CD274)

Seung-Jin Lee[a,1], Byeong-Churl Jang[b,1], Soo-Woong Lee[c], Young-Il Yang[d], Seong-Il Suh[b], Yeong-Min Park[e], Sangtaek Oh[f], Jae-Gook Shin[f], Sheng Yao[g,h], Lieping Chen[g,h], In-Hak Choi[a,c,*]

[a] *Department of Microbiology, Inje University College of Medicine, Busan 614-735, Republic of Korea*
[b] *Chronic Disease Research Center and Institute for Medical Science, Keimyung University School of Medicine, Daegu 700-12, Republic of Korea*
[c] *Center for Viral Disease Research, Inje University College of Medicine, Busan 614-735, Republic of Korea*
[d] *Paik Institute for Clinical Research, Inje University College of Medicine, Busan 614-735, Republic of Korea*
[e] *Department of Microbiology and Immunology, and Medical Research Institute, and Laboratory of Dendritic Cell Differentiation and Regulation, Pusan National University College of Medicine, Busan 614-735, Republic of Korea*
[f] *PharmcoGenomic Research Center, Inje University College of Medicine, Busan 614-735, Republic of Korea*
[g] *Department of Dermatology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA*
[h] *Department of Oncology and the Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA*

Received 22 October 2005; revised 22 December 2005; accepted 23 December 2005

Available online 9 January 2006

Edited by Beat Imhof

Abstract Majority of cancer cells upregulate co-inhibitory molecule B7-H1 which confers resistance to anti-tumor immunity, allowing cancers to escape from host immune surveillance. We addressed the molecular mechanism underlying the regulation of cancer-associated B7-H1 expression in response to interferon-γ (IFN-γ). Using promoter constructs in luciferase assay, the region between 202 and 320 bp from the translational start site is responsible for B7-H1 expression. Electrophoretic mobility shift assay, site-directed mutagenesis and knockdown experiment using siRNA revealed that interferon regulatory factor-1 (IRF-1) is primarily responsible for the constitutive B7-H1 expression as well as for the IFN-γ-mediated B7-H1 upregulation in a human lung cancer cell line A549. Additionally, AG490, a Janus activated kinase/signal transducer and activator of transcription inhibitor, greatly abolished the responsiveness of A549 cells to IFN-γ by reducing the IRF-1 transcription. Our findings support a critical role of IRF-1 in the regulation of constitutive and IFN-γ-induced expression of B7-H1 in cancer cells.
© 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: B7-H1; IRF-1; IFN-γ; JAK/STAT pathway

1. Introduction

B7-H1 (CD274, PD-L1), a member of B7 family, is a co-signaling molecule that could be stimulatory or inhibitory for T cell immunity through engagement of different receptors expressed on T cells [1]. Small fraction of antigen-presenting myeloid dendritic cells and Kupffer cells in liver express a low level of endogenous B7-H1 on the surface. However, since the B7-H1 expression in peripheral tissues is tightly regulated in vivo, it is hard to detect B7-H1 expression in a variety of normal tissues through immunohistochemical analysis [2,3]. Unlike normal cells of epithelial origin, human cancer cells, including lung cancer, ovarian cancer, and melanoma, express constitutively B7-H1 on the surface, and its expression is greatly upregulated by interferon-γ (IFN-γ) [4].

IFN-γ is a pro-inflammatory cytokine that is produced mainly by activated type 1 $CD4^+$ helper T cells (Th1), $CD8^+$ cytotoxic T lymphocytes (CTLs), macrophages, and natural killer (NK) cells, and regulates anti-viral and anti-tumor immunity [5]. Collectively, in cancer microenvironment, cancer-specific or non-specific immune cells that are recruited into cancer sites induce inflammation, a cellular process involving the release of a wide variety of inflammatory mediators including IFN-γ [6], subsequently ensuing induction of various cellular proteins such as B7-H1 in cancer cells.

Overwhelming data indicate that cancer-associated B7-H1 in murine cancer model facilitates apoptosis of cancer-reactive CTLs or forms a molecular shield, resulting in suppression of antitumor immunity [4,7]. In addition to the blockade of cancer-associated B7-H1 using antagonistic B7-H1 antibody, downregulation of B7-H1 expression by genetic or pharmacological methods may offer alternative promising approaches for cancer treatment. To manipulate B7-H1 expression genetically or pharmacologically, it is necessary to understand how its expression is regulated within cancer cells [8,9]. In a recent publication, Loke et al. [10] reported that B7-H1 expression was enhanced on inflammatory macrophages by classical activation by LPS and IFN-γ in a STAT1/3-dependent manner. However, it still remains unclear what molecular mechanisms are involved in the constitutive and IFN-γ-induced B7-H1 expression in cancer cells.

In this study, we demonstrated that endogenously expressed interferon regulatory factor-1 (IRF-1), a transcription factor, is primarily responsible for the constitutive B7-H1 expression as well as for the early induction of B7-H1 within 45 min after IFN-γ stimulation. Additionally, de novo synthesized IRF-1 through Janus activated kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is involved in the late induction of B7-H1 expression in response to IFN-γ.

*Corresponding author. Fax: +82 51 891 6004.
E-mail address: miccih@inje.ac.kr (I.-H. Choi).

[1] These authors contributed equally to this work.

Abbreviations: IRF-1, interferon regulatory factor-1; IFN-γ, interferon-γ; JAK, Janus activated kinase; STAT, signal transducer and activator of transcription

0014-5793/$32.00 © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2005.12.093

2. Materials and methods

2.1. Cell lines and reagents

A549 human lung carcinoma cell line, colorectal carcinoma (Colo201 and WiDr), and HeLa cells were purchased from American Type Culture Collection (Manassas, VA). SNU739 hepatocellular carcinoma cell line was purchased from Korean Cell Line Bank (Seoul, Korea). Cells were grown in RPMI 1640 medium (GIBCO, NY) supplemented with 10% heat inactivated fetal bovine serum (FBS). IFN-γ and AG490 were purchased from R&D Systems (Minneapolis, MN) and Calbiochem (Ra Jolla, CA), respectively. PD98059, SB203580, SP600125, and LY294002 were purchased from Biomol (Plymoth, PA, USA). Rapamycin, PP1, Rottlerin, Staurosporine, and Genistein were purchased from Sigma–Aldrich (St. Louis, MO, USA). pGL3 basic vector, pRL CMV vector and dual luciferase reporter assay kit were purchased from Promega (Madison, WI). Restriction enzymes and modified enzyme were purchased from Promega and TaKaRa (Seta, Japan). Antibodies specific for IRF-1 and IRF-2 were purchased from Cell Signaling Technology (Beverly, MA). [γ-^{32}P]-ATP and poly(dI-dC)$_2$ were obtained from Amersham Biosciences (Piscataway, NJ). PE-conjugated streptavidin was obtained from Jackson ImmunoResearch (West Grove, PA). Anti-human B7-H1 (5H1) was generated in our laboratory, and was described previously [11,12].

