pp32, an INHAT component, is a transcription machinery recruiter for maximal induction of IFN-stimulated genes

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Summary
Type I interferon (IFN) plays a crucial role in establishing the cellular antiviral state by inducing transcription of IFN-stimulated genes (ISGs). Generally, histone acetyltransferases (HATs) are positive regulators of transcription, but histone deacetylase (HDAC) activity is essential for transcriptional induction of ISGs. pp32 is known to be a key component of the inhibitor of acetyltransferase (INHAT) complex that inhibits HAT-dependent transcriptional activation. Here, we show that pp32 is involved in the positive regulation of IFN transcription. pp32 interacted with signal transducer and activator of transcription 1 (STAT1) and STAT2 in an IFN-dependent manner. pp32 was not required for tyrosine phosphorylation and nuclear translocation of STATs, but was needed for binding of transcriptional complexes with ISG promoters and, thereby, for maximal transcription activation. pp32 was found to be associated with ISG promoters in IFN-untreated cells, and its binding amount fluctuated as a function of time after IFN treatment. Short interfering RNA (siRNA)-mediated knockdown of pp32 expression reduced the histone acetylation level on ISG promoters, suggesting that pp32 plays a role in ISG transcription by a function other than that of INHAT. Taking these findings together, we propose that pp32 is involved in the formation of ISG transcription initiation complexes, possibly as their recruiter.

Key words: Acetylation, Histone, IFN-stimulated gene, INHAT, Interferon, pp32, Signal transduction, STAT, Transcription

Introduction
Interferon (IFN) plays a crucial role in the cellular antiviral mechanism by inducing immediate transcription of IFN-stimulated genes (ISGs), which encode proteins involved in antiviral activities, through the IFN signaling pathway called the JAK-STAT pathway. In the case of stimulation by type I IFN, then IFN-α and IFN-β, signal transducers and activators of transcription 1 (STAT1) and STAT2 are direct targets of the receptor-associated Janus kinases (JAKs) and are phosphorylated at tyrosine residues in the src homology 2 (SH2) domain, through which they form a STAT1–STAT2 heterodimer. The STAT1–STAT2 heterodimer together with IFN regulatory factor 9 (IRF9) forms the IFN-stimulated gene factor 3 (ISGF3) complex. This complex rapidly translocates from the cytoplasm into the nucleus, binds to IFN-stimulated response element (ISRE) sequences on the ISG promoter, facilitates a transcription initiation complex, and thereby promotes transcription of ISGs (Lau and Horvath, 2002; Platanias, 2005).

Several transcriptional coactivators have roles in the regulation of type I IFN-inducible transcription (Platanias, 2005). Histone acetyltransferases (HATs), such as CBP/p300 and GCN5, interact with STAT1 and/or STAT2 and positively regulate IFN-α/β- and IFN-γ-inducible transcription (Bhattacharya et al., 1996; Horvai et al., 1997; Paulson et al., 1999; Paulson et al., 2002; Zhang et al., 1996). The overexpression of EIA, which binds to CBP/p300 and inhibits its HAT activity, represses IFN-α- and IFN-γ-inducible transcription (Bhattacharya et al., 1996; Zhang et al., 1996). GCN5 interacts specifically with STAT2 and regulates transient acetylation of histones on the ISG promoter, thereby inducing ISG transcription (Paulson et al., 2002). In addition, recent studies have shown that lysine acetylation is one of the major post-translational modifications, not only for histones but also for non-histone proteins including the components of the IFN signaling pathway. In response to type I IFN, the type I IFN receptor 2 (IFNαR2) recruits CBP, and CBP acetylates IFNαR2 at lysine 399, which in turn serves as a docking site for IRF9. In addition, acetylation of IRF9 and STAT2 is also induced by type I IFN stimulation. Acetylation of IRF9 at lysine 81 is required for IRF9 dimerization and DNA binding, and acetylation of STAT2 at lysine 390 might be crucial for its heterodimerization with STAT1 or for formation of the ISGF3 complex (Tang et al., 2007). Interestingly, histone-deacetylase (HDAC) activity has been shown to be required for IFN-dependent gene transcription (Chang et al., 2004; Nusinzon and Horvath, 2003; Sakamoto et al., 2004). Inhibition of the HDAC activity leads to global impairment of ISG expression, although the exact molecular mechanism of the HDAC function during ISG induction remains undefined. These findings seem to contradict the observation that the ISG transcription mediated by STAT1 and STAT2 is positively regulated by histone acetyltransferases (HATs) and/or HAT activity.

