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Positive and Negative Regulation of the Innate Antiviral Response and Beta Interferon Gene Expression by Deacetylation†

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Beta interferon (IFN-β) gene expression in response to virus infection relies on the dynamic assembly of a multiprotein enhanceosome complex that is initiated by the activation of two inducible transcription factors, interferon regulatory factor 3 (IRF3) and NF-κB. Virus or double-stranded RNA-induced activation of IFN-β gene expression is prevented by the addition of protein deacetylase inhibitors. The isolated IRF-responsive positive regulatory domain was found to require deacetylation for its activity, but IRF3 protein activation leading to its nuclear translocation and DNA binding was not impaired by deacetylase inhibition. In contrast, NF-κB activity was not affected by deacetylase inhibitors. RNA interference indicated that several deacetylase enzymes, including histone deacetylase 1 (HDAC1), HDAC8, and HDAC6, influence IFN-β gene expression with opposing effects. While HDAC1 and HDAC8 repress IFN-β expression, HDAC6 acts as a coactivator essential for enhancer activity. Virus replication is enhanced in HDAC6-depleted cells, demonstrating HDAC6 is an essential component of innate antiviral immunity.

Virus infection or molecular replication intermediates, like double-stranded RNA (dsRNA), can elicit the production of beta interferon (IFN-β), a crucial component of innate antiviral immunity. The human IFN-β gene is controlled by a dynamic multicomponent enhancer regulatory complex known as an enhanceosome (20). Infection or dsRNA treatment trigger the activation of latent NF-κB and interferon regulatory factor 3 (IRF3) transcription factors, which enter the nucleus and in collusion with ATF2/c-Jun and HMG I(Y) initiate gene transcription. These transcription factors combine to recruit several coactivators, notably the histone acetyltransferase (HAT) proteins CBP, p300, and GCN5, the SWI/SNF chromatin remodeling complex, and basal transcription factors, all of which participate in altering histone posttranslational modifications, remodeling chromatin architecture, repositioning nucleosomes, and activating RNA polymerase II (Pol II) assembly and subsequent elongation (13).

The newly synthesized IFN-β is secreted and can engage specific IFN receptors on nearby cells to induce activation of a JAK-STAT signaling cascade. Type I IFN signaling results in the formation of the STAT1- and STAT2-containing heterotrimeric transcription regulator, IFN-stimulated gene factor 3 (ISGF3), which regulates the expression of IFN-stimulated genes (ISGs). ISGs produce a broadly effective cellular antiviral state refractory to virus replication (18). As with the proteins involved in IFN-β enhanceosome activity, ISGF3 specifically interacts with several coactivators that are essential for transcriptional induction of target gene promoters, including HAT proteins, nucleosome remodeling factors, and the Mediator complex (2, 8, 12, 16, 21). In addition to these transcriptional partners commonly associated with gene activation, recent reports have described histone deacetylase (HDAC) proteins as essential ISGF3 coactivators (4, 15, 17). Although HDACs are widely believed to act as corepressors rather than coactivators of gene transcription, their roles as positive acting factors in cytokine-induced transcription have been clearly established in the IFN system as essential for inducible gene expression and innate antiviral defense (4, 15). Named for their importance in modifying histone tails, HDACs regulate a wide variety of protein substrates, and contemporary studies are beginning to elucidate additional HDAC functions outside the nucleus that are required for signaling and gene regulation both directly and indirectly (9–11).

Given the importance of deacetylation in the IFN-mediated antiviral system, the potential role of deacetylation in the initial cellular response to virus infection and dsRNA was tested. Results indicate that HDAC activity is required for the induction of cellular antiviral defenses induced by virus infection or by dsRNA. Inhibition of antiviral responses was attributed to a failure of IFN-β production in cells, and data indicate that IFN-β gene activation requires HDAC activity. In particular, the IFN regulatory factor (IRF)-dependent transcriptional activity required deacetylation, but IRF3 nuclear translocation and DNA binding were not altered by deacetylase inhibition. Targeted RNA interference (RNAi) analysis of individual HDAC proteins revealed distinct effects of lowered HDAC levels that were either the same as or different from pharmacological HDAC inhibition. Notably, while HDAC1 and HDAC8 function as repressors of IFN-β gene expression, HDAC6 functions as a coactivator of IRF3-dependent transcription. These findings indicate that HDAC activity is essential for IFN-β gene induction in human cells and demonstrate that HDAC proteins can have unique and opposing roles in the regulation of individual genes.

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MATERIALS AND METHODS

Cell culture, dsRNA and drug treatments, and transfection. Human 2fTGH, HeLa, and A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% cosmic calf serum (HyClone). DMEM containing 10% fetal bovine serum was used in the RNAi assay, and DMEM with 2% cosmic calf serum was used for virus infection. A549 stable cell lines were generous gifts of Tso-Pang Yao, Duke University. Transfection of 2fTGH cells was carried out using Superfect reagent (QIAGEN) and transfection of A549 cells was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. Treatment of cells with poly(I) · poly(C) (dsRNA; Amersham) was performed by direct addition to the medium at a final concentration of 100 μg/ml. Trichostatin A (TSA; Upstate Biotechnology) was added at 400 ng/ml simultaneously with dsRNA, with the exception of the experiment shown below in Fig. 1A, where TSA was diluted as indicated. Cycloheximide was added at 100 μg/ml simultaneously with dsRNA as indicated.