2.2. Flow cytometric analysis

Cells were stained with biotin-conjugated anti-human B7-H1 (5H1), detected with PE-conjugated streptavidin, and analyzed using flow cytometry and CellQuestPro software (BD science, Franklin Lakes, NJ). Cells were washed between the reactions in FACS buffer (1% FCS and 0.1% NaN_3 in PBS). B7-H1 expression on the surface was represented as mean fluorescence intensity (MFI).

2.3. Western blot

Whole cell extracts were prepared in the lysis buffer as described previously [13]. Protein extracts were subject to SDS–PAGE (10% gel), transferred to nitrocellulose membranes (Schleicher & Schuell, NH), and probed with antibodies. The respective bands were detected with HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology Inc., CA). The amount of chemiluminescence was measured by LAS-3000 SYSTEM (Fuji Photo Film, Japan).

2.4. Preparation of promoter constructs

Human genomic DNA was isolated from human embryonic kidney-293 (HEK-293) cell line using a DNeasy Tissue Kit (Qiagen, GmhH, Germany). The 3-kb of 5′-flanking promoter region was amplified from genomic DNA by PCR using upstream primer, 5′-TGA <u>CTC GAG</u> ACA CAT ATA GGA TGT GAG-3′; downstream primer, 5′-TCT <u>CTC GAG</u> CCC AAA GAA AGG GTG TAG-3′; The amplified 3036 bp fragment was cloned into TA-vector (pTA-3036) (Promega, WI). The luciferase constructs were generated by digesting pTA-3036 DNA with appropriate restriction enzymes mapped on promoter region and by subcloning into pGL3 basic (Promega). Restriction enzymes used for generation of promoter constructs were as follows; *Bam*HI/*Xho*I (pH1-2405), *Ssp*I/*Bgl*II (pH1-1101), *Dra*I/*Bgl*II (pH1-633), *Hin*dIII/*Hin*dIII (pH1-202), and *Pvu*II/*Hin*dIII (pH1-88). Exceptionally, pH1-457 and pH1-320 plasmids were created by PCR cloning using specific forward primers (pH1-457: 5′-ACG CC<u>G AGC TC</u>A TAA AGG TTA AGG-3′, 457 bp; pH1-320: 5′-ATC TTC G<u>GA GCT C</u>TT CCC GGT G-3′, 320 bp), and the downstream primer used for PCR of 3036 bp fragment. The B7-H1 promoter sequence was analyzed for transcription factor binding sites within the promoter region using a sequence motif search program of GenomeNet (http://motif.genome.jp).

2.5. Transient transfection and luciferase assay

Luciferase assay was performed using Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction. Luciferase assays were carried out using Wallac 1420 multilabeling counter (Perkin–Elmer, CT). All firefly luciferase values were normalized to Renilla luciferase in order to compare the transfection efficiencies. Results were represented as means ± S.D. of a representative experiment performed in triplicate.

2.6. Site-directed mutagenesis

Mutagenesis of IRF-1 in B7-H1 promoter region was generated using a Quick Change site-directed mutagenesis system (Stratagene, CA). The mutated primers used are listed in Table 1. PCR was performed using two antiparallel primers with the required nucleotide substitutions and the PCR product was used to transform XL1-Blue supercompetent *Escherichia coli* cells (Stratagene). The sequences of the mutation constructs were confirmed by bi-directional DNA sequencing.

2.7. EMSA analysis

Sense and antisense oligonucleotides encompassing the IRF-1 binding site in B7-H1 promoter were synthesized (Table 1). Electrophoretic mobility-shift assay (EMSA) probes were made by annealing the oligonucleotides and end-labeling the resulting double-stranded oligonucleotides using T4 polynucleotide kinase and [γ-^{32}P]-ATP. EMSA analysis was performed using the Gel Shift Assay System (Promega) with A549 nuclear extracts, prepared as previously described [14,15]. For supershift assays, protein extracts were preincubated with 2 μg of anti-IRF-1, anti-IRF-2, and mouse IgG antibody for 4 h at 4 °C prior to binding reaction, and then subject to EMSA assay. The resultant protein/DNA complexes were electrophoretically resolved on 5% polyacrylamide gels in 0.5 × TBE buffer for 3 h at 120 V, and analyzed by autoradiography. The relative band intensity was analyzed using Image Acquisition and Analysis Program (UVP, Cambridge, UK).

2.8. RT-PCR

Total RNAs were isolated using Trizol reagent (Invitrogen, CA), according to the manufacturer's instructions. cDNA was generated using ImProm-II Reverse Transcription System (Promega), which was then amplified by PCR using specific primers for B7-H1 (forward, 5′-GAC CTG AAG GTT CAG CAT AG-3′; reverse, 5′-GTA TCT TGG ATG CCA CAT TT-3′, 613 bp), IRF-1 (forward, 5′-GTA AGG AGG AGC CAG AAA TTG ACA GC-3′; reverse, 5′-CTA CGG TGC ACA GGG AAT GGC CTG-3′, 157 bp), and IFN-γR (forward, 5′-AGT ACC AGA TCA TGC CAC AGG TCC-3′; reverse, 5′-ATC GCT AAC TGG CAC TGA ATC TCG-3′, 465 bp). The various inhibitors were used at working concentration such as AG490 (50 μM), PD98059 (50 μM), SP600125 (25 μM), SB203580 (25 μM), LY294002 (25 μM) Rapamycin (6 nM), PP1 (2 μg/ml), Rottlerin (10 μM), Staurosporine (0.1 μM) and Genistein (10 μM).