pp32, alternatively designated Anp32a/Lanp/PHAPI, is a member of the Anp32 family, which encode leucine-rich repeat (LRR) domains and an acidic domain in their N-terminal and C-terminal regions, respectively (Matsuoka et al., 1994). pp32 is a multifunctional protein that is implicated in mRNA transport, the cell cycle, apoptosis, cellular morphology, and multiple cellular pathways regulated by protein phosphatase 2A (Matilla and Radrizzani, 2005). pp32 is also known as a component of the inhibitor of acetyltransferase (INHAT) complex (Kutney et al.,
Here, we examine the role of pp32 in the regulation of ISG transcription. Our findings show that pp32 interacts with phosphorylated STAT1 and STAT2. The knockdown of pp32 expression reduced promoter loading of the transcriptional complex, and reduced acetylation of histones on ISG promoters in response to IFN stimulation. Because the interaction between pp32 and STAT was found to be functionally important in the regulation of ISG transcription, we carried out immunoprecipitation assays. We found that pp32 was associated with ISG promoter and that the binding level of pp32 fluctuates as a function of time after IFN stimulation. Taking these results together, we propose that pp32 plays an important role in ISG transcription, possibly as a recruiter of the transcription initiation complex to the ISG promoter.

Results

Interaction of pp32 with STATs

It has been reported that in the course of IFN-stimulated transcription, not only HAT activity but also HDAC activity is required. We tried to understand these apparently contradictory observations by examining the role of INHAT. First, to examine the regulatory involvement of pp32, a component of the INHAT complex, in IFN-stimulated transcription, we carried out immunoprecipitation assays. HeLa S3 cells were transfected with an expression vector encoding STATs and pp32, and immunoprecipitated proteins were analyzed by western blotting with antibodies against STAT1 and STAT2. We found that the STATs that interacted with FLAG-tagged pp32 were the phosphorylated forms (Fig. 1C). These results suggest that pp32 is involved in regulation of IFN-stimulated transcription by interacting with STATs in the nucleus.

Knockdown of pp32 decreases the transcription induced by IFN stimulation

To examine the role of pp32 in the transcriptional induction of ISGs, knockdown (KD) of pp32 expression was performed using small interfering RNA (siRNA) specific for pp32. Control cells were transfected with nonspecific siRNA. In the pp32 KD cells, the expression level of pp32 was less than 25% that in the control cells (Fig. 2A, lane 4). To examine the effect of the reduction of pp32 on transcription, we compared the knockdown of pp32 with the control cells (Fig. 2A, lane 4). To examine the effect of the reduction of pp32 on transcription, we compared the knockdown of pp32 with the control cells (Fig. 2A, lane 4). To examine the effect of the reduction of pp32 on transcription, we compared the knockdown of pp32 with the control cells (Fig. 2A, lane 4). To examine the effect of the reduction of pp32 on transcription, we compared the knockdown of pp32 with the control cells (Fig. 2A, lane 4). To examine the effect of the reduction of pp32 on transcription, we compared the knockdown of pp32 with the control cells (Fig. 2A, lane 4).
collected and subjected to quantitative RT-PCR using specific primer sets for mRNAs encoding *IFITM1*, *ISG15*, *ISG56* and *ISG54* (Fig. 2B). *GAPDH* mRNA was analyzed using the same samples as those of the internal control. The mRNA level of all ISG mRNAs in the pp32 KD cells decreased more than that in the control cells. This was also the case in cells expressing siRNA against pp32, which is generated from a transfected siRNA-expression vector and targets a different region from that of the siRNA used for the analysis in Fig. 2 (see also supplementary material Fig. S1). These results suggest that pp32 positively regulates ISG transcriptional induction by IFN.

**pp32 affects ISG transcription but not phosphorylation and translocation of STATs**

Transcriptional induction of ISGs depends on activation of the JAK–STAT pathway, which can be dissected into distinct steps, including steps for tyrosine phosphorylation of STAT1 and STAT2, nuclear translocation of ISGF3, and transcription of ISGs (Lau and Horvath, 2002). The effect of pp32 on ISG promoter-dependent transcription was measured using a reporter gene assay. Control cells and pp32 KD cells were transfected with pISRE-TA-*Luc*, which contains the luciferase (*Luc*) gene under the control of the ISRE sequence responsible for type I IFN stimulation, and with the pSEAP-control, followed by IFN-β treatment. Whole cell lysates were subjected to the luciferase assay, and luciferase activities were normalized by the SEAP activities in each sample (Fig. 3A). The luciferase activity was suppressed by transfection with pp32-specific siRNA, indicating that ISG transcription induced by IFN is impaired at the transcriptional level in pp32 KD cells. To investigate which step is altered by reduction of the pp32 level, we carried out western blotting to measure the tyrosine phosphorylation level of STAT1 and STAT2 in pp32 KD cells. Control cells and pp32 KD cells were treated with IFN-β, and whole cell lysates