Virus infection. Sendai virus (Cantell strain; gift of Tom Moran, Mount Sinai School of Medicine), vesicular stomatitis virus (VSV; Indiana strain), or recombinant VSV harboring a green fluorescent protein (GFP) transgene (VSV-GFP; gift of John Hiscott, Montreal, Canada) infections were performed at a multiplicity of infection (MOI) of 10 PFU/cell or as indicated. Specifically, virus was diluted in serum-free DMEM and added to cells in the absence or presence of simultaneous TSA for 1 to 2 h to allow adsorption. Virus was then removed, and DMEM with 2% cosmic calf serum was added for the remaining time.

Antiviral assays. Antiviral responses measured by cytopathic effect (CPE) assay (6) were conducted as follows: in the experiment shown below in Fig. 1A, cells were cultured in 96-well plates and infection with Sendai virus was performed at the indicated concentrations. TSA was added during virus adsorption as described above. At 24 h postinfection, cells were fixed and stained with methylene blue (3% in 50% ethanol). In the experiment shown in Fig. 1C, below, cells were pretreated with dsRNA with or without simultaneous TSA for 8 h and then infected with VSV at the indicated MOI. At 18 h postinfection, cells were fixed and stained as described above. In VSV-GFP infection assays, cells were transfected with small interfering RNA (siRNA) (or stable knockdown cells were used), and at 48 h posttransfection cells were treated with dsRNA for 8 h or with Sendai virus for 5 h. After treatment, cells were infected with VSV-GFP and photographed using a Zeiss inverted fluorescence microscope at 18 h post-VSV-GFP infection.

Plasmids and reporter gene assays. Luciferase assays were carried out according to the manufacturer's instructions (Promega) by cotransfecting the firefly luciferase reporter gene and *Renilla* luciferase (dual-luciferase reporter assay) to normalize for transfection efficiency. Luciferase reporters −110Luc, 3xPRDIII/I, and 4xPRDII were gifts of Dimitris Thanos (A. Fleming). At 24 h after transfection, cells were treated with dsRNA and TSA for the indicated times. For all luciferase assays, data represent the average firefly luciferase value of three to six samples, normalized by *Renilla* luciferase activity, ± the standard deviation.

RNA analysis. Total RNA was prepared by using TRIzol reagent (Invitrogen), digested with DNase I, and subjected to reverse transcriptase with SuperScript II RNase H reverse transcriptase (Invitrogen) and PCR analysis. One-tenth of the resulting cDNA product was used as a template for 25 cycles of PCR in the presence of [α-^{32}P]dATP (Perkin-Elmer Life Sciences) using specific primers as indicated. Following gel electrophoresis, products were detected by phosphorimaging.

Primer sequences used were as follows: *IFN*βT, 5′-TAGTCATTCACTGAAACTTTA-3′; *IFN*βB, 5′-AGGTTGCAGTTAGAATGTC-3′; *GAPDH*T, 5′-CCCTTCATTGACCTCAACT-3′; and *GAPDH*B, 5′-GACGCCAGTGGACTCCA-3′.

Cell fractionation and DNA binding analysis. Following dsRNA treatment, nuclear and cytoplasmic fractions were separated using nuclear extract buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 0.15 mM spermine, 0.5 mM spermidine). Nuclei were lysed using cellular extract buffer (50 mM Tris, pH 8.0, 280 mM NaCl, 0.5% IGEPAL, 0.2 nM EDTA, 2 mM EGTA, 10% glycerol, 1 mM dithiothreitol) containing protease and phosphatase inhibitors. Cytoplasmic and nuclear IRF3 was analyzed by Western blotting. Nuclear IRF3 DNA binding was analyzed using the oligonucleotide–biotin-streptavidin purification system with the following oligonucleotides annealed to make a double-stranded affinity probe: biotinylated *PRDIII*/*I*T, 5′GATCGAAAACTGAAAGGGAGAAGTGAAAGTG-3′; unmodified *PRDIII*/*I*B, 5′GATCCACTTTCACTTCTCCCTTTCAGTTTTC-3′.

Nuclear extracts were incubated with the biotinylated affinity probe for 1 h, and DNA-protein complexes were collected using ImmunoPure immobilized streptavidin (Pierce). Proteins were eluted with sodium dodecyl sulfate (SDS) loading buffer and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed, and chemiluminescence detection was carried out according to the manufacturer's protocol (NEN Renaissance).

RNA interference. Individual wells of a 24-well culture dish containing 2fTGH cells were transfected with 60 pmol of siRNA alone or together with a luciferase reporter and *Renilla* luciferase plasmid by using the siLent-Fect reagent (Bio-Rad) following the manufacturer's recommendations. siRNAs (SMARTpool) specific for individual HDACs or scrambled control were obtained from Dharmacon. Treatment with dsRNA or Sendai virus, followed by reporter gene analysis, reverse transcription-PCR (RT-PCR), or VSV-GFP infection was performed 48 h after transfection.

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed in HeLa cells by using the ChIP assay kit (Upstate Biotechnology). Protein-DNA complexes were cross-linked with formaldehyde, cells were lysed in cellular extract buffer (50 mM Tris, pH 8.0, 280 mM NaCl, 0.5% IGEPAL, 0.2 nM EDTA, 2 mM EGTA, 10% glycerol, 1 mM dithiothreitol), and DNA was sonicated extensively. Pol II protein-DNA complexes were precipitated by using anti-Pol II antibody (N-20; Santa Cruz). Precipitated DNA was amplified by radioactive PCR using the following primers encompassing 200 bp around the minimal IFN-β promoter: *IFNbProm*T, 5′ TAGTCATTCACTGAAACTTTA 3′; *IFNbProm*B, 5′ AGGTTGCAGTTAGAATGTC 3′.

PCR products were separated on a 5% polyacrylamide gel and detected by phosphorimaging. Signals were quantified and normalized by the input material.

