Requirement of Histone Deacetylase Activity for Signaling by STAT1*Ś

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STAT1 is a transcription factor that plays a crucial role in signaling by interferons (IFNs). In this study we demonstrated that inhibitors of histone deacetylase (HDAC) activity, butyrate, trichostatin A, and suberoylanilide hydroxamic acid, prevented IFNγ-induced JAK1 activation, STAT1 phosphorylation, its nuclear translocation, and STAT1-dependent gene activation. Furthermore, we showed that silencing of HDAC1, HDAC2, and HDAC3 through RNA interference markedly decreased IFNγ-driven gene activation and that overexpression of HDAC1, HDAC2, and HDAC3 enhanced STAT1-dependent transcriptional activity. Our data therefore established the essential role of deacetylase activity in STAT1 signaling. Induction of IRF-1 by IFNγ requires functional STAT1 signaling and was abrogated by butyrate, trichostatin A, suberoylanilide hydroxamic acid, and STAT1 small interfering RNA. In contrast, silencing of STAT1 did not interfere with IFNγ-induced expression of STAT2 and caspase-7, and HDAC inhibitors did not preclude IFNγ-induced expression of STAT1, STAT2, and caspase-7, suggesting that HDAC inhibitors impede the expression of IFNγ target genes whose expression depends on STAT1 but do not interfere with STAT1-independent signaling by IFNγ. Finally, we showed that inhibitors of deacetylase activity sensitized colon cancer cells to IFNγ-induced apoptosis through cooperative negative regulation of Bel-x expression, demonstrating that interruption of the balance between STAT1-dependent and STAT1-independent signaling significantly alters the biological activity of IFNγ.

IFNγ is a pluripotent cytokine produced by activated T lymphocytes and natural killer cells that has a crucial role in regulating the antiviral response, cell proliferation, apoptosis, immune surveillance, and tumor suppression (1–5). Binding of IFNγ to cell surface receptors initiates signaling through transphosphorylation and activation of the Janus kinases, JAK1/JAK2, which leads to tyrosine phosphorylation of STAT1 (6). The JAK1/JAK2-mediated tyrosine phosphorylation of STAT1 is required for STAT1 dimerization, its nuclear translocation, DNA binding, and transcriptional activation (7). Serine phosphorylation of STAT1 is required for its full transcriptional activity (8), but the kinase responsible for the phosphorylation of STAT1 on Ser-727 remains to be identified.

In addition to its role in mediating ligand-induced gene expression, STAT1 also supports the basal expression of several genes, such as caspase-1 (9), LMP2, and major histocompatibility complex class I (10). In contrast to the inducible gene regulation, STAT1 phosphorylation and its nuclear translocation are not required for the constitutive expression of these genes, demonstrating that unphosphorylated STAT1 has an important role in the regulation of gene expression. Tyrosine-phosphorylated STAT1 and unphosphorylated STAT1 appear to shuttle to the nucleus through independent pathways and modulate the expression of distinct sets of target genes (11).

Although STAT1 has been considered an obligatory transcription factor required for signaling by IFNγ, recent data demonstrated that IFNγ induces a plethora of genes independently of STAT1 activation and exerts biological activities in STAT1-deficient cells (12–14). IFNγ is known to activate several other signal transduction pathways, such as ERK1/ERK2 (15), the src family kinase Fyn (16), AKT (17), and SHP-1 and SHP-2 (18, 19). Nevertheless, STAT1 deficiency significantly modifies the expression profile of IFN-regulated genes and thereby alters the biological activity of IFNγ. For example, IFNγ inhibits c-Myc in STAT1+/− cells and inhibits proliferation, but it activates c-Myc and accelerates proliferation in STAT1−/− cells (20). The nature of signaling by IFNγ that bypasses STAT1 and the biological consequences of STAT1-independent signaling by IFNγ are not well understood.

IFNγ is secreted by lamina propria cells, and its levels are strongly up-regulated in the intestinal mucosa from patients with inflammatory bowel disease (IBD) (21–24). Furthermore, elevated levels of STAT1, as well as the other STAT family members (STAT3, STAT5, and STAT6), were detected in the colonic mucosa of patients with ulcerative colitis and Crohn’s disease (25). In addition, STAT1 has been shown to be constitutively activated in inflammatory intestinal diseases such as ulcerative colitis and pouchitis (26, 27). The expression of STAT1 has been reported to be reduced (28–32) or constitutively activated in tumors (33–35), pointing to a complex role of STAT1 in tumorigenesis. It is noteworthy that patients with ulcerative colitis and Crohn’s disease are at increased risk to develop colorectal cancer (36, 37).