![Fig. 2. Suppression of ISG transcription by knockdown of pp32.](image)

(A) Expression level of pp32 in pp32 KD cells. HeLa S3 cells were transfected with siRNA specific for pp32 (lane 4) or negative control (NC) siRNA (lanes 1–3). Whole cell lysates were prepared 72 hours post-transfection and analyzed by western blotting with antibodies for pp32, STAT2 and STAT1. (B) Effect of pp32 KD on ISG induction. Control cells and pp32 KD cells were prepared as for A and treated with or without IFN-β for the indicated periods. Total RNA was subjected to reverse transcription followed by quantitative PCR using specific primer sets for ISG mRNAs and *GAPDH* mRNA. The amount of ISG mRNAs was normalized as a relative amount of *GAPDH* mRNA. Results are represented as mean values + s.d. from three independent experiments.

![Fig. 3. pp32 function is required for transcription but not STAT activation.](image)

(A) Requirement of pp32 for ISG transcription. Hep G2 cells were transfected with siRNA specific for pp32 or negative control siRNA. At 72 hours post-transfection, cells were transfected with pISRE-TA-*Luc* together with pSEAP-control and then treated with or without IFN-β for 6 hours, followed by the luciferase assay. The luciferase activity was normalized with the SEAP activity in each sample and represented as fold-induction relative to that from untreated control cells. Results are represented as mean values + s.d. from three independent experiments. (B) IFN-responsive STAT phosphorylation in pp32 KD cells. HeLa S3 cells were transfected with siRNA specific for pp32 (lanes 6–10) or control siRNA (lanes 1–5). At 72 hours post-transfection, cells were treated with or without IFN-β for the indicated period. Whole cell lysates were analyzed by western blotting with antibodies specific for tyrosine phosphorylated (pY) STAT1 and STAT2, and total STAT1 and STAT2. (C) Translocation of STATs in the nucleus in response to IFN treatment. HeLa S3 cells were transfected with siRNA specific for pp32. At 72 hours post-transfection, cells were treated with or without IFN-β for 30 minutes and processed for indirect immunofluorescence to detect pp32 (green), STAT1 (upper panels) and STAT2 (lower panels). Control cells and pp32 KD cells are shown by yellow arrowheads and white arrowheads, respectively.
were prepared and subjected to western blotting using antibodies specific for tyrosine-phosphorylated STAT1 and STAT2. Both STAT1 and STAT2 became tyrosine-phosphorylated in response to IFN, and the phosphorylation level remained unchanged in the pp32 KD cells compared with that in the control cells (Fig. 3B). This suggests that pp32 is not involved in the phosphorylation step of STATs and thus has no effect on the formation of the transcriptional complex because the level of tyrosine phosphorylation of STAT1 and STAT2 required to dimerize and form ISGF3 was unaltered (Improtta et al., 1994; Qureshi et al., 1995). Next, to examine whether pp32 has a role in the subcellular localization of ISGF3 components, indirect immunofluorescence assays were performed in pp32 KD cells. The expression level of pp32 was decreased in pp32 KD cells, whereas the distribution patterns of STAT1 and STAT2 were not changed upon IFN stimulation as compared with that in control cells (Fig. 3C). These results suggest that pp32 has no effect on translocation of STATs from the cytoplasm to the nucleus upon IFN stimulation. Taken altogether, these results indicate that upstream steps prior to transcriptional induction are not pp32 targets.