Generation of stable cell lines. Stable cell lines with lowered HDAC1 expression were generated using RNA interference pGSU6-GFP vectors (Gene Therapy Systems, Inc.) containing hairpin loop sequences specific for HDAC1. A double-stranded DNA fragment was generated by annealing oligonucleotides with the following sequences and ligating it into the vector using standard methodology: *siHDAC1*T, 5′GATCCAAGCAGATGCAGAGATTCAACGAAGCTTGGTTGAATCTCTGCATCTGCTTTTTTTTGGAAGC-3′; *siHDAC1*B, 5′GGCCGCTTCCAAAAAAAAGCAGATGCAGAGATTCAACCAAGCTTCGTTGAATCTCTGCATCTGCTTG-3′.

2fTGH cells were transfected with the plasmids, and stable transformants were selected using G418 and screened for HDAC1 levels by Western blotting using the anti-HDAC1 antibody (Santa Cruz).

RESULTS

TSA inhibits IFN-β production. To examine the role of deacetylation in the establishment of cellular antiviral defense mechanisms, an assay for virus-induced CPE was performed (Fig. 1A). 2fTGH cells were infected with serial dilutions of Sendai virus in the absence or presence of the deacetylase inhibitor TSA. In the absence of TSA, the CPE endpoint was observed at an MOI of 12.5 PFU/cell. The presence of TSA increased cellular susceptibility to Sendai virus in a dose-dependent manner. Treatment with 400 ng/ml TSA reduced the CPE endpoint by 25-fold, to 0.5 PFU/cell. This biological response assay provides a demonstration that HDAC activity is required for optimal cellular defense against virus infection.

While many factors contribute to the establishment of an antiviral state in cells, an immediate primary component of the innate antiviral system is the inducible production of IFN-β. To assess whether the production of IFN in response to virus is impaired in the absence of HDAC activity, IFN-β mRNA levels were analyzed by RT-PCR in 2fTGH cells infected with Sendai virus in the absence or presence of TSA (Fig. 1B). IFN-β mRNA was rapidly and transiently induced, reaching a maximum at 5 h postinfection. Treatment with TSA completely prevented the accumulation of IFN-β mRNA, indicating that deacetylase activity is required for IFN-β production in response to virus infection.

One mechanism by which viruses can induce IFN-β biosynthesis is through the replication intermediate, double-stranded RNA. To determine whether deacetylation is also required for the dsRNA-induced cellular antiviral protection, a cytopathic effect assay was performed (Fig. 1C). Cells were treated with

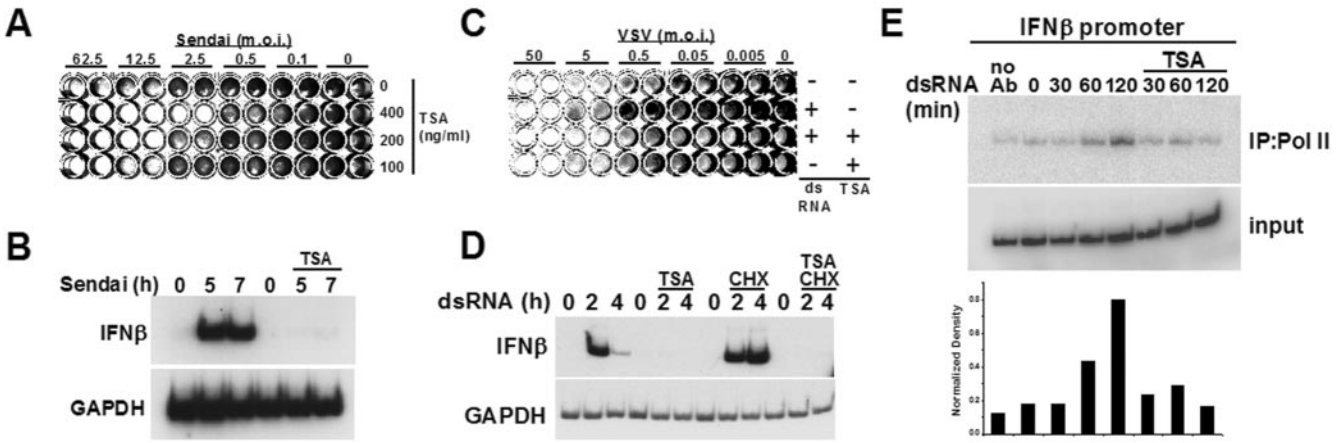

FIG. 1. Deacetylase activity is required for innate antiviral defense. A. Duplicate wells of 2fTGH cells were infected with dilutions of Sendai virus to achieve MOIs of 62.5, 12.5, 2.5, 0.5, 0.1, and 0 PFU/cell. Simultaneously with inoculation, cells were treated with TSA at the indicated concentrations. Cells were stained and analyzed 24 h postinfection. B. 2fTGH cells were infected with Sendai virus (MOI, 10 PFU/cell) for the indicated times in the presence or absence of TSA. RT-PCR was carried out using primers for IFN-β or the control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). C. 2fTGH cells were pretreated with dsRNA for 8 h with or without TSA and then infected with VSV at MOIs of 50, 5, 0.5, 0.05, 0.005, and 0 PFU/cell. Cells were stained and analyzed 18 h postinfection. D. 2fTGH cells were treated with dsRNA, TSA, and cycloheximide (CHX) as indicated, and RT-PCR for IFN-β and GAPDH was performed. E. (Top) HeLa cells were treated with dsRNA and TSA as indicated, and ChIP assays were performed using antiserum specific for RNA Pol II. Coprecipitated DNA was amplified by PCR using primers specific for the IFN-β promoter. (Bottom) Phosphorimaging was used for quantitative analysis, and values were normalized to the input.