Table 1
Sequence of sense oligonucleotides used in site-directed mutagenesis and EMSAs

Oligonucleotides	Sequence (5′ → 3′)	Location
IRF-1α		
Wild-type	ACT GGA CTG ACA TGT TTC ACT TTC TGT TTC	−291 to −262
Mutant	ACT GGA CTG ACA TGT <u>g</u>T<u>a</u> AC<u>c</u> TTC TGT TTC	
IRF-1β		
Wild-type	CTA GAT ACC TAA ACT GAA AGC TTC CGC CGA	−222 to −199
Mutant	CTA GAT ACC TAA <u>g</u>CT <u>t</u>A<u>c</u> AGC TTC CGC CGA	

Mutated bases are underlined [16,17]. Locations are relative to the first translation start site.

2.9. Preparation of small interfering RNA and transfection

Sense and antisense strands of small interfering RNA (siRNA) oligonucleotides directed against IRF-1 mRNA were synthesized as a duplex format (sense, 5′-CCAAGAACCAGAGAAAAGA*dTdT*-3′; antisense, 5′-UCUUUUCUCUGGUUCUUGG*dTdT*-3′) [16,17]. A549 cells were plated at a density of 6×105 cells onto 6-well plate. After 24 h, the cells were transfected with various concentrations of IRF-1 siRNA or control siRNA using Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's protocol.

2.10. Statistical analysis

Student's *t* test was performed wherever applicable. Mean ± S.D. is shown unless otherwise stated.

3. Results

3.1. B7-H1 is upregulated in A549 cells in response to IFN-γ

RT-PCR analyses showed detectable expressions of constitutive B7-H1 mRNA in most cancer cells tested, including Colo201, WiDr, HeLa, and A549 cells. Exceptionally, SNU739, a hepatoma cell line, expressed higher level of endogenous B7-H1 mRNA. Treatment of the various cancer cells with 100 U/ml IFN-γ for 24 h resulted in the induction of high levels of B7-H1 mRNA expression (Fig. 1A). This finding was consistent with protein expression as evidenced by FACS analysis (Fig. 1B). We also observed the upregulated B7-H1 expression in other cancer cell lines such as lung cancer cells (A427 and Calu-3), hepatoma (HepG2 and SNU449), and colon cancer cells (LS513) (Fig. S1). We found that the constitutive expression of B7-H1 protein was more likely prominent than B7-H1 mRNA expression in cancer cells. The finding that the same treatment did not seem to affect the expression of the IFN-γ receptor (IFN-γ R) in any of the cancer cells tested suggest that B7-H1 upregulation in response to IFN-γ does not occur by modulation of its receptor. Given the similarity of responses in the different cancer cell lines, A549 was used in this study. To determine the concentration of IFN-γ revealing a maximum expression of B7-H1, we treated A549 cells with various concentrations of IFN-γ for 24 h. B7-H1 mRNA (Fig. 1C) and protein (Fig. 1D) was maximally increased at 100–200 U/ml IFN-γ. As shown in Fig. 1E and F, B7-H1 mRNA was slightly increased after 1 h of IFN-γ treatment and was further increased in a time-dependent manner thereafter. The maximal induction of B7-H1 mRNA and protein occurred at 9–12 and 24 h, respectively. From the experiment to demonstrate a stability of IFN-γ-induced B7-H1 after withdrawal of INF-γ at 24 h of culture, we found that B7-H1 expression was decreased time-dependently at a similar rate to that under IFN-γ stimulation (Fig. S2).

3.2. Cloning and characterization of the B7-H1 promoter

A 3.0-kb fragment of the B7-H1 promoter was amplified from human genomic DNA by PCR and was cloned into a luciferase reporter plasmid, pGL3 basic vector. The luciferase activity of pH1-2405 was induced 3.0 (±0.6)-fold higher following IFN-γ treatment compared to that of untreated control. Reporter constructs containing promoter regions covering from nt −2405 to nt −320 remained highly inducible in response to IFN-γ (Fig. 2A). There was a dramatic loss of promoter activity in pH1-202 and pH1-88. While these constructs showed greatly reduced luciferase activity, they still displayed B7-H1 promoter activity compared to pGL vector (Fig. 2, inset). Specifically, luciferase activity of pH1-202 construct that contains NF-κB

