Interferon-stimulated gene factor 3 complex is required for the induction of sterile α motif and HD domain-containing protein 1 expression by interferon-α in SMMC-7721 cells

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Abstract. Sterile α motif and HD domain-containing protein 1 (SAMHD1) is a novel intrinsic restriction factor that inhibits the replication of certain retroviruses and DNA viruses through its deoxynucleoside triphosphate triphosphohydrolase activity. A previous study by our group showed that SAMHD1 restrained hepatitis B virus replication and interferon (IFN)-α induced SAMHD1 expression in liver cells. However the mechanisms of SAMHD1 upregulation by IFN-α in liver cells have remained elusive. The present study demonstrated that IFN-α treatment increased SAMHD1 mRNA levels in SMMC-7721 cells in a time-dependent manner. Knockdown of STAT1 inhibited the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells. STAT2 silencing also suppressed the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells. Furthermore, the induction of SAMHD1 expression in SMMC-7721 cells by IFN-α was found to be dependent on IFN-regulatory factor 9 (IRF9). In conclusion, these results suggested that the interferon-stimulated gene factor 3 complex, which consists of STAT1, STAT2 and IRF9, is required for the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells.

Introduction

Primates and humans possess intrinsic immunity to inhibit viral replication immediately and directly (1,2). The sterile α motif and HD domain-containing protein 1 (SAMHD1) is a newly identified anti-viral factor in this immunity (3). SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase (dNTPase) that depletes the intracellular pool of deoxynucleoside triphosphates (dNTPs) and restricts the replication of human immunodeficiency virus type 1 (HIV-1) in non-cycling myeloid cells (4-8). Most recently, it was discovered that SAMHD1 prevented HIV-1 infection by directly degrading HIV-1 RNA through its ribonuclease activity (9).

Besides HIV-1, SAMHD1 has the ability to restrict other retroviruses, including simian immunodeficiency virus, feline immunodeficiency virus, bovine immunodeficiency virus, equine infectious anemia virus, murine leukemia virus (MLV), Mason Pfizer monkey virus, Rous sarcoma virus and human T-cell leukemia virus type 1 (10-12). In addition, it has been discovered that SAMHD1 restricts two DNA viruses, herpes simplex virus type 1 (HSV-1) and vaccinia virus, in non-dividing myeloid cells (13,14). A previous study by our group showed that SAMHD1 also restrained the replication of another DNA virus, hepatitis B virus (HBV) (15). Most recently, porcine SAMHD1 was demonstrated to block the replication of porcine reproductive and respiratory syndrome virus, a positive-stranded RNA virus, in MARC-145 cells (16). Thus, SAMHD1 is a relatively broad-spectrum anti-viral factor against numerous types of virus.

Interferons (IFNs) often strongly induce the expression of restriction factors during the anti-viral state (17). It is well known that type I IFNs may induce the expression of IFN-stimulated genes (ISGs) through the canonical and non-canonical signaling pathway (18,19). In the canonical pathway, the binding of IFN-α to the IFN-α receptor results in the activation of Janus kinase (JAK) members Tyk2 and JAK1, which phosphorylate signal transducer and activator of transcription 1 (STAT1) and STAT2. Phosphorylated STAT1 and STAT2 dimerize and further assemble with IFN-regulatory factor 9 (IRF9) to form a transcription factor complex called IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to IFN-stimulated response elements (ISRE) and directly activates the transcription of ISGs. Accumulating evidence has

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shown that non-canonical IFN-α signaling pathways exist and function beyond ISGF3 (19).

Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G protein is a well-studied anti-viral factor that may be induced by IFN-α in liver cells through a novel STAT1-independent signaling pathway (20). A previous study by our group reported that IFN-α induced SAMHD1 expression in liver cells (15). However, the mechanism of how SAMHD1 expression is upregulated by IFN-α in liver cells has remained elusive. The present study found that ISGF3 complex was required for the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells, suggesting that IFN-α induced SAMHD1 expression in liver cells through the canonical IFN-α signaling pathway.

Materials and methods

Cell culture, IFN-α stimulation and transfection. SMMC-7721 cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (Zhejiang Tianhang Biological Technology Co., Ltd., Hangzhou, China), in a 37˚C incubator containing 5% CO_2. SMMC-7721 cells were plated in six-well plates ($4x10^5$ cells/well) and grown to 80-90% confluency. Then cells were treated with 1,000 IU/ml IFN-α (Anhui Anke Biotechnology Co., Ltd., Hefei, China) for 0, 2, 4, 6, 8, 10 or 24 h, respectively, and were then harvested for reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis. SMMC-7721 cells were plated in 12-well plates ($2x10^5$ cells/well) and transfected with small interfering (si)RNAs (negative control siRNA, STAT1 siRNA, STAT2 siRNA or IRF9 siRNA) using Lipofectamine™ 2000 (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. 48 h post-transfection, cells were further treated with 1,000 IU/ml IFN-α for 10 h. Cells were harvested for western blot and RT-qPCR analyses.