dsRNA in the absence or presence of TSA, followed by infection with serially diluted VSV. Treatment with dsRNA protected cells from virus-induced CPE with the endpoint of 5 PFU/cell, and this protection was completely abolished by TSA. TSA treatment alone did not alter cellular susceptibility to virus, demonstrating a specific requirement for deacetylation in inducible antiviral protection. RT-PCR analysis revealed endogenous IFN-β mRNA accumulated in response to dsRNA treatment, peaking at 2 h of treatment and subsiding by 4 h, but IFN-β mRNA failed to accumulate in the presence of TSA. This requirement for deacetylase activity is provided by preexisting proteins, as no IFN was induced by dsRNA in the presence of TSA if the cells were simultaneously treated with cycloheximide. The kinetics of IFN-β activation by dsRNA are different from those of Sendai virus infection. Sendai virus treatment results in a more sustained response, potentially reflecting differences in activation mechanisms. Deacetylation, however, is similarly required for both stimuli.

Because a defect in IFN-β mRNA accumulation was observed in the presence of TSA, the requirement for deacetylation in transcription of IFN-β was tested. Cross-linking chromatin immunoprecipitation assays revealed increased Pol II binding to the IFN-β promoter after 60 min and 120 min of dsRNA treatment (Fig. 1D). No increase in Pol II binding was evident in the presence of TSA. This indicates that deacetylase activity is required at a step prior to RNA polymerase loading onto the IFN-β promoter.

TSA affects IRF but not NF-κB activity. The primary activated transcription factor targets of dsRNA contributing to IFN-β gene induction are IRF3 and NF-κB, which bind to positive regulatory domains (PRDs) PRDIII/I and PRDII, respectively. The sensitivities of these factors to HDAC inhibition were tested in luciferase reporter gene assays. Three luciferase reporters were used containing either the full IFN-β enhanceosome sequence (−110Luc), PRDIII/I, or PRDII (Fig. 2A). dsRNA stimulation increased luciferase activity of the −110Luc reporter, and TSA prevented this induction (Fig. 2A, left). Opposing effects of TSA were observed with the IRF- and NF-κB-dependent reporter genes (Fig. 2A, middle and right). The IRF reporter was induced by dsRNA and, similarly to −110Luc, this induction was prevented by TSA. In contrast to the effects on −110Luc and the IRF reporter gene, HDAC inhibition enhanced the activity of the NF-κB reporter gene. These results demonstrate that (i) dsRNA-induced IRF signaling requires deacetylase activity, (ii) HDAC inhibition enhances NF-κB activity, and (iii) the native enhanceosome function can be limited by inhibition of one of its components, in this case, IRF.

Inhibition of IRF3 by RNA interference resulted in complete abolishment of IFN-β induction, while inhibition of IRF7 had a minor effect (data not shown). Since IRF3 is the primary regulator of IFN-β in these cells, IRF3 nuclear translocation and DNA binding were tested to evaluate nontranscriptional effects of TSA in the dsRNA system. Cell fractionation revealed dsRNA-induced IRF3 nuclear translocation is not inhibited by TSA (Fig. 2B). The fact that nuclear preparation is uncontaminated with the cytoplasmic fraction is demonstrated by the complete absence of IRF3 in the nucleus without stimulation. Furthermore, the nuclear IRF3 can bind PRDIII/I DNA irrespective of HDAC activity (Fig. 2C). These results indicate that the deacetylation step required for IRF3 activity lies downstream of IRF3 DNA binding. Together, the results suggest a role for deacetylation enzymes as positive coactivators required at a regulatory step between IRF3 and RNA polymerase II recruitment that is essential for IFN-β gene expression.

HDAC1 and HDAC8 repress IFN-β expression. The dependence of IFN-β gene activation on deacetylation implies the in-