**pp32 regulates promoter binding of transcriptional complexes and histone modification on ISG promoters**

On the basis of the above-mentioned results, we considered the possibility that pp32 is involved in transcription complex formation and/or chromatin regulation around the site of the ISG promoter. To examine whether pp32 regulates transcriptional complex formation on the ISG promoter, we performed chromatin immunoprecipitation (ChIP) assays. pp32 KD cells and control cells were treated with or without IFN-β for 30 minutes and then fixed with formaldehyde, and ChIP assays using antibodies against STAT1, STAT2 and RNA polymerase II (Pol II) were carried out. Immunoprecipitated DNA was subjected to quantitative PCR using primer sets corresponding to the promoters for IFITM1 and ISG15 (Fig. 4A). The binding of STAT2, STAT1 and Pol II to the IFITM1 and ISG15 promoters was decreased in pp32 KD cells compared with that in the control cells (Fig. 4A). The decreased level of transcription factors bound to each ISG promoter in pp32 KD cells was in good agreement with the decreased level of ISG transcription in pp32 KD cells (Fig. 2B). These results strongly suggest that pp32 affects recruitment of transcription factors to the ISG promoters. pp32 has been reported to be a component of INHAT, which interacts with histones and inhibits histone acetylation by HDACs (Seo et al., 2002; Seo et al., 2001). In addition, the HDAC activity has been shown to be required for IFN-dependent gene transcription (Chang et al., 2004; Nusinzon and Horvath, 2003; Sakamoto et al., 2004). ChIP assays were performed using antibodies against histone H3 (H3) and acetylated histone H3 (AcH3) (Fig. 4B). We found that the amount of H3 on the ISG promoters was unchanged in the pp32 KD cells compared with that in the control cells, irrespective of mock- or IFN-treatment. The AcH3 level was not changed in pp32 KD cells compared with that in control cells in the absence of IFN treatment, whereas the amount of AcH3, when induced by IFN treatment, was suppressed in pp32 KD cells compared with that in the control cells. These results suggest that pp32 is required for IFN-dependent histone acetylation on the ISG promoter. It is worthwhile noting that this function of pp32 is unexpected given that pp32 is an INHAT component.

**pp32 targets the transcriptional complex**

We have shown that pp32 affects not only recruitment of transcription factors but also histone modification status on ISG promoters. However, it is unclear which step in ISG transcription is the primary target of pp32. To address this, we tried to determine the target of pp32 using a mutant pp32 protein. It has been shown that pp32 contains two INHAT domains in its C-terminal region and that INHAT domain I alone acts as an INHAT through its...
histone binding ability (Seo et al., 2002). We constructed FLAG-tagged pp32ΔC lacking the C-terminal region between amino acid positions 181 and 249, corresponding to INHAT domain II (Fig. 5A). We confirmed that FLAG-tagged pp32ΔC was expressed at the same level as that of FLAG-tagged wild-type pp32 (Fig. 5B). Indirect immunofluorescence assays were performed to visualize the subcellular localization of FLAG-tagged pp32ΔC (Fig. 5C). We found that FLAG-tagged pp32ΔC was predominantly localized in the nucleus.

Next, to examine whether pp32ΔC binds to STAT1 and STAT2, immunoprecipitation assays were performed. Cells transfected with expression vectors encoding FLAG-tagged pp32 or FLAG-tagged pp32ΔC were treated with IFN-β for 30 minutes. Whole cell lysates were prepared, and subjected to immunoprecipitation using the agarose-conjugated antibody against FLAG. We found that FLAG-tagged pp32ΔC does not co-precipitate STAT1 and STAT2 in either IFN- or mock-treated cells (Fig. 5D), indicating that pp32ΔC, which retains its ability as an INHAT, cannot interact with a transcription complex containing STAT1 and STAT2. To confirm that pp32ΔC loses the ability to rescue ISG transcription, we performed rescue experiments with FLAG-tagged pp32ΔC using reporter gene assays in pp32 KD cells. The pp32 KD cells were transfected with plISRE-TA-Luc, pSEAP-control, and expression vectors encoding either FLAG-tagged pp32 or FLAG-tagged pp32ΔC and then treated with IFN-β. Whole cell lysates were subjected to luciferase assay, and the luciferase activity was normalized by the SEAP activity in each sample (Fig. 5E). The transcriptional suppression caused by pp32 KD was rescued by expression of wild-type pp32, but not of pp32ΔC. These results strongly suggest that the target of pp32 is the transcriptional complex containing STAT1 and STAT2 but not histones in the chromatin structure. STAT2 has been reported to recruit GCN5, a HAT protein, through its transactivation domain, resulting in transient acetylation of histones on the ISG promoter in an IFN-dependent manner (Paulson et al., 2002). The decrease in the formation on the ISG promoter of the transcription complex including STAT2 that is able to recruit HAT might result in the decreased level of histone acetylation upon IFN stimulation in pp32 KD cells (Fig. 4B).