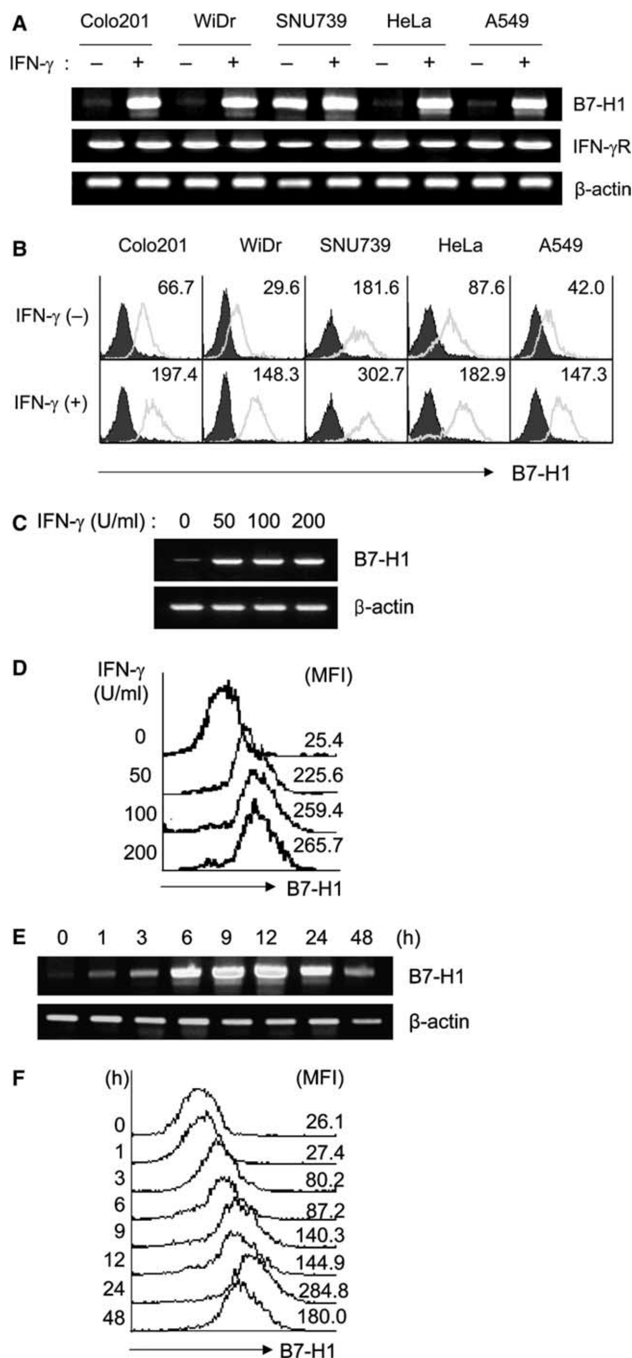

Fig. 1. Effect of IFN-γ on the expression of B7-H1. (A) Total RNAs were isolated from various human cancer cell lines grown in the presence or absence of 100 U/ml IFN-γ for 24 h and analyzed for the relative levels of mRNA of B7-H1, IFN-γ receptor (IFN-γR) or β-actin by RT-PCR. (B) Human cancer cell lines treated as in Fig. 1A were stained with anti-B7-H1 (thin line) and control antibody (shaded area) and analyzed by flow cytometry. (C) A549 cells were stimulated with the indicated concentrations of IFN-γ for 24 h. Total RNAs were isolated from the cells and analyzed for B7-H1 mRNA expression by RT-PCR. (D) The IFN-γ-stimulated A549 cells were stained with biotin-conjugated anti-B7-H1 (5H1) antibody, detected with PE-conjugated streptavidin, and analyzed by flow cytometry. A549 cells were stimulated with 100 U/ml IFN-γ for the indicated times (0–48 h). Time-course effect of IFN-γ on the expression of B7-H1 mRNA (E) or protein (F) in A549 cells was analyzed by RT-PCR and flow cytometry, respectively, as in C and D. All B7-H1 expressions on the cell surface are represented as a MFI in B, D, and F.

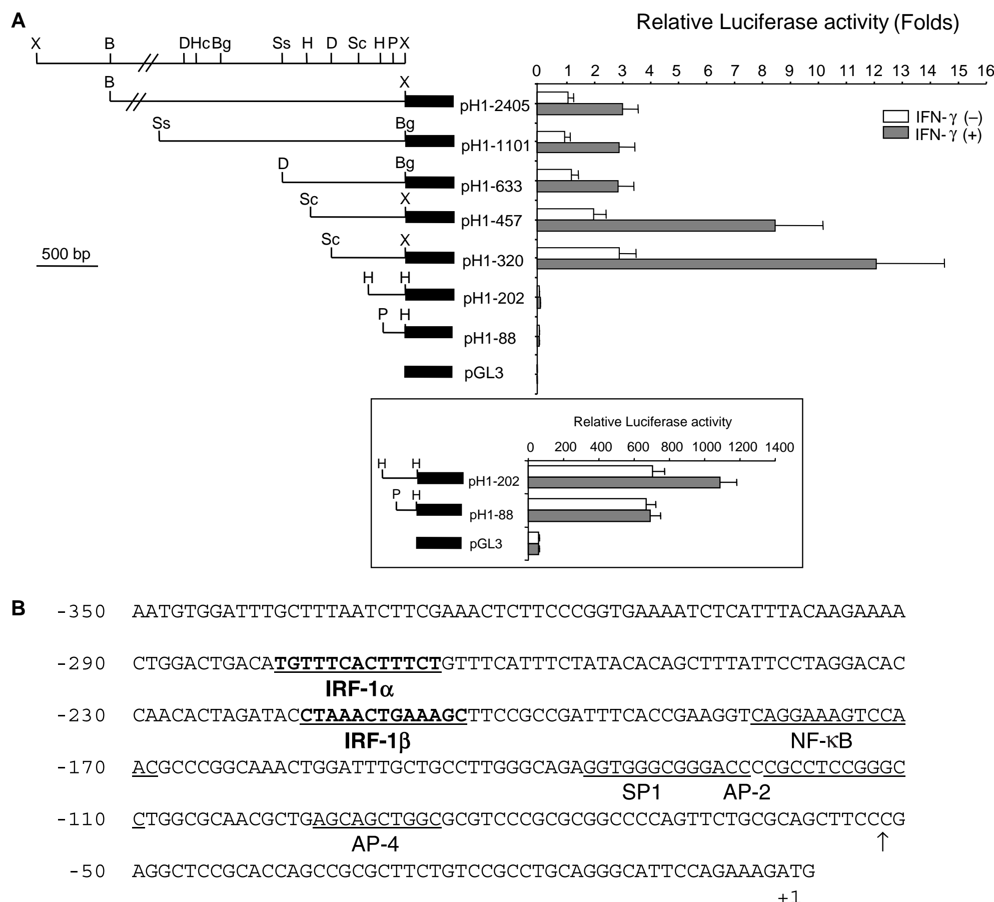

Fig. 2. Delineation of human B7-H1 promoter region. (A) Promoter constructs and restriction enzyme map of human B7-H1 promoter region. A549 cells were transiently cotransfected with various promoter constructs and empty luciferase vector pRL CMV for 36 h to compare transfection efficiencies. The cells were left untreated or treated with 100 U/ml IFN-γ for the last 9 h. Relative luciferase activity was determined as described under Section 2. Results are represented as means ± S.D. of a representative experiment performed in triplicate. B, *Bam*HI; Bg, *Bgl*II; D, *Dra*I; Hc, *Hin*cII; H, *Hin*dIII; P, *Pvu*II; Sc, *Sac*I; Ss, *Ssp*I; X, *Xho*I. (B) Nucleotide sequence of the promoter region of human B7-H1 gene. The 350-bp sequence of the 5′-flanking region of B7-H1 is shown. The translation start site is indicated by +1. The arrow indicates the transcription start site. Underlined sequences are possible transcription factor binding sites, as predicted by GenomeNet.

was likely induced by IFN-γ stimulation, but the relative luciferase activity is significantly lower than that of pH1-320. These results suggest that the promoter region between nt −320 and nt −202 is more likely responsible for the basal or IFN-γ-induced B7-H1 promoter activity in A549 cells. A sequence motif search of GenomeNet further identified transcription factor binding sites in that region, including two IRF-1 sites (IRF-1α, -1β) (Fig. 2B).