RNA interference. RNA interference against STAT1, STAT2 and IRF9 was performed using known siRNAs which had been used in previous studies (21-23). The siRNAs had the following sequences: STAT1 sense, 5'-r(CACGAGACCAAU GGUGUGG)d(TT)-3' and anti-sense, 5'-r(CCACACCAU UGGUCUCGUG)d(TT)-3'; STAT2 sense, 5'-GGACUG AGUUGCCUGGUUAUU-3' and anti-sense, 5'-(P)UAA CCAGGCAACUCAGUCCUU-3'; IRF9 sense, 5'-GCAGAG ACUUGGUCAGGUAUU-3' and anti-sense, 5'-(P)UACCUG ACCAAGUCUCUGCUU-3'; negative control sense, 5'-UUC UCCGAACGUGUCACGUTT-3' and anti-sense, 5'-ACG UGACACGUUCGGAGAATT-3'. All of these siRNAs were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). SMMC-7721 cells were transfected with the respective siRNAs and incubated for 48 h. After further stimulation with IFN-α, cells were harvested for western blot and RT-qPCR.

Western blot analysis. After RNA interference and IFN-α treatment, SMMC-7721 cells were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The protein concentration of the supernatants was determined using a bicinchoninic acid kit (cat. no. P00125; Beyotime Institute of Biotechnology) after centrifugation at 12,000 g for 5 min, 20 μg protein was loaded onto each lane and separated by 10% SDS-PAGE. Then proteins were transferred onto Immuno-Blot polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Following blocking, the membranes were incubated with rabbit anti-STAT1 polyclonal antibody (cat. no. 10144-2-AP; 1:1,000), rabbit anti-STAT2 polyclonal antibody (cat. no. 16674-1-AP; 1:1,000) or rabbit anti-IRF9 polyclonal antibody (cat. no. 14167-1-AP; 1:1,000) at 4˚C overnight, respectively. These primary antibodies were purchased from Proteintech Group (Wuhan, China). β-actin was used as a loading control and mouse anti-β-actin monoclonal antibody (cat. no. TA-09; 1:500) was obtained from Beijing ZSGB-Biotechnology Co., Ltd. (Beijing, China) and incubated at 4˚C overnight. The following secondary antibodies were used in the present study: Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin IgG (cat. no. BL003A; 1:10,000), incubated at room temperature for 1 h, and goat anti-mouse IgG (cat. no. BL001A; 1:10,000), incubated at room temperature for 1 h (Biosharp Co., Hefei, China). Immunoreactive proteins were visualized using the Super Signal West Femto kit (cat. no. 34094; Thermo Fisher Scientific, Waltham, MA, USA) and images were captured using the digital gel image analysis system (4500SF; Tanon Science & Technology Co., Ltd., Shanghai, China).

RT-qPCR analysis. Total RNA was isolated from SMMC-7721 cells after RNA interference and IFN-α treatment using TRIzol® reagent (Life Technologies). cDNAs were then prepared by reverse transcription from total RNA using M-MLV Reverse Transcriptase (Life Technologies). Real-time qPCR experiments were performed in 0.2 ml 96-well PCR plates using TaqMan® Gene Expression Master Mix (Life Technologies). Each reaction well in the 96-well PCR plates contained a total volume of 20 μl: 1 μl 20xTaqMan® Gene Expression Assay, 10 μl 2X TaqMan® Gene Expression Master Mix, 4 μl cDNA template (500 ng) and 5 μl RNase-free water. The following primer/probe sets were utilized in the present study: SAMHD1 (Hs00210019_m1), ISG15 (Hs00192713_m1) and GAPDH (Hs99999905_m1) (Life Technologies). Real-time PCR reactions were performed using StepOnePlus™ Real-time PCR System (Life Technologies). Thermal cycling conditions were as follows: 2 min at 50˚C and 10 min at 95˚C, followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. Data analysis and quantification were performed using the $2^{-\Delta\Delta CT}$ comparative method (24).