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‡ The abbreviations used are: IFN, interferon; JAK, Janus kinase; STAT, signal transducer and activator of transcription; IBD, inflammatory bowel disease; PPARY, peroxisome proliferator-activated receptor γ; HDAC, histone deacetylase; TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid; LUC, luciferase; GAS, gamma-activated sequence(s); siRNA, small interfering RNA; Bu, butyrate; Na-Bu, sodium butyrate.
The JAK/STAT signaling pathway therefore represents a potential target for therapeutic intervention in inflammatory diseases as well as in cancer. PPARγ agonists, such as 15d-PGJ2 and rosiglitazone, recently have been shown to suppress JAK-STAT signaling via induction of SOCS1 and SOCS3, possibly underlying the anti-inflammatory activity of these agents (38). Accordingly, PPARγ ligands inhibited a subset of IFN-γ-dependent genes, such as inducible nitric oxide synthase and IP-10 (39).

The HDAC inhibitors butyrate, TSA, and SAHA modulate the expression of several genes by inhibiting the activity of HDACs, which results in acetylation of histones and subsequent nucleosome remodeling (40). As a general rule condensed chromatin mediates transcriptional repression, whereas transcriptionally active genes are in the areas of open chromatin (41). Inhibition of HDAC activity therefore often results in the relaxing of the heterochromatin and thereby increases the expression of several genes. However, global analysis of gene expression has clearly established that inhibition of HDAC activity results both in induction and in repression of gene expression (42). The inhibition of gene expression by HDAC inhibitors is not well understood but may be linked to the findings that several transcription factors are also regulated by acetylation/deacetylation.

We recently reported that butyrate, an HDAC inhibitor and a dietary chemopreventive agent for colon cancer, inhibits signaling by IFN-γ (43). We showed that butyrate inhibits activation of STAT1, a transcription factor required for signaling by IFN-γ, through inhibition of its tyrosine and serine phosphorylation, nuclear translocation, and DNA binding activity and suggested that this activity underlies both the anti-inflammatory and chemopreventive activities of butyrate. HDAC1 has recently been reported to associate with both STAT1 and STAT2 (44).

In this study we demonstrated that HDAC1, HDAC2, and HDAC3 are required for IFN-γ-induced STAT1-dependent transcription. Consistently, we showed that HDAC inhibitors negatively regulate the expression of a subset of IFN-responsive genes and thereby alter the biological activity of IFN-γ. HDAC inhibitors are promising new anticancer agents that alter gene expression and induce cell cycle arrest, differentiation, and apoptosis (45, 46). Understanding of their biological activity will help to predict the long term effects of these drugs on normal cells and to avoid potential side effects in patients.

MATERIALS AND METHODS

Cell Culture and Western Blot Analysis—The HCT116 colorectal carcinoma cell line, its clonal derivative Hke-3 that lacks the mutant h-ras allele (47), and HT29 cells were cultured under standard conditions in minimum Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. Western blot analysis was performed using standard procedures. Briefly, 50 μg of total cell lysates were fractionated in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with antibodies for 1 h at room temperature or overnight at 4 °C, and ECL (Amersham Biosciences) was used for visualization of immune complexes.

Immunofluorescence—Cells grown on chamber slides were serum-starved for 16 h and were treated with IFN-γ for 30 or 60 min either alone or after a 16-h preincubation with HDAC inhibitors. Cells were fixed in ice-cold methanol/acetic acid solution (95:5 (v/v)) for 20 min at −20 °C. Incubation with anti-STAT1 antibodies (Upstate Biotechnology) was performed for 1 h at 37 °C. Slides were washed with phosphate-buffered saline and incubated with a secondary anti-rabbit antibody conjugated to fluorescein isothiocyanate for 45 min at 37 °C. Samples were examined with a fluorescent microscope, and images were acquired with a SPOT CCD camera and analyzed by SPOT software.