3.3. IRF-1 binds to the putative binding sites in B7-H1 promoter

To test the binding of IRF-1 to B7-H1 promoter region, we performed EMSA analyses. As shown in Fig. 3A, IFN-γ increased the protein complex formation between IRF-1 and the DNA probes. The formation of detectable complex was competed out with 50-fold excess of unlabeled cold probe (Fig. 3A). This result provides evidence that IRF-1 is induced by IFN-γ and binds to a specific site in the B7-H1 promoter. Additionally, signal generated by the IRF-1–DNA complex was supershifted in the presence of anti-IRF-1, not anti-IRF-2, antibody, strongly indicating that the nuclear protein complex that binds to the putative IRF-1 binding sequence in B7-H1 promoter region contains IRF-1 protein. Furthermore, a time course analysis showed that maximum binding activity of IRF-1 to B7-H1 promoter appeared in two phases; the first peak of binding activity within 5 min and the second peak at 7 h after IFN-γ stimulation (Fig. 3B).

3.4. IRF-1 is prerequisite to constitutive and IFN-γ-induced B7-H1 transcription

In order to examine the effect of IRF-1 on B7-H1 transcription, we mutagenized the predicted transcription factor binding sites, IRF-1α and IRF-1β, in the pH1-320 promoter construct. Luciferase assay using those mutants revealed that mutation at the IRF-1α and -1β binding sites resulted in a great reduction of constitutive- as well as IFN-γ-induced B7-H1 promoter activity by ∼80–90% (Fig. 4A), implying the importance of these *cis*-acting elements and their involvement in B7-H1 promoter activation. This notion was further supported by the finding that IRF-1 siRNA significantly decreased the IFN-γ-mediated IRF-1 expression and subsequently suppressed IFN-γ-induced B7-H1 mRNA and protein expression by about 80% and 60%, respectively (Fig. 4B). Additionally, IRF-1 siRNA greatly diminished B7-H1 promoter activity in response to IFN-γ in luciferase assay.

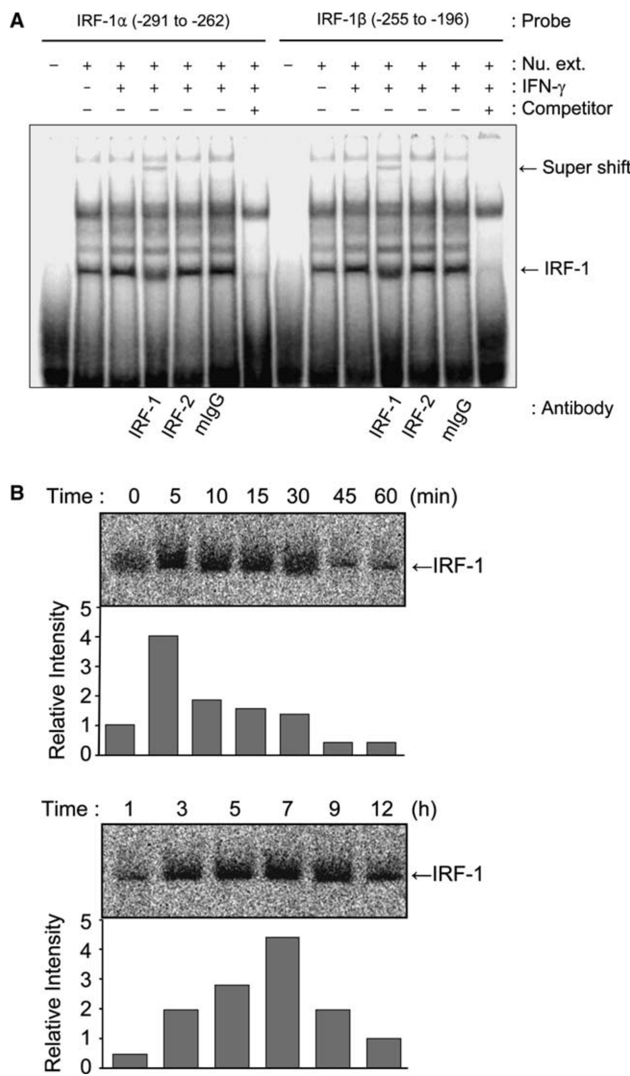

Fig. 3. EMSA of putative IRF-1 binding sites. (A) EMSA was performed with nuclear extract obtained from A549 cells left untreated or treated with 100 U/ml IFN-γ for 9 h, indicated ^{32}P labeled probes, and indicated antibodies. The competition assay was performed with 50-fold excess of unlabeled cold probes. (B) Similar EMSA was performed using nuclear extracts obtained from A549 cells stimulated with 100 U/ml IFN-γ for the indicated times. The relative band intensity was analyzed by densitometry. Results shown are representative of at least three independent experiments.

Interestingly, IRF-1 siRNA also reduced the basal expression of IRF-1, and subsequently declined the constitutive expression of B7-H1 mRNA and protein (Fig. 4B), and downregulated basal B7-H1 promoter activity in luciferase assay (Fig. 4C). In line with this observation, we found that IRF-1 siRNA largely reduced the constitutive B7-H1 expression in SNU739 that expresses high level of basal B7-H1 and greatly diminished the IFN-γ-induced B7-H1 expression in SNU739 and HeLa cells (Fig. 4D). Collectively, the data suggest that IRF-1 plays a key role in constitutive B7-H1 expression and in IFN-γ-induced B7-H1 transcription in A549 cells. This