**pp32 is associated with ISG promoters by IFN stimulation.** To discuss the dynamics of pp32 in ISG transcription, we investigated whether pp32 is associated with the ISG promoter in an IFN-dependent manner. Cells transfected with the expression vector encoding FLAG-tagged pp32 or FLAG-tagged pp32ΔC were treated with IFN-β, and ChIP assays were carried out. Immunoprecipitated DNA was subjected to quantitative PCR using primer sets corresponding to the promoters for *IFITM1*, *ISG15* and

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**Fig. 5. Targets of pp32.** (A) Representation of FLAGV–pp32ΔC. The INHAT domain I and INHAT domain II are indicated by gray and black boxes, respectively. (B) Expression levels of FLAG–pp32 and FLAG–pp32ΔC. HeLa S3 cells were transfected with expression vectors encoding FLAG-tagged pp32 (lane 2) and FLAG-tagged pp32ΔC (lane 3). At 24 hours post-transfection, whole cell lysates were prepared and analyzed by western blotting with the antibody for FLAG. (C) Nuclear localization of FLAG–pp32 and FLAG–pp32ΔC. Cells were prepared as for B and analyzed by indirect immunofluorescence to detect FLAG–pp32 (in upper panels) and FLAG–pp32ΔC (in lower panels). (D) HeLa S3 cells were transfected with FLAG-tagged pp32 (lanes 3 and 4) or FLAG-tagged pp32ΔC (lanes 5 and 6). At 24 hours post-transfection, cells were treated with or without IFN-β for 30 minutes. Whole cell lysates were subjected to immunoprecipitation with the agarose-conjugated antibody against FLAG, and immunoprecipitated proteins were analyzed by western blotting with antibodies for STAT1, STAT2 and FLAG. (E) Transcription rescue by FLAG–pp32 but not FLAG–pp32ΔC. Hep G2 cells were transfected with siRNA specific for pp32 or control siRNA. At 72 hours post-transfection, cells were further transfected with plISRE-TA-Luc, pSEAP-control, and either FLAG–pp32 (wt) or FLAG–pp32ΔC (ΔC). Controls were transfected with empty vector. Samples were then treated with or without IFN-β for 6 hours followed by the luciferase and SEAP assays. The luciferase activity was normalized with the SEAP activity in each sample and represented as fold-induction relative to that from untreated control cells. Results are represented as mean values ± s.d. from three independent experiments.
β-actin (Fig. 6A). pp32 was associated with each ISG promoter and with the β-actin promoter in both IFN- and mock-treated cells, and the amount of pp32 on the ISG promoter, but not on the β-actin promoter, was transiently increased after IFN stimulation, followed by gradual decrease after the onset of ISG transcription. pp32ΔC was also associated with the ISG promoter in both IFN- and mock-treated cells, but the amount of pp32ΔC on the ISG promoter remained unchanged, irrespective of mock or IFN treatment. To examine whether pp32ΔC has the ability to recruit transcription factors to the ISG promoters, we conducted rescue experiments in pp32 KD cells (Fig. 6B). ChIP assays revealed that the suppression of transcription factor recruitment to the ISG promoters in pp32 KD cells was rescued by expression of wild-type pp32, but not of pp32ΔC. These results suggest that the transient increase of pp32 on the ISG promoter depends on its interaction with STATs and that this interaction is important in transcription factor recruitment to the ISG promoter in an IFN-dependent manner. In addition, the amount of pp32 on the ISG promoter is regulated along with the progression of ISG transcription, and the pp32 function could be required for the initial stage of ISG transcription.

Discussion

We have shown that pp32 is required for positive transcriptional regulation of type I IFN-stimulated genes (Fig. 2B and Fig. 3A). After IFN stimulation, the ISGF3 complex composed of tyrosine-phosphorylated STAT2 and STAT1 and the non-STAT protein, IRF9, which predominantly interacts with STAT2 in an IFN-independent manner (Banninger and Reich, 2004; Dale et al., 1991; Horvath et al., 1996; Lau et al., 2000; Levy et al., 1989; Martinez-Moczygemba et al., 1997), translocates rapidly into the nucleus, binds to the ISRE promoter, and recruits Pol II to initiate ISG transcription. We have shown that pp32 interacts with both tyrosine-phosphorylated STAT2 and STAT1 in an IFN-dependent manner (Fig. 1C) and that the pp32–STAT2–STAT1 complex is found only in the nucleus (Fig. 1B), in agreement with reports that pp32 is localized in the nucleus and type I IFN stimulates the nuclear translocation of STAT2 and STAT1 (Matilla et al., 1997; Matsuoka et al., 1994; Schindler et al., 1992). Our results suggest that pp32 regulates the formation of the transcriptional complex in the nucleus. In fact, we have shown that less Pol II is recruited to the ISG promoters in pp32 KD cells than in control cells (Fig. 4A). pp32 KD suppressed the level of histone acetylation on the ISG promoters induced by IFN stimulation, whereas the histone acetylation level remained unchanged in IFN-untreated cells (Fig. 4B), suggesting that pp32 might not act as an INHAT for histones on the ISG promoter. This notion was supported by rescue experiments using pp32ΔC (Fig. 5).