Transient Transfections and Reporter Gene Assays—To examine the effect of HDAC inhibitors on STAT1-dependent transcriptional activity, we used plasmid 8×GAS-pGL3LUC, which was kindly provided to us by Dr. C. Glass (48). It contains eight GAS (gamma-activated sequence) STAT1 binding sites cloned upstream of the luciferase reporter gene. Cells were transfected with 1 μg of DNA/12-well plate using the calcium phosphate method (Profection mammalian transfection system, Promega, Madison, WI) as we described previously (49). Transfection efficiency was normalized by cotransfection with pTK-Renilla (Dulbecco luciferase reporter assay system, Promega). Cells were either left untreated or were treated with IFN-γ (10 ng/ml) alone or in the presence of HDAC inhibitors. To determine the effect of HDACs on the STAT1-dependent transcriptional activity, the GAS-LUC reporter gene was cotransfected with increasing concentrations of plasmids expressing HDAC1, HDAC2, HDAC3, or HDAC4 from 0.05–0.75 μg/12-well plate. The basal and IFN-γ-inducible transcriptional activity was determined 8 h after treatment. Results are expressed as the fold-induction of LUC activity, calculated from the ratio between the activity of the reporter plasmid in treated and untreated cells. The siRNAs specific for STAT1, HDAC1, HDAC2, and HDAC3 (Dharmacon, Lafayette, CO) were transfected into Hke-3 cells using the calcium phosphate method as described above.

Apoptosis Assay—Cells were resuspended in hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate) and stained with propidium iodide (50 μg/ml) for 4 h at 4 °C as described previously (50). Samples were filtered through a nylon mesh (40-micron pore size) and analyzed by flow cytometry. Cell cycle distribution and the extent of apoptosis (cells with subG1 DNA content) were analyzed by Modfit software. The apoptotic nature of butyrate-treated cells was confirmed with annexin V staining (Molecular Probes) (51). Briefly, 5 μl of the annexin V-fluorescein isothiocyanate conjugate was added to 100 μl of cell suspension, and samples were analyzed by flow cytometry 15 min after incubation at room temperature.

RESULTS

HDAC Inhibitors Impede IFN-γ-induced Phosphorylation of STAT1 on Tyrosine and Serine—We recently demonstrated that the short chain fatty acid butyrate interferes with nuclear translocation and subsequent DNA binding of STAT1 through inhibition of STAT1 phosphorylation on tyrosine and serine residues (43). Inhibition of HDAC activity represents an important biological activity of butyrate (52). To define the significance of HDAC activity in signaling by IFN-γ, we first examined whether two additional HDAC inhibitors, TSA and SAHA, also inhibit STAT1 tyrosine phosphorylation. Treatment of cells with each of the three inhibitors of HDAC activity induced dose-dependent acetylation of histone H3 and histone H4 in these cells (Supplemental Fig. 1). In subsequent experiments we used concentrations of HDAC inhibitors that were effective in inducing acetylation of histones. Hke-3 cells were treated with IFN-γ for 15 min alone or after a 16-h preincubation with butyrate (3 mM), TSA (1 μM), or SAHA (2 μM). Cell lysates were examined by immunoblotting for the expression of total STAT1 and for the activation of STAT1, the latter assessed by its phosphorylation on tyrosine 701. Untreated Hke-3 cells had no detectable levels of STAT1 phosphorylated on tyrosine. As expected, IFN-γ induced a rapid tyrosine phosphorylation of STAT1 (Fig. 1A). Although the levels of total STAT1 were not modulated by the treatment of cells with butyrate, SAHA, or TSA (Fig. 1A), IFN-γ-induced tyrosine phosphorylation of STAT1 was completely prevented by each of the three inhibitors of HDAC activity. The estimated EC50 was 1 mM for butyrate, 5 μM for TSA, and 3 μM for SAHA. We demonstrated that butyrate inhibits STAT1 phosphorylation in a time- and concentration-dependent manner (Supplemental Fig. 2, A, B, and C). Although we did not detect the inhibitory effect of HDAC inhibitors on STAT phosphorylation 1 h after treatment, pretreatment of cells with HDAC inhibitor for 4 h was sufficient to significantly inhibit STAT1 phosphorylation (Supplemental Fig. 2). This suggests that the effect of HDAC inhibitors on STAT1 phosphorylation is not direct but may involve activation of an inhibitor of STAT1 phosphorylation or inhibition of the factor required for STAT1 phosphorylation.
JAK1 and JAK2 are the upstream kinases responsible for the tyrosine phosphorylation of STAT1 (53). We have reported previously that butyrate inhibits STAT1 signaling through inhibition of JAK2 activation (43). In this study we examined whether JAK1 is also a target of HDAC inhibitors. Cells were treated with IFN-γ/H9253 for 5 or 15 min alone or after preincubation with HDAC inhibitors as indicated in Fig. 1B. This experiment demonstrated that butyrate, TSA, and SAHA strongly inhibited IFN-γ/H9253-induced activation of JAK1 (Fig. 1B) indicating that HDAC inhibitors negatively regulate STAT1 tyrosine phosphorylation through inhibition of JAK1 activation. The levels of total JAK1 and JAK2 were not modulated by HDAC inhibitors at the mRNA (42) nor at the protein levels (Supplemental Fig. 3).