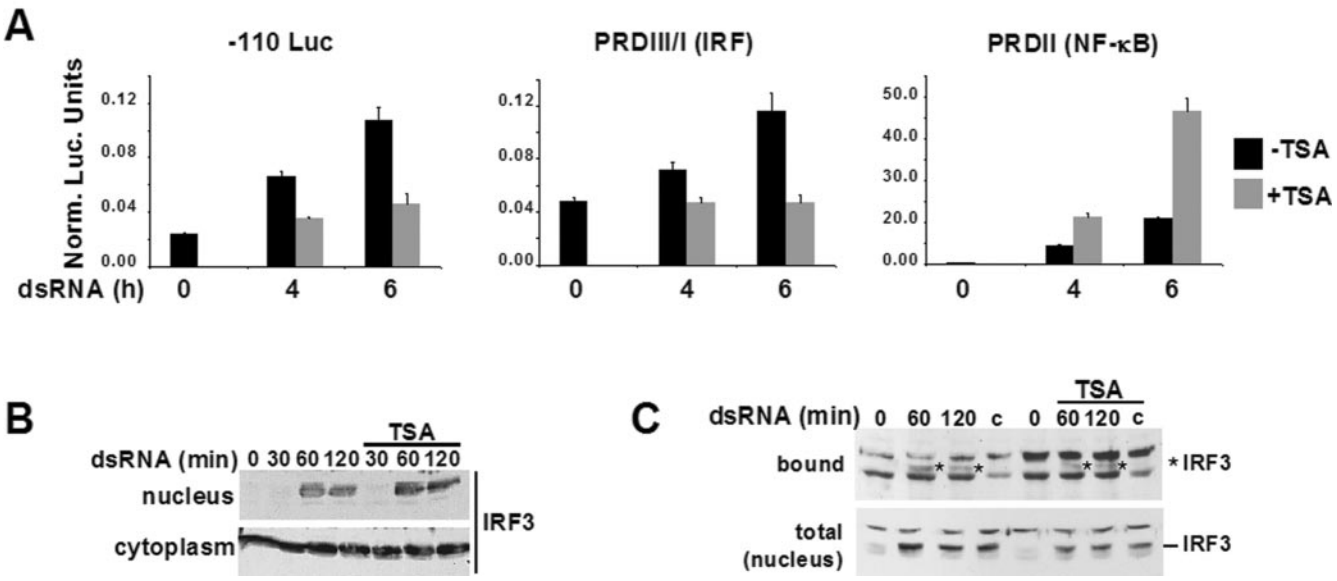

FIG. 2. Deacetylase activity is required for IFN-β enhanceosome activity. A. Luciferase assays were performed in 2fTGH cells using the indicated reporter genes, and cells were treated with dsRNA and TSA for the indicated times. B. 2fTGH cells were treated with dsRNA and TSA as indicated, and nuclear and cytoplasmic fractions were separated followed by SDS-PAGE and Western blotting with antibodies for IRF3. C. 2fTGH cells were treated with dsRNA and TSA as indicated, nuclei were purified, and nuclear lysates were incubated with a biotinylated double-stranded oligonucleotide containing IRF3 binding sites. IRF3 was eluted from DNA and visualized by SDS-PAGE and Western blotting. Asterisks indicate the IRF3 band. Bound, IRF3 that was bound to oligonucleotide; total, total nuclear IRF3; c, control containing no oligonucleotide.

volvement of deacetylase enzymes. As the deacetylase HDAC1 was previously demonstrated to be involved in coactivation of genes induced by the IFN signaling system (15), its importance in the regulation of IFN-β was tested. Reporter gene assays were performed in the presence or absence of siRNA specific for HDAC1 (Fig. 3A). Interference with HDAC1 elevated luciferase activity for all three dsRNA-responsive reporter genes. Unlike its role as a coactivator of IFN-induced transcription, HDAC1 functions as a repressor of IFN-β expression and of enhancer components IRF3 and NF-κB. To verify these results, stable cell lines expressing hairpin RNAi sequences specific for HDAC1 were created (Fig. 3B). In accordance with our previous observations, the HDAC1-deficient cells exhibited decreased IFN-stimulated gene expression (data not shown) (15). In response to dsRNA, these cell lines exhibited a greater accumulation of IFN-β mRNA (Fig. 3C), consistent with the reporter gene data implicating HDAC1 as a repressor of IFN-β gene expression. It is apparent that HDAC1 has unique and opposing roles in the regulation of the two related pathways of innate antiviral immunity: HDAC1 represses IFN-β biosynthesis but enhances IFN-induced transcriptional responses.

To determine if a single HDAC protein might satisfy the criteria for being the particular deacetylase enzyme required for optimal IFN-β gene transcription, an siRNA screen targeting the known HDACs (HDAC1 to HDAC10) was performed (see Fig. S1A in the supplemental material). HDAC1 to HDAC9 mRNAs were readily detected and reduced with specific siRNA pools, and HDAC10 mRNA was not detected in these cells. In this screen, inhibition of HDAC1 confirmed previous results, and two other HDACs (HDAC8 and HDAC6) had an effect on IFN-β reporter gene transcription. Reporter gene assays revealed that HDAC8 acted similarly to HDAC1, functioning as a repressor of IFN-β gene expression (Fig. 3D). It may be relevant that HDAC1 and HDAC8 belong to the same HDAC family (class I), and the significance of this association is under further investigation.

HDAC6 activates IFN-β expression. While the functions of HDAC1 and HDAC8 in IFN-β gene regulation do not explain the ability of TSA to suppress gene induction, the siRNA screen revealed one deacetylase protein, HDAC6, that acted in the predicted fashion. Interference with HDAC6 resulted in decreased IFN-β–luciferase activity, indicating HDAC6 acts as an activator for IFN-β transcription (Fig. 4A, left). Interference with HDAC6 had little effect on NF-κB activity in response to dsRNA but effectively inhibited the activation of the IRF3-dependent reporter gene (Fig. 4A, middle and right). The effects of HDAC6 interference are similar to those of TSA treatment (Fig. 2A) and suggest that HDAC6 imposes its IFN-β-activating effects through IRF3. To demonstrate the effects of HDAC6 interference on the endogenous IFN-β gene, an RT-PCR assay was performed (Fig. 4B). Lowering HDAC6 protein levels (Fig. 4B, top) resulted in decreased IFN-β mRNA accumulation consistent with defective IFN-β gene activation (Fig. 4B, bottom).

To confirm these results in a biological assay, the role of HDAC6 in an antiviral response was tested. Cells were transfected with siRNA specific for HDAC6 or scrambled control and then treated with dsRNA or Sendai virus to induce IFN biosynthesis (Fig. 4C). To evaluate the IFN response, the treated cells were infected with a reporter virus, VSV, harboring a GFP transgene. Control cells were uniformly fluorescent