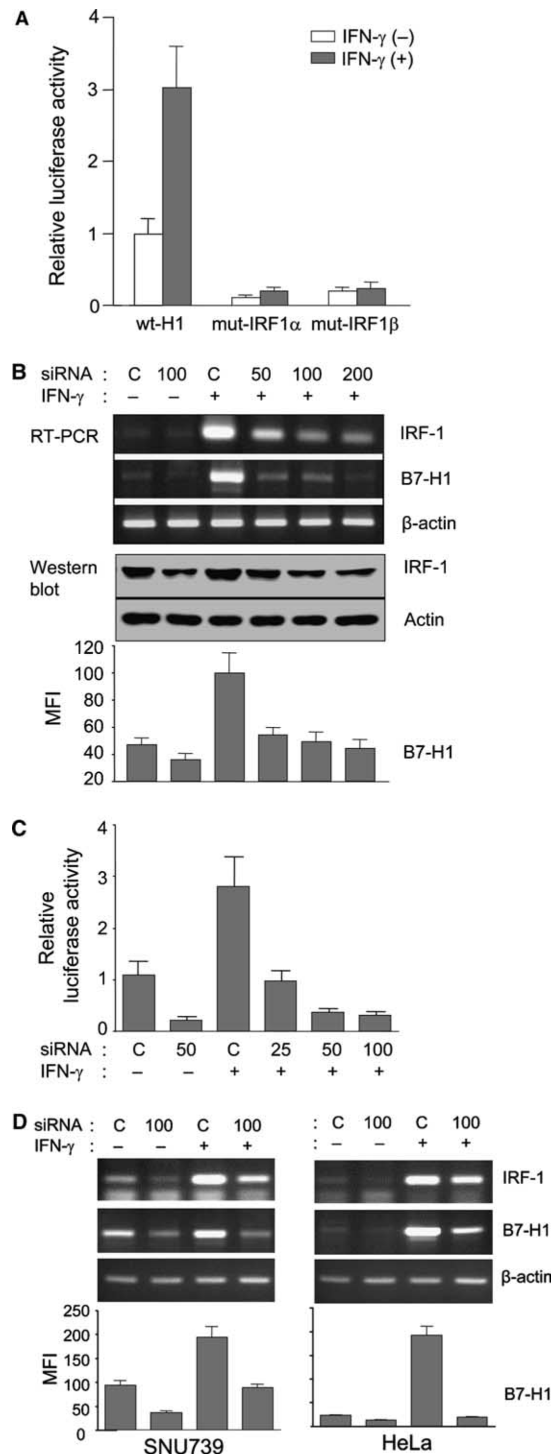

Fig. 4. Contribution of IRF-1 to B7-H1 transcription. (A) A549 cells were transfected for 36 h with promoter construct (pH1-320) containing mutation at IRF-1 binding sites; wild-type pH1-320 (wt-H1), IRF-1α mutant (mut-IRF-1α), IRF-1β mutant (mut-IRF-1β). The cells were left untreated or treated with 100 U/ml IFN-γ for the last 9 h. The cells were then lysed and assayed for relative luciferase activity under Section 2. (B) A549 cells were transfected for 36 h with 100 nM of control siRNA or various concentrations (50–200 nM) of IRF-1 siRNA and then stimulated with 100 U/ml IFN-γ for the last 9 h (for mRNA) and 24 h (for protein). B7-H1 mRNA and protein expression was analyzed using RT-PCR and flow cytometry, respectively. (C) A549 cells were cotransfected with control siRNA or various concentrations of IRF-1 siRNA and pH1-320 construct for 36 h, and then stimulated with 100 U/ml IFN-γ for the last 9 h. Promoter activity was represented as a relative luciferase activity. Results shown are representative of at least three independent experiments. (D) Transfection of control or IRF-1 siRNA into SNU739 and HeLa cells and the analysis of B7-H1 and IRF-1 expression were done as in B.

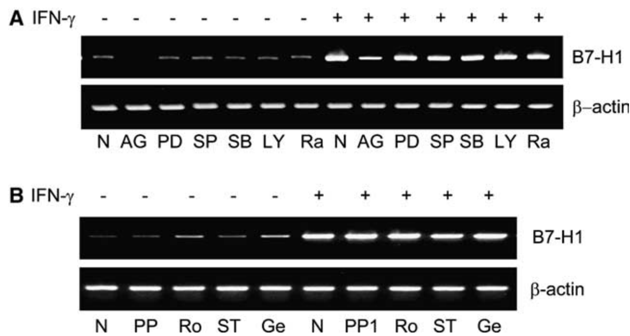

Fig. 5. Effect of various signaling inhibitors on IFN-γ-induced B7-H1 expression. (A) A549 cells were pretreated for 1 h with the indicated inhibitors and then stimulated with 100 U/ml IFN-γ in the presence or absence of the same inhibitor for an additional 9 h. Total RNAs were prepared and analyzed by RT-PCR for B7-H1 or β-actin. N, no treatment; AG, AG490 (JAKII inhibitor); PD, PD98059 (ERK inhibitor); SP, SP600125 (JNK inhibitor); SB, SB203580 (p38 inhibitor); LY, LY294002 (PI3K inhibitor); Ra, rapamycin (mTOR/p70S6K inhibitor). (B) The same experiment as in Fig. 6A except for PP, PP1 (Src kinase inhibitor); RO, rottlerin (PKC inhibitor); ST, staurosporin (PKC inhibitor); Ge, genistein (protein kinase inhibitor).

notion was the case with primary normal cells including human dermal fibroblasts and adipose stem cells (Fig. S2).

3.5. *JAK/STAT pathway is responsible for regulation of IFN-γ-induced B7-H1 expression*

It has been reported that IFN-γ activates various intracellular signaling proteins such as JAK/STAT, PI3K, MTOR/p70-S6K, ERK, JNK, p38, and protein kinases C (PKCs) [18]. We sought to examine whether inhibition of these signaling proteins affects IFN-γ-mediated B7-H1 expression in A549 cells. We found that treatment with AG490 (an inhibitor of JAK/STAT) greatly reduced the transcription of B7-H1 in the presence or absence of IFN-γ compared to other inhibitors for signaling proteins (Fig. 5A and B). Interestingly, there was a detectable upregulation of B7-H1 transcription in A549 cells treated with rottlerin and genistein (Fig. 5B). Taken together, JAK/STAT pathway may be predominantly responsible for the IFN-γ-mediated B7-H1 transcription. This postulate was further supported by the finding that pretreatment with AG490 strongly reduced both basal expression and IFN-γ-induced upregulation of B7-H1 mRNA and protein in dose-dependent mode (Fig. 6A). This was the case with IRF-1 transcription in the presence of AG490 (Fig. 6A). In consistent with this finding, basal and IFN-γ-induced B7-H1 promoter activity was substantially diminished in the presence of AG490 (Fig. 6B), an observation suggesting that JAK/STAT pathway may be a key signaling pathway involved in basal expression and IFN-γ-induced upregulation of B7-H1 in A549 cancer cells.