Since histones on the ISG promoter are transiently acetylated by HAT in an IFN-dependent manner (Paulson et al., 2002), the chromatin structure through histone modification could be important in the IFN-inducible regulation of transcription. The decrease in the formation on the ISG promoter of the transcription complex that recruits HATs might result in suppression of histone acetylation upon IFN stimulation (Fig. 4B). pp32 is associated with ISG promoters in IFN-untreated cells. After IFN stimulation, the amount of pp32 on the ISG promoter was transiently increased but then became dissociated from the ISG promoter after the onset of ISG transcription (Fig. 6A). The transient recruitment of pp32 to the ISG promoter depends on its interaction with STATs. However, this dissociation mechanism of pp32 is still unclear. pp32 binds specifically to unacetylated and hypoacetylated histones, but not to hyperacetylated histones (Kutney et al., 2004; Schneider et al., 2004). On this line, the amount of pp32 on the ISG promoter might be autogenously regulated by the histone acetylation status itself. Thus, it could be hypothesized that (i) IFN stimulation deposits STAT1, STAT2 and pp32 on the ISG promoter; (ii) the STAT1–STAT2 complex recruits functional transcription complexes, including HATs, with the aid of pp32; and (iii) the resultant histone acetylation dissociates pp32 from the ISG promoter. Once the fully active transcription complex is formed on the ISG promoter, it might maintain the IFN-responsive transcription for a given period and gradually decrease as a function of time. The positive effect of pp32 is relatively modest, although pp32 is required for the maximal induction of ISG transcription (Fig. 2B). pp32 is a member of the Anp32 family of proteins, so it...
is possible that other family proteins might be involved in the regulation of the ISG transcription coordinate with or independently of pp32. HDAC activity has been reported to be required for IFN-dependent gene transcription (Chang et al., 2004; Nusinzon and Horvath, 2003; Sakamoto et al., 2004). Inhibition of HDAC activity using HDAC inhibitors such as trichostatin A leads to global impairment of ISG expression, although the molecular mechanism of the HDAC function upon ISG induction remains unresolved. Tyrosine-phosphorylation of STAT as well as nuclear transport and DNA binding of ISGF3 is not altered in cells treated with the HDAC inhibitor (Chang et al., 2004; Nusinzon and Horvath, 2003), whereas Pol II recruitment to the ISG promoter is (Sakamoto et al., 2004). On the basis of these observations, it seems likely that HDAC requirement lies downstream of STAT activation and assembly on chromatin, but prior to Pol II recruitment and/or initiation of transcription. Recently, it has been shown that CBP modifies several STAT family proteins at lysine residues. Acetylation of STATs is important for the regulation of phosphorylation turnover, complex formation and/or DNA binding (Kramer and Heinzel, 2010; Kramer et al., 2009; Tang et al., 2007; Yuan et al., 2005). In addition, HDACs interact with STATs and regulate STAT-mediated transcription (Nusinzon and Horvath, 2003). Levy and colleagues (Chang et al., 2004) discussed how deacetylation of target proteins is required during initiation of ISG transcription and a potential target could be the transactivation domain of STAT2. However, they failed to detect any significant acetylation of STAT2 and we also confirmed this (data not shown).

Because the apparent function of pp32 clarified in this study resembles the function of HDAC in the IFN signaling pathway, it is possible that pp32 cooperates with HDAC to regulate the initiation of ISG transcription. It is also possible that non-STAT transcription proteins are deacetylated by HDAC and that the level of the nonacetylated form is maintained by pp32 during the initiation of ISG transcription initiation.