Statistical analysis. Values are expressed as the mean ± standard deviation. Statistical significance of differences between STAT1, STAT2 or IRF9 siRNA-transfected groups with IFN-α-treatment and the control siRNA-transfected group with IFN-α treatment were analyzed by Student's *t* test using GraphPad Prism 5 (GraphPad Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

IFN-α treatment increases SAMHD1 mRNA levels in SMMC-7721 cells in a time-dependent manner. A previous

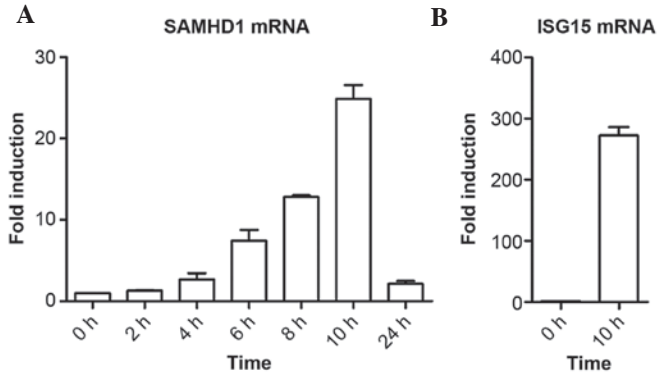

Figure 1. IFN-α induces SAMHD1 expression at the mRNA level in SMMC-7721 cells in a time-dependent manner. (A) SMMC-7721 cells were treated with 1,000 IU/ml IFN-α and harvested following incubation for 0, 2, 4, 6, 8, 10 or 24 h for assessment of SAMHD1 mRNA levels using RT-qPCR. (B) SMMC-7721 cells were stimulated with 1,000 IU/ml IFN-α for 10 h and the mRNA levels of the known IFN-α-responsive ISG15 were detected using RT-qPCR method. Results are representative of at least three independent experiments with triplicate samples, and are expressed as the mean ± standard deviation. IFN, interferon; SAMHD1, sterile α motif and HD domain-containing protein 1; ISG15, interferon-stimulated gene factor 3; RT-qPCR, reverse-transcription quantitative polymerase chain reaction.

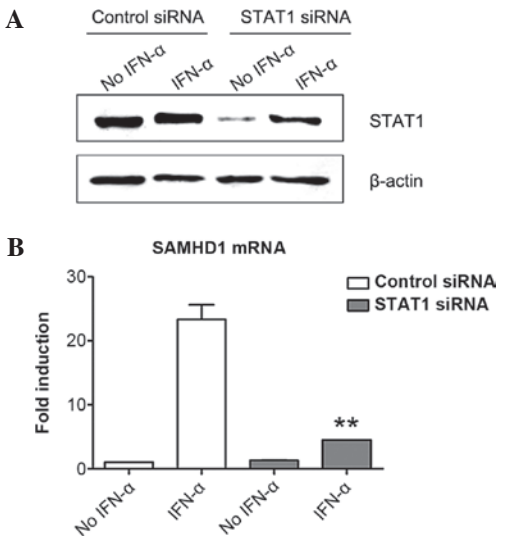

Figure 2. STAT1 downregulation blocks the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells. STAT1 siRNA or control siRNA was transfected into SMMC-7721 cells for 48 h, which were subsequently incubated with or without 1,000 IU/ml IFN-α for 10 h. The cells were harvested and (A) STAT1 protein expression was detected by western blot analysis using anti-STAT1 polyclonal antibody and (B) SAMHD1 mRNA levels were determined by reverse-transcription quantitative polymerase chain reaction. Results are representative of at least three independent experiments with triplicate samples, and are expressed as the mean ± standard deviation. **P<0.01, STAT1 siRNA-transfected group with IFN-α treatment vs. control siRNA-transfected group with IFN-α treatment. IFN, interferon; siRNA, small interfering RNA; STAT, signal transducer and enhancer of transcription; SAMHD1, sterile α motif and HD domain-containing protein 1.

study by our group identified that IFN-α induced SAMHD1 expression at the protein level in liver cells by using western blot analysis (15). The present study further assessed whether IFN-α induced SAMHD1 expression at the mRNA level using RT-qPCR analysis. The results showed that IFN-α treatment increased the mRNA levels of SAMHD1 in SMMC-7721 cells in a time-dependent manner (Fig. 1A). The fold induction of SAMHD1 mRNA expression by IFN-α was ~25-fold following 10 h of incubation, while at 24 h, it was reduced to ~two-fold of the levels at the beginning of the experiment. As the positive control, ISG15, a the well-defined IFN-α-responsive gene, was used. ISG15 mRNA levels in SMMC-7721 cells were markedly upregulated by IFN-α following 10 h of incubation (Fig. 1B). Together with the results of the previous study by our group (15), the results of the present study demonstrated that IFN-α induced SAMHD1 expression in SMMC-7721 cells at the mRNA as well as the protein level in a time-dependent manner.