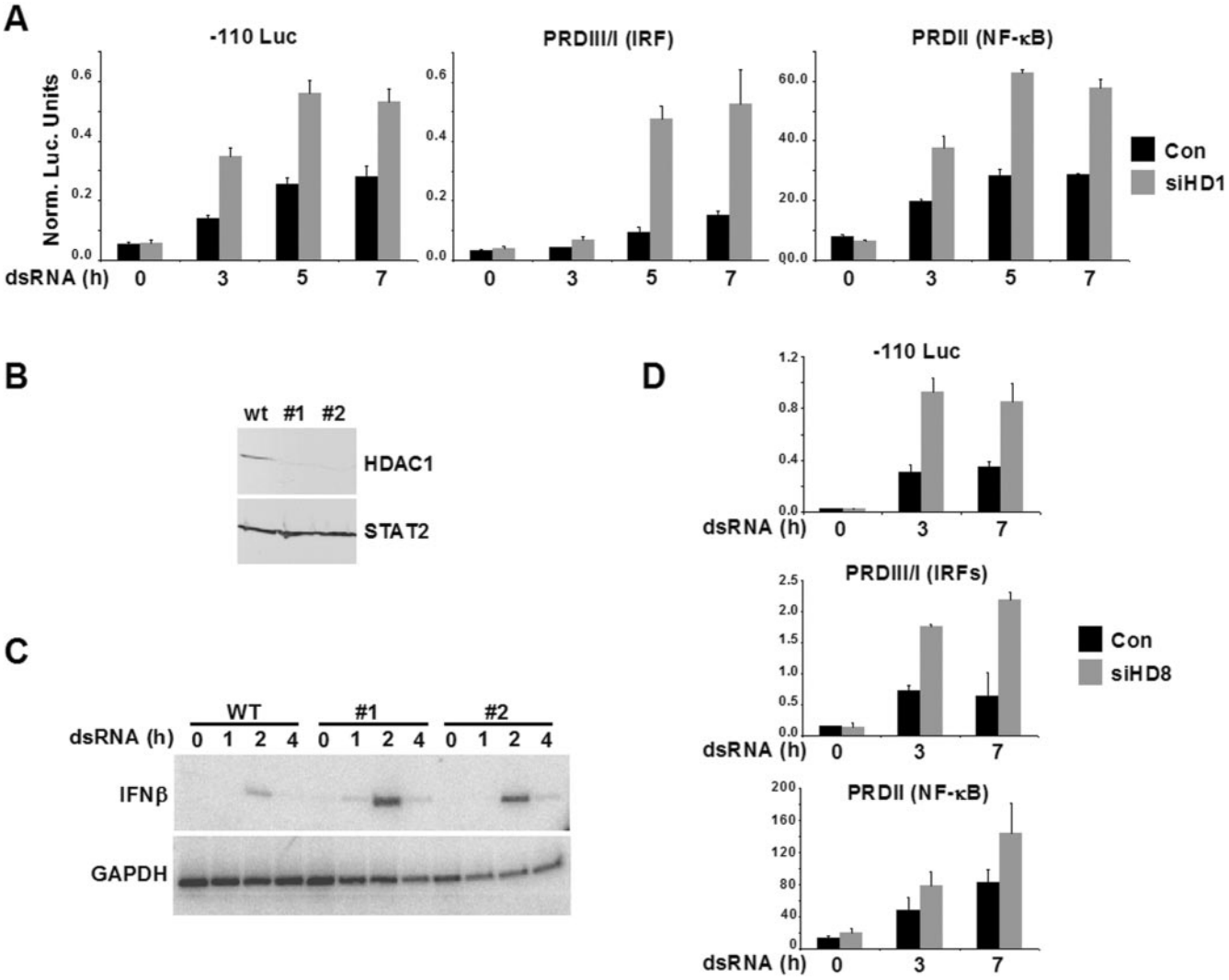

FIG. 3. HDAC1 and HDAC8 inhibit IFN-β expression. A. Luciferase assays with the indicated reporters were performed in the presence of siRNA for HDAC1 (siHD1) or scrambled control (Con). At 48 h after transfection with siRNA and reporter, cells were treated with dsRNA for the indicated times. B. Two clones stably expressing RNA interference vectors specific for HDAC1 were analyzed by Western blotting. wt, wild-type cells. C. The stable clones were treated with dsRNA as indicated, and RT-PCR was performed for IFN-β or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). WT, wild-type cells. D. Similar to the experiment in panel A, except siRNA specific for HDAC8 was used.

18 h postinfection, and the GFP fluorescence was brighter after HDAC6 interference, indicating enhanced virus replication in the absence of HDAC6. Similar effects of HDAC6 interference were observed in cells that had been induced to synthesize IFN-β by pretreatment with dsRNA or Sendai virus. The overall VSV-GFP fluorescence intensity was decreased compared with untreated cells due to the IFN-induced antiviral response created by pretreatment. However, in all cases of HDAC6 interference, fluorescence intensity was increased as a result of greater VSV-GFP replication, an effect attributable to a defect in IFN-β production in these cells in addition to possible defects in other IFNs as well.

The effects of HDAC6 silencing were confirmed in stable cell lines expressing hairpin RNAi sequences specifically targeting HDAC6 (10). These cells exhibited diminished IFN-β production in response to dsRNA compared to the control cell line (Fig. 4D), and VSV-GFP replication was in all cases increased in the HDAC6-silenced cells (Fig. 4E). Together, these findings indicate HDAC6 plays a positive regulatory role in the activation of human IFN-β gene transcription independent of cell type and establish HDAC6 as an essential component of the innate antiviral immune response.

DISCUSSION

The results of this study demonstrate a requirement for protein deacetylation in activation of innate antiviral responses. Susceptibility to virus-induced cytopathic effects was increased in the absence of deacetylase activity, and this effect was attributed to a requirement for deacetylation in IFN biosynthesis as well as the IFN response. Activation of IFN-β gene expression by virus or dsRNA was greatly reduced in the presence of HDAC inhibitors, and this defect was attributed to failure in the progression of the IRF component.