Materials and Methods

Cell culture and antibody

Human cervical carcinoma cell line HeLa S3, human hepatoma cell line Hep G2 cells and Hela 293 cells were grown in Dulbecco’s modified essential medium supplemented with 10% fetal calf serum. Antibodies used in this study were as follows: anti-STAT1α/β antibody (sc-346; Santa Cruz Biotechnology), anti-STAT2 (sc-476; Santa Cruz Biotechnology), anti-phospho-(Y701) STAT1 (9171; Cell Signaling Technology), anti-phospho-(Y698) STAT2 (907-224; Upstate Biotechnology), anti-pp32 (sc-5652; Santa Cruz Biotechnology), anti-Histone H3 (ab1791; Abcam), anti-acetyl-Histone H3 (06-599; Millipore) and anti-FLAG (F3165; Sigma) antibodies.

Preparation of plasmids

To generate pcDNA–FLAG–pp32 and pcDNA–FLAG–pp32C, fragments of pp32 cDNA and pp32AC cDNA encoding full-sized pp32 and a portion between amino acid positions 1 and 180 of pp32, respectively, were amplified by PCR using His-pp32 cDNA and pp32AC cDNA as templates and primers 5’-GCGGATCCGGGATGGAGTTGGCGACAGGATGTC-3’ and 5’-GCGGATCC-TATTGATCTGACTTCTCCTCCTCT-3’ for pp32, and 5’-GCGGATCCGGGATGGTGATGATAGT-3’ and 5’-GCGGATCCGGGATGGTGATGATAGT-3’, respectively, for pp32AC. The amplified cDNA fragments were digested with BamHI and cloned into pcDNA3.1(+)–FLAG vector (Nishie et al., 2007) that had been digested with the same enzyme.

Immunoprecipitation assay

In order to examine the interaction ofFLAG–pp32 or FLAG–pp32AC with STAT proteins, HeLa S3 cells were transfected with pcDNA–FLAG–pp32 or pcDNA–FLAG–pp32AC using Gene Juice (Novergen). Cells were treated with 1000 IU/ml of human IFN-β for the indicated time periods. Cells were lysed by sonication in IP lysis buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM EDTA, 10% glycerol, 5 mM β-D-glyceroephosphate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM PMSF) and incubated at 4°C for 30 minutes with constant rotation. After centrifugation, the supernatant fraction was incubated with anti-FLAG M2 affinity gel (Sigma) slurred at 4°C for overnight. Proteins associated with complexes were washed on 7.5–10% SDS-PAGE and electroblotted to a nitrocellulose membrane. Proteins were detected by western blotting using a specific antibody, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Amersham), biotinylated anti-rabbit Ig (Amersham), or biotinylated anti-mouse Ig (Amersham). Streptavidin–alkaline phosphatase (Amersham) or streptavidin–horseradish peroxidase (Amersham) were used for detection of biotinylated Iggs.

siRNA for pp32

The Stealth siRNA Stealth 905; Invitrogen) and negative control Stealth siRNA (12935-200; Invitrogen) were introduced into HeLa S3 cells with Lipofectamine 2000 (Invitrogen) or Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol. Furthermore, we used pU6-puro-sipp32 and pU6-puro-EGFP plasmids, which express 21-nucleotide hairpin-type siRNAs against pp32 and EGFP, respectively, under the control of the U6 promoter (Haruki et al., 2006; Naito et al., 2007). The target sequence of pU6-puro-sipp32, 5’-AGGA-ACCTTCTCCGGACACAGCATG-3’, differs from that of p32 Stealth siRNA, 5’-AACCTCTATGTTAATGCTTGTTT-3’.

Indirect immunofluorescence assay

Cells on glass coverslips treated with or without IFN-β (1000 IU/ml) for 45 minutes were fixed with phosphate-buffered saline (PBS) containing 3% paraformaldehyde and then permeabilized with PBS containing 0.2% Triton X-100. The coverslips were soaked in PBS containing 1% BSA, and then incubated at 37°C for 1 hour with a primary antibody. After incubation for 1 hour, the coverslips were washed three times with PBS containing 0.2% BSA for 5 minutes and then subjected to incubation for 1 hour with Alexa-Fluor-488-conjugated donkey anti-goat IgG antibody (Molecular Probes) or Cy3-conjugated AffiniPure donkey anti-rabbit IgG antibody (Jackson ImmunoResearch). The coverslips were washed with PBS containing 0.1% NP-40, and then incubated for 10 minutes with 10 mM 4’,6-diamidino-2-phenyindole (DAPI) or TO-PRO 3(III) iodide (F3635; Invitrogen). The coverslips were finally mounted on glass plates, and cells were observed using a confocal laser-scanning microscope (Carl Zeiss).