4. Discussion

IRF-1, a member of IRF family of 10 mammalian transcription factors (IRF-1-10), reveals a great functional diversity in the regulation of cellular response in host defense [19]. IRF-1 mRNA is expressed in a variety of cell types, and its expression is dramatically upregulated upon viral infection or IFN stimulation [20]. In this study, we demonstrated that IRF-1 is a

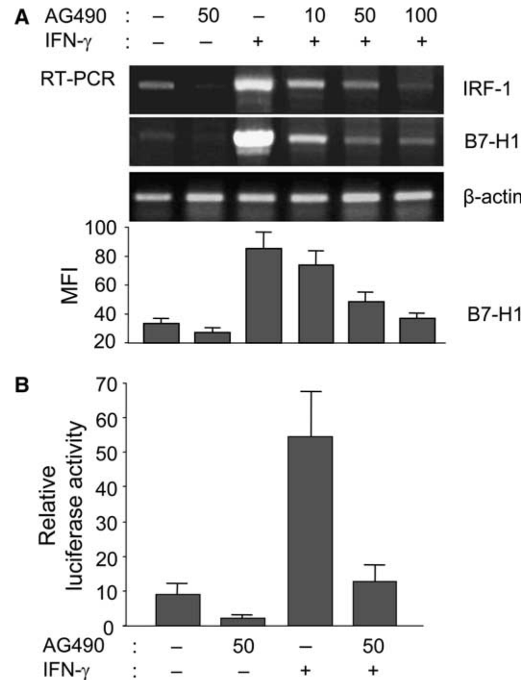

Fig. 6. The effect of JAK/STAT pathway on B7-H1 expression. (A) A549 cell were pretreated with various concentration of the JAKII inhibitor AG490 for 1 h before stimulation with 100 U/ml IFN-γ, and then incubated in the presence or absence of AG490 for an additional 9 and 24 h, and then B7-H1 mRNA and protein expression were analyzed. (B) A549 cells were transfected with promoter construct pH1-320 and pretreated with 50 μM of AG490 for 1 h before 9 h-IFN-γ stimulation. Relative luciferase activity was determined. Results shown are representative of at least three independent experiments.

critical transcription factor for both constitutive and IFN-γ-induced B7-H1 expression. This result was evidenced by the observations from a site-directed mutagenesis study, in which mutations of the IRF-1 binding site in B7-H1 promoter greatly diminished the basal transcription of B7-H1 in A549 cells that were unstimulated with IFN-γ. Additionally, those mutations appeared to completely abolish the responsiveness of A549 cells to IFN-γ. In support of this finding, knockdown of IRF-1 using siRNA greatly decreased both the constitutive and IFN-γ-induced transcription and translation of B7-H1 in A549 cells. The data provide evidence that IRF-1 serves as an essential transcription factor involved not only in the constitutive but also in IFN-γ-induced B7-H1 expression.

B7-H1 transcription and translation was induced as early as 1 h post-IFN-γ stimulation. This early induction of B7-H1 expression may be largely mediated by the basally expressed IRF-1, as demonstrated by the finding that cytoplasmic IRF-1 was significantly mobilized into a nucleus and to bind B7-H1 promoter region as early as 5 min after IFN-γ stimulation, a time period during which IFN-γ stimulation is probably not sufficient to de novo synthesize IRF-1 through JAK/STAT pathway [21,22]. In addition, the late IFN-γ-mediated B7-H1 upregulation at 12–24 h after IFN-γ stimulation may occur through de novo synthesized IRF-1, which shows a maximal binding activity to B7-H1 promoter at 7 h following IFN-γ stimulation. Based on the result of IRF-1 and B7-H1 transcription in the presence of AG490, JAK/STAT can be demonstrated as key signaling molecules responsible for basal and IFN-γ-induced transcription of IRF-1 and B7-H1. From the

data on PKC inhibitor-induced upregulation of B7-H1 in the absence of IFN-γ, however, we cannot exclude the possibility that other signaling pathway such as PKC might be involved in basal expression of B7-H1.

From the promoter activity assay using various promoter constructs, we found that promoter region from nt −457 and nt −2405 was implicated in an inhibition of promoter activity in response to IFN-γ compared to pH1-457 and pH1-320 which showed maximal B7-H1 promoter activity. This observation suggests that promoter region upstream to nt −457 may contain binding site(s) for some inhibitory transcription factor(s). It remains to be illustrated in the future study. NF-κB is a well-known transcription factor involved in many immunological events, such as inflammatory cytokine production, TCR-mediated T cell activation, and regulation of co-signaling molecules [12,23,24]. Interestingly, although B7-H1 promoter contains an NF-κB binding site at nt −182, NF-κB is unlikely to play a key role in the transcription of B7-H1 in A549 lung cancer cells, as evidenced by the luciferase assay in which promoter construct containing NF-κB binding site greatly abolished the responsiveness of A549 cells to IFN-γ. Recently, a publication suggested that NF-κB is essential for IFN-γ-induced B7-H1 upregulation in normal dermal fibroblast, and PI3K is involved in this upregulation [25]. We suppose that this discrepancy in terms of transcription factor and signal pathway involving B7-H1 transcription may result from the different cell types, cancer and normal cells, in which IFN-γ may trigger activation of different signaling proteins, whose activation may differently involve B7-H1 expression.

It is conceivable that tumor cells that are exposed to IFN-γ in the cancer microenvironment, which can exert harsh conditions for cancer cells, can escape immune surveillance by suppressing cancer-specific CTLs through upregulation of a co-inhibitory molecule B7-H1 on the surface. Therefore, one should be cautious to the use of IFN-γ as a therapeutic agent for cancer treatment. Thus understanding the molecular mechanism of regulation of B7-H1 expression in cancers may shed a light on a development of alternative strategies for cancer treatment including cytokines or pharmaceutical compounds downregulating the cancer-associated B7-H1 expression.

Acknowledgments: This study was supported by National R&D Program Grant (M10417060006-04N1706-00610) of The Ministry of Science and Technology and 2,000 Inje University Grant (I.-H.C), and by National Institutes of Health Grant CA97085 and CA106861 (L.C.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.12.093.

References

[1] Chen, L. (2004) Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat. Rev. Immunol. 4, 336–347.

[2] Liang, S.C., Latchman, Y.E., Buhlmann, J.E., Tomczak, M.F., Horwitz, B.H., Freeman, G.J. and Sharpe, A.H. (2003) Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. Eur. J. Immunol. 33, 2706–2716.

[3] Ichikawa, M. and Chen, L. (2005) Role of B7-H1 and B7-H4 molecules in downregulating effector phase of T-cell immunity: novel cancer escaping mechanisms. Front. Biosci. 10, 2856–2860.