Induction of SAMHD1 expression in SMMC-7721 cells by IFN-α is inhibited by STAT1 knockdown. ISGF3 transcription factor complex consists of STAT1, STAT2 and IRF9, which are involved in the canonical type I interferon signaling pathway (19). To explore the role of ISGF3 in the induction of SAMHD1 expression by IFN-α, the present study firstly downregulated the expression of STAT1 in SMMC-7721 cells by RNA interference and then evaluated its influence in the induction of SAMHD1 expression by IFN-α using RT-qPCR. The results showed that STAT1 was expressed in SMMC7-7721 cells and that IFN-α treatment induced its expression (Fig. 2A). This phenomenon was similar to that reported by a previous study (25). STAT1-specific siRNA efficiently silenced STAT1 expression in SMMC-7721 cells, although IFN-α treatment partially abrogated the effects of STAT1 siRNA, suggesting that STAT1-knockdown was efficient (Fig. 2A). Of note, STAT1 siRNA treatment significantly reduced the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells, while control siRNA had no effect on IFN-α-induced SAMHD1 expression (Fig. 2B). These results indicated that STAT1 was required for the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells.

STAT2 silencing suppresses the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells. Next, the present study determined the role of STAT2 in the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells. Similarly to the results on STAT1, it was shown that STAT2 was also expressed in SMMC-7721 cells and that IFN-α treatment induced its expression (Fig. 3A). STAT2 expression was almost completely silenced by STAT2-specific siRNA in the group without IFN-α treatment. With IFN-α treatment, STAT2 protein levels in the STAT2 siRNA-transfected cells was obviously lower than those in control siRNA-transfected cells (Fig. 3A). These results demonstrated that STAT2 siRNA successfully inhibited STAT2 expression in SMMC-7721 cells. Of note, the results showed that silencing of STAT2 expression markedly reduced the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells (Fig. 3B), indicating that STAT2 was involved in the signaling pathway of IFN-α-induced SAMHD1 expression.

Induction of SAMHD1 expression by IFN-α is IRF9-dependent in SMMC-7721 cells. The present study further tested whether IRF9 was also involved in the induction of the expression of SAMHD1 by IFN-α in SMMC-7721 cells. The

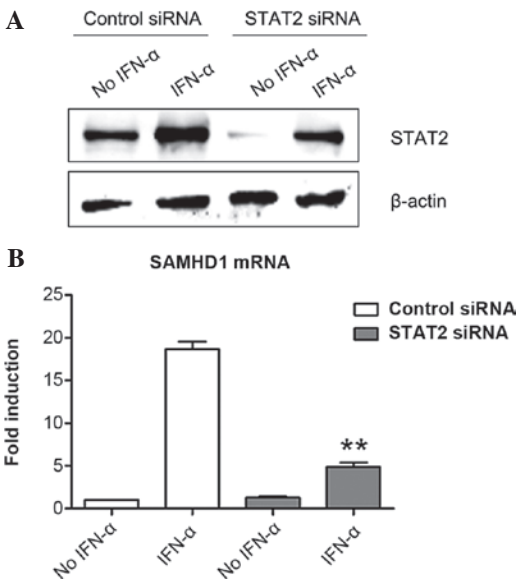

Figure 3. STAT2 silencing reduces the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells. STAT2 siRNA or control siRNA was transfected into SMMC-7721 cells for 48 h and cells were subsequently incubated with or without 1,000 IU/ml IFN-α for 10 h. The cells were harvested and (A) STAT2 protein expression was detected by western blot analysis using anti-STAT2 polyclonal antibody and (B) SAMHD1 mRNA levels were determined by reverse-transcription quantitative polymerase chain reaction. Results are representative of at least three independent experiments with triplicate samples, and are expressed as the mean ± standard deviation. **P<0.01, STAT2 siRNA-transfected group with IFN-α treatment vs. control siRNA-transfected group with IFN-α treatment. IFN, interferon; siRNA, small interfering RNA; STAT, signal transducer and enhancer of transcription; SAMHD1, sterile α motif and HD domain-containing protein 1.