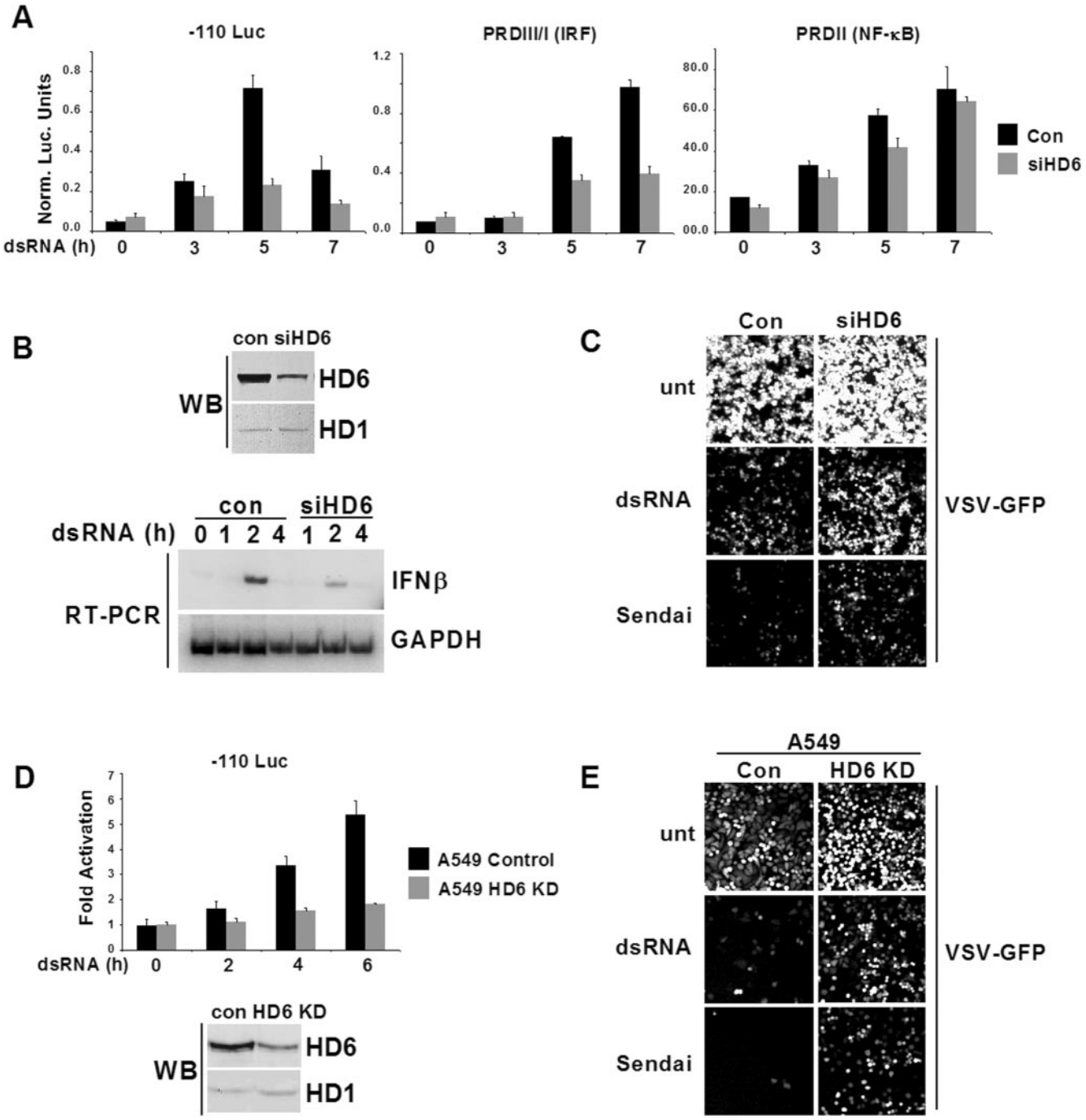

FIG. 4. HDAC6 is required for IFN-β expression and innate antiviral immunity. A. Luciferase assays with the indicated reporters were performed in the presence of siRNA for HDAC6 (siHD6) or scrambled control (Con). At 48 h after transfection with siRNA and reporter, cells were treated with dsRNA for the indicated times. B. (Top) Western blot for HDAC6 in 2fTGH cells transfected with HDAC6-specific siRNA. (Bottom) 2fTGH cells transfected with siRNA were treated with dsRNA, and RT-PCR was performed for IFN-β and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). WB, Western blot. C. 2fTGH cells were transfected with siRNA and 48 h later were treated with dsRNA (8 h) or Sendai virus (5 h) or left untreated (unt) and then infected with VSV-GFP for 18 h. D. (Top) Luciferase assay similar to the experiment shown in panel A, but with A549 cells (control) or an HDAC6-silenced A549 stable cell line (HD6 KD). (Bottom) Western blot for HDAC6 in the A549 cells above. E. Similar to the experiment in panel C, but with A549 cells (Con) or a HDAC6-silenced A549 stable cell line (HD6 KD).

While isolated individual components IRF3 and NF-κB exhibit opposing responses to HDAC inhibitors, in the context of the intact enhancer, inhibition of a single component will prevent proper enhanceosome function. Based on the IRF3- and NF-κB-dependent reporter gene assays, we hypothesize that IRF3 is the susceptible element in IFN-β activation that fails to function in the absence of HDAC activity.

Analysis of individual HDAC family members revealed that

several HDAC proteins can modulate IFN-β expression. HDAC1 and HDAC8 apparently function as repressors for this gene, as their absence increases IFN-β mRNA levels. It is interesting that HDAC1 plays a more conventional role as a transcriptional repressor for IFN-β activation by dsRNA while functioning as a transcriptional coactivator for ISGF3, a transcription factor downstream of IFN-β required for IFN-stimulated promoter activation. This function of HDAC1 as an activator and as a repressor in these two distinct but related branches of the innate antiviral system underscores the notion that HDACs, like most protein-modifying enzymes, can exert both positive and negative effects depending on the overall cellular context and substrate specificity.