Total RNA extraction and RT-PCR

Total RNA was prepared from cells treated with or without IFN-β (1000 IU/ml) using RNeasy minikit (Qiagen) and RNase-free Dnase I (Qiagen), and subjected to reverse transcription with oligo-dT primer and SuperScript III Reverse Transcriptase (Invitrogen). Synthesized cDNA was used for quantitative PCR using FastStart SYBR Green Master (Roche) with specific primer sets; 5’-GTATCGTGC-6 ATTCGGCTCTG-3’ and 5’-GTTGTTGGTTGATATAACGGC-3’, for IFITM1, 5’-TACCTGAAGGCCGACAATGGAGGA-3’ and 5’-TGGCGATCTGCTGCGCAGG-3’, for ISG56, 5’-GACACGTTGAATGTTGGAG-3’ and 5’-GGCTAGTTGTTGTCAGATCTC-3’, for ISG54, 5’-CAGATCACCAGCAAGATC-3’ and 5’-CTCCCTGATTTACTCCAGC-3’, for ISG15, and 5’-AGCAGAAAATTCTACATCT-3’ and 5’-GGACTGTGTGCTAGATC-CTTC-3’, for GAPDH.

Chromatin immunoprecipitation assay

Cells were treated with or without IFN-β (1000 IU/ml) for 30 minutes followed by fixation with 1% formaldehyde at room temperature for 15 minutes. Fixation was quenched by the addition of glycine at the final concentration of 125 mM, then cells were washed twice with PBS. Cells were swollen for 10 minutes in a hypotonic lysis buffer (20 mM HEPES–NaOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, and 0.2% NP-40) and collected in a 1.5 ml tube. After centrifugation, nuclear pellets were lysed in ChIP lysis buffer (50 mM Tris–HCl, pH 7.9, 1% Triton X-100, 0.1% SDS, 0.1% deoxycholic acid, 10 mM EDTA, 5 mM β-mercaptoethanol, and 1 mM PMSF). Nuclear lysates were sonicated to shear the chromatin DNA to ~500 base-pairs in size and then diluted with four volumes of ChIP dilution buffer (12.5 mM Tris–HCl, pH 7.9, 187.5 mM NaCl, and 1% Triton X-100). Lysates clarified by centrifugation were incubated with each antibody at 4°C overnight. Antibody–protein DNA complexes were incubated with Protein A Sepharose 4 Fast Flow (Amersham), and immunoprecipitates collected onto Protein A Sepharose were washed three times with a high salt wash buffer (20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100), three times with LiCl buffer (10 mM Tris–HCl, pH 7.9, 250 mM LiCl, 1% NP-40, 1% deoxycholic acid, and 1 mM EDTA), and twice with 10 mM Tris–HCl, pH 7.9, 1 mM EDTA) successively. Then, bound proteins were eluted from the beads in elution buffer (1% SDS and 100 mM NaHCO₃) by incubation at room temperature for 15 minutes. Crosslinking was reversed by incubation at 65°C overnight. All samples were treated with 40 μg/ml of proteinase K at 55°C for 2 hours, followed by extraction with phenol/chloroform/isomyl alcohol and precipitation with ethanol. DNA fragments were subjected to quantitative PCR using FastStart SYBR Green Master (Roche) with specific primer sets: 5’-TGGTCCCTGGGAATCAC-3’ and 5’-AAGATGGTGTTTGGTCCG-3’, for IFITM1, 5’-CATGGCCTGGAAGAGAAGG-3’ and 5’-GTGACATCTGCTTACATGG-
3' for ISG15, and 5'-ATGCTGCACTGTCGGCGAAG-3' and 5'-ATGGCCA-AAGGCGAGGCTC-3' for β-actin.

Reporter gene assay
Hep G2 cells were transfected with pp32 Stealth siRNA or negative control Stealth siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. At 72 hours after siRNA transfection, cells were further transfected with pSRE-TA-Luc (Clontech) containing ISRE sequences, pSEAP-Control (Clontech), and one of the following: empty vector, pcDNA-FLAG–pp32 or pcDNA–FLAG–pp32AC. At 24 hours post-transfection, cells were treated with or without IFN-β (1000 IU/ml) for 6 hours. Cell lysates were used for assays of the luciferase activity using the luciferase assay system (Promega) and a MiniLumat LB9506 luminometer (Berthold). To monitor the transfection efficiency, a portion of each cell supernatant was assayed for secreted alkaline phosphatase (SEAP) by using the SEAP assay kit (ToyoBio).

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