In addition to inducing tyrosine phosphorylation of STAT1, IFN-γ is known to induce serine phosphorylation of STAT1, and both modifications appear to be required for the optimal transcriptional activity of STAT1 (8). We examined the effect of HDAC inhibitors on STAT1 serine phosphorylation by immunoblotting using an antibody specific for STAT1 phosphorylated on Ser-727 (Upstate Cell Signaling Solutions). As reported earlier, we consistently detected low basal phosphorylation of STAT1 on serine 727 in resting Hke-3 cells (43), and treatment of cells with IFN-γ for 30 min further increased serine phosphorylation of STAT1 (Fig. 1C). The HDAC inhibitors butyrate, TSA, and SAHA did not modulate the basal serine phosphorylation of STAT1 but clearly inhibited the IFN-γ-induced increase in serine phosphorylation of STAT1 (Fig. 1C). These data therefore demonstrate that inhibition of HDAC activity negatively regulates STAT1 activity through the inhibition of its tyrosine and serine phosphorylation.

To rule out the possibility that HDAC inhibitors interfere with IFN/STAT signaling through the inhibition of the expression of IFN-γ receptors, we examined the effect of butyrate, TSA, and SAHA on the expression of both subunits of the IFN-γ receptor. Analysis of cell lysates by immunoblotting revealed that HDAC inhibitors did not regulate the expression of the
IFN-γ receptor α or β chains (data not shown), excluding the possibility that these agents interfere with IFN-γ signaling through inhibition of the expression of IFN-γ receptors.

**HDAC Inhibitors Restrain IFN-γ-induced STAT1 Nuclear Translocation**—To determine whether inhibition of STAT1 phosphorylation by HDAC inhibitors results in impaired STAT1 nuclear translocation, we examined the effect of HDAC inhibitors on the subcellular localization of STAT1. Hke-3 cells were cultured on chamber slides, were serum-starved overnight, and were treated with IFN-γ (10 ng/ml) for 30 min alone or after a 16-h preincubation of the cells with 3 mM butyrate, 5 μM TSA, or 2 μM SAHA. Treatment of cells with HDAC inhibitors did not induce substantial apoptosis as assessed by 4′,6-diamidino-2-phenylindole staining (Fig. 2).

The cells were stained with STAT1 antibody as described under "Materials and Methods." STAT1 was localized to both the cytoplasm and the nuclei in untreated cells, confirming a constitutive nucleocytoplasmic trafficking of STAT1, which results in partial nuclear localization of unphosphorylated STAT1 in the absence of IFN-γ stimulation (54). Treatment of cells with IFN-γ for 30 min altered the equilibrium between the nuclear and cytoplasmic STAT1 and induced a complete nuclear translocation of STAT1 (Fig. 2). Treatment of cells with HDAC inhibitors alone did not alter the subcellular localization of STAT1, it prevented IFN-γ-induced nuclear translocation of STAT1 (Fig. 2).

Our data therefore demonstrate that inhibition of STAT1 phosphorylation by inhibitors of HDAC activity results in abrogated nuclear translocation of STAT1 upon treatment of cells with IFN-γ.

**HDAC Inhibitors Interfere with STAT1-dependent Transcriptional Activity**—Phosphorylation of STAT1 on tyrosine and serine residues is required for its full transcriptional activity (8). Because we demonstrated that both serine and tyrosine phosphorylation of STAT1 are inhibited by HDAC inhibitors