In contrast with HDAC1 and HDAC8, HDAC6 functions as an activator of IFN-β. Results indicate that HDAC6 exerts this effect through the PRDIII/I elements, compatible with the effects of generalized deacetylase inhibition by TSA. It is prudent to note that the effect of specific RNAi inhibition of HDAC6 is not as dramatic as that of pharmacological HDAC inhibition. This difference is likely due to the incomplete reduction of HDAC6 levels in the cell by siRNA (Fig. 4B, top) compared to very efficient inhibition of HDAC activity by TSA. Incomplete delivery of siRNA into every cell may contribute to the partial effect. It is noteworthy that the hyperactivation of IFN-β reporter gene activity observed upon HDAC1 inhibition is attenuated by simultaneous inhibition of HDAC6 (see Fig. S1B in the supplemental material). This suggests that these HDACs function at different steps in the IFN induction pathway.

None of the other HDACs tested matched the effects of TSA (see Fig. S1A in the supplemental material), suggesting that HDAC6 is a relevant TSA target for this promoter's regulation. HDAC6 is a class II deacetylase protein, and it possesses tandemly duplicated catalytic domains which appear to function independently of each other (7). Unlike other HDAC family members, HDAC6 possesses a Cys/His-rich C-terminal domain that specifically interacts with polyubiquitin while maintaining the protein's deacetylase activity (19). HDAC6 has been associated with α-tubulin deacetylation and can dynamically interact with the microtubule motor dynein to regulate intracellular transport of misfolded proteins. HDAC6 can also regulate distribution of T-cell receptor components during assembly of the immunological synapse between T cells and antigen-presenting cells (5, 9, 10). Evidently, HDAC6 is a multifunctional protein involved in immune functions and stress responses that provides an intriguing link between protein ubiquitination and deacetylation. The relationship between these activities of HDAC6 and its role in IFN-β gene regulation is not clear. The substrates of HDAC6 important for IFN-β gene expression have not been determined, but detailed analysis of the IFN-β promoter demonstrates lysine residues on histone H4 (K5, K8, and K12) are rapidly acetylated and then deacetylated prior to TBP recruitment and IFN-β mRNA accumulation (1).

To reconcile the opposing effects of HDAC1, HDAC8, and HDAC6 on IFN-β gene expression, we hypothesize that these enzymes act independently during the early response to dsRNA and strike a balance of deacetylase activities needed for optimal IFN-β gene regulation (Fig. 5A). It is likely that these enzymes exert their effects at different times or places during IFN-β activation and could potentially have distinct or multiple substrates. Inhibition of total deacetylase activity by TSA or other HDAC inhibitors blocks the activity of HDAC1 and HDAC8 as well as the essential HDAC6 coactivator activity, resulting in defective IFN-β expression (Fig. 5B). Specific inhibition of HDACs by RNA interference can have opposite effects. Suppression of HDAC1 or HDAC8 derepresses IFN-β (Fig. 5C), but HDAC6 inhibition prevents IFN-β expression (Fig. 5D).

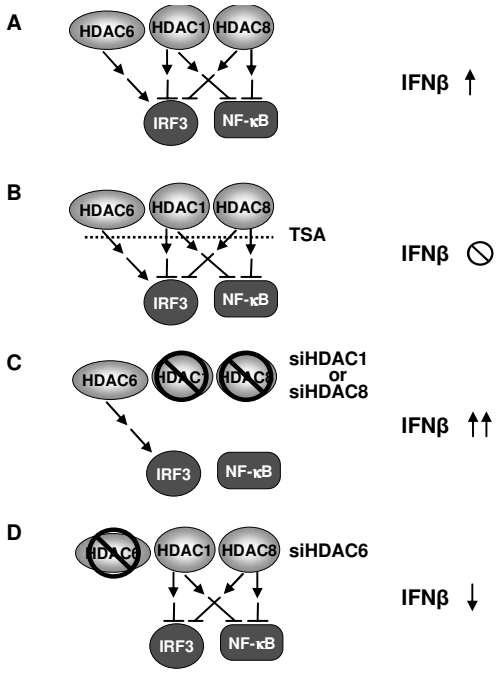

FIG. 5. HDACs are activators and repressors of IFN-β. A. HDAC6 activates IRF3, while HDAC1 and HDAC8 repress both IRF3 and NF-κB. Together, these HDACs strike a balance for optimal activation of IFN-β. B. TSA inhibits all HDAC activity, thereby blocking IFN-β activation. C. siRNA specific for either HDAC1 or HDAC8 relieves the inhibitory actions of these proteins, resulting in a heightened transcriptional response. D. siRNA specific for HDAC6 removes the activating effect of this deacetylase, causing lower IFN-β expression.

The role of deacetylation as an activating signal seems to undermine the general paradigm that associates HDACs with transcriptional repression. However, many studies indicate that this convention is not generally applicable (14). For example, gene expression profiling studies indicate that a similar proportion of genes are activated in response to HDAC inhibitor treatments as that of genes repressed, together constituting only a minor fraction of the total genes tested (3). Deacetylation is also required for rapid activation of some inducible promoters, such as those of the innate immune system. Given the multiple and opposing roles for deacetylation in specific regulation of inducible gene expression, it is anticipated that the type of regulation patterns described here will prove to be widely applicable for many cases of genetic and epigenetic regulation. Complete understanding of the relationship between different HDAC and HAT proteins in the cell and particularly in the innate antiviral system will provide insights into the precise mechanisms of cellular regulation by protein acetylation.

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