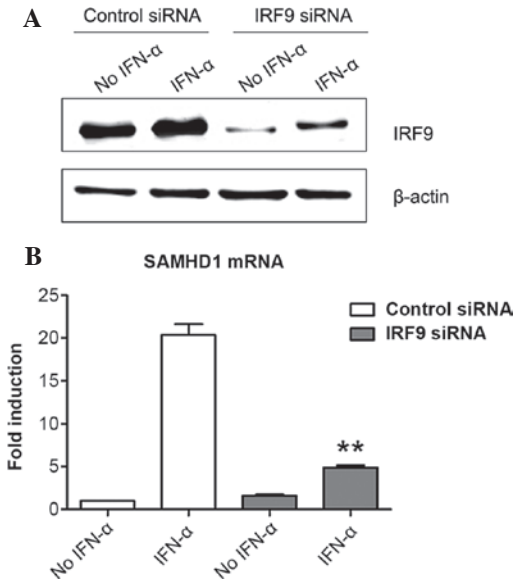

Figure 4. Induction of SAMHD1 expression by IFN-α is IRF9-dependent in SMMC-7721 cells. IRF9 siRNA or control siRNA was transfected into SMMC-7721 cells for 48 h and cells were subsequently incubated with or without 1,000 IU/ml IFN-α for 10 h. Then cells were harvested and (A) IRF9 protein expression was detected by western blot analysis using anti-IRF9 polyclonal antibody and (B) SAMHD1 mRNA levels were determined by reverse-transcription quantitative polymerase chain reaction. Results are representative of at least three independent experiments with triplicate samples, and are expressed as the mean ± standard deviation. **P<0.01, IRF9 siRNA-transfected group with IFN-α treatment vs. control siRNA-transfected group with IFN-α treatment. IFN, interferon; siRNA, small interfering RNA; STAT, signal transducer and enhancer of transcription; SAMHD1, sterile α motif and HD domain-containing protein 1; IRF9, IFN-regulatory factor 9.

results showed that IRF9 was expressed in SMMC-7721 cells and that IFN-α treatment markedly enhanced its expression (Fig. 4A). Of note, IRF9 siRNA markedly inhibited IRF9 expression in the presence or absence of IFN-α (Fig. 4A). More importantly, siRNA-mediated knockdown of IRF9 largely suppressed the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells. These results revealed that IRF9 was required for the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells.

Discussion

It has been demonstrated that IFN-α induces SAMHD1 expression in monocytic cells (26), U87-MG cells (27), human embryonic 293T cells and HeLa cells (28). A previous study showed that SAMHD1 expression was obviously induced by IFN-α at the protein level in SMMC-7721 and BEL-7402 hepatoma cell lines (15). However, the signaling pathway via which the induction of SAMHD1 expression by IFN-α is mediated has remained elusive. The formation of ISGF3 transcription factor complex comprising STAT1, STAT2 and IRF9 is a hallmark of the canonical type I IFN signaling pathway (19). The present study revealed that silencing of STAT1, STAT2 and IRF9 by their specific siRNAs blocked IFN-α-induced SAMHD1 expression in SMMC-7721 cells, indicating that the ISGF3 complex was required for the induction of SAMHD1 expression by IFN-α in SMMC-7721 cells.

Previous studies showed that STAT1, STAT2 and IRF9 mRNA as well as protein levels were upregulated by IFN-α treatment in human hepatoma HepG2 cells (29), human peripheral blood mononuclear cells and macrophages (30). Consistent with these results, the present study indicated that IFN-α upregulated STAT1, STAT2 and IRF9 in human hepatoma SMMC-7721 cells. However, STAT1, STAT2 and IRF9 expression levels in the specific siRNA-transfected groups were markedly lower than those in the control siRNA-transfected groups even after IFN-α stimulation, indicating that these siRNAs were efficient regardless of the presence or absence of IFN-α.

In the classical type I IFN signaling pathway, ISGF3 binds to the consensus ISRE DNA sequence and then activates gene expression. Whether ISRE is located upstream of the SAMHD1 gene requires further investigation. It has been reported that STAT1 may be phosphorylated by inhibitor of nuclear factor κB kinase ε (IKKε) and certain type I IFN-stimulated genes remain inactive in the absence of IKKε, indicated by ISGF3 not binding to the promoter elements of these genes (31). Further studies are required to explore whether IKKε or JAKs are engaged in the formation of ISGF3 in IFN-α-induced SAMHD1 expression in SMMC-7721 cells.

Zhang *et al* (29) reported that the ISGF3 complex has a key role in IFN-α-mediated anti-HBV responses in human hepatoma cells. The present study demonstrated that IFN-α induced SAMHD1 expression through the ISGF3 complex in SMMC-7721 cells. Together with the results of a previous study by our group (15), it may be concluded that SAMHD1 is induced by IFN-α in liver cells through the canonical IFN-α signaling pathway and inhibits HBV replication during IFN-α treatment of HBV-infected patients.

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