[4] Dong, H., Strome, S.E., Salomao, D.R., Tamura, H., Hirano, F., Flies, D.B., Roche, P.C., Lu, J., Zhu, G., Tamada, K., Lennon, V.A., Celis, E. and Chen, L. (2002) Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat. Med. 8, 793–800.

[5] Kalvakolanu, D.V. and Borden, E.C. (1996) An overview of the interferon system: signal transduction and mechanisms of action. Cancer Invest. 14, 25–53.

[6] Matsuura, H., Sakaue, M., Subbaramaiah, K., Kamitani, H., Eling, T.E., Dannenberg, A.J., Tanabe, T., Inoue, H., Arata, J. and Jetten, A.M. (1999) Regulation of cyclooxygenase-2 by interferon gamma and transforming growth factor alpha in normal human epidermal keratinocytes and squamous carcinoma cells. Role of mitogen-activated protein kinases. J. Biol. Chem. 274, 29138–29148.

[7] Dong, H. and Chen, L. (2003) B7-H1 pathway and its role in the evasion of tumor immunity. J. Mol. Med. 81, 281–287.

[8] Thompson, R.H., Gillett, M.D., Cheville, J.C., Lohse, C.M., Dong, H., Webster, W.S., Krejci, K.G., Lobo, J.R., Sengupta, S., Chen, L., Zincke, H., Blute, M.L., Strome, S.E., Leibovich, B.C. and Kwon, E.D. (2004) Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. Proc. Natl. Acad. Sci. USA 101, 17174–17179.

[9] Kim, J., Myers, A.C., Chen, L., Pardoll, D.M., Truong-Tran, Q.A., Lane, A.P., McDyer, J.F., Fortuno, L. and Schleimer, R.P. (2005) Constitutive and inducible expression of b7 family of ligands by human airway epithelial cells. Am. J. Respir. Cell. Mol. Biol. 33, 280–289.

[10] Loke, P. and Allison, J.P. (2003) PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. Proc. Natl. Acad. Sci. USA 100, 5336–5341.

[11] Curiel, T.J., Wei, S., Dong, H., Alvarez, X., Cheng, P., Mottram, P., Krzysiek, R., Knutson, K.L., Daniel, B., Zimmermann, M.C., David, O., Burow, M., Gordon, A., Dhurandhar, N., Myers, L., Berggren, R., Hemminki, A., Alvarez, R.D., Emilie, D., Curiel, D.T., Chen, L. and Zou, W. (2003) Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. Nat. Med. 9, 562–567.

[12] Yamazaki, T., Akiba, H., Iwai, H., Matsuda, H., Aoki, M., Tanno, Y., Shin, T., Tsuchiya, H., Pardoll, D.M., Okumura, K., Azuma, M. and Yagita, H. (2002) Expression of programmed death 1 ligands by murine T cells and APC. J. Immunol. 169, 5538–5545.

[13] Jang, B.C., Lim, K.J., Paik, J.H., Kwon, Y.K., Shin, S.W., Kim, S.C., Jung, T.Y., Kwon, T.K., Cho, J.W., Baek, W.K., Kim, S.P., Suh, M.H. and Suh, S.I. (2004) Up-regulation of human beta-defensin 2 by interleukin-1beta in A549 cells: involvement of PI3K, PKC, p38 MAPK, JNK, and NF-kappaB. Biochem. Biophys. Res. Commun. 320, 1026–1033.

[14] Kim, S.Y., Choi, E.C., Jo, Y.W., Henson, J.W. and Kim, H.S. (2004) Transcriptional activation of JC virus early promoter by phorbol ester and interleukin-1beta: critical role of nuclear factor-1. Virology 327, 60–69.

[15] Perkins, K.J., Burton, E.A. and Davies, K.E. (2001) The role of basal and myogenic factors in the transcriptional activation of utrophin promoter A: implications for therapeutic up-regulation in Duchenne muscular dystrophy. Nucleic Acids Res. 29, 4843–4850.

[16] Kanazawa, N., Kurosaki, M., Sakamoto, N., Enomoto, N., Itsui, Y., Yamashiro, T., Tanabe, Y., Maekawa, S., Nakagawa, M., Chen, C.H., Kakinuma, S., Oshima, S., Nakamura, T., Kato, T., Wakita, T. and Watanabe, M. (2004) Regulation of hepatitis C virus replication by interferon regulatory factor 1. J. Virol. 78, 9713–9720.

[17] Oshima, S., Nakamura, T., Namiki, S., Okada, E., Tsuchiya, K., Okamoto, R., Yamazaki, M., Yokota, T., Aida, M., Yamaguchi, Y., Kanai, T., Handa, H. and Watanabe, M. (2004) Interferon regulatory factor 1 (IRF-1) and IRF-2 distinctively up-regulate

gene expression and production of interleukin-7 in human intestinal epithelial cells. Mol. Cell. Biol. 24, 6298–6310.
[18] Platanias, L.C. (2005) Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat. Rev. Immunol. 5, 375–386.
[19] Taniguchi, T., Ogasawara, K., Takaoka, A. and Tanaka, N. (2001) IRF family of transcription factors as regulators of host defense. Annu. Rev. Immunol. 19, 623–655.
[20] Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T. and Taniguchi, T. (1988) Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. Cell 54, 903–913.
[21] Schindler, C., Shuai, K., Prezioso, V.R. and Darnell, J.E. (1992) Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. Science 257, 809–813.
[22] Silvennoinen, O., Ihle, J.N., Schlessinger, J. and Levy, D.E. (1993) Interferon-induced nuclear signalling by Jak protein tyrosine kinases. Nature 366, 583–585.
[23] Kim, J.O., Kim, H.W., Baek, K.M. and Kang, C.Y. (2003) NF-kappaB and AP-1 regulate activation-dependent CD137 (4-1BB) expression in T cells. FEBS Lett. 541, 163–170.
[24] Ruefli-Brasse, A.A., Lee, W.P., Hurst, S. and Dixit, V.M. (2004) Rip2 participates in Bcl10 signaling and T-cell receptor-mediated NF-kappaB activation. J. Biol. Chem. 279, 1570–1574.
[25] Lee, S.K., Seo, S.H., Kim, B.S., Kim, C.D., Lee, J.H., Kang, J.S., Maeng, P.J. and Lim, J.S. (2005) IFN-gamma regulates the expression of B7-H1 in dermal fibroblast cells. J. Dermatol. Sci. 40, 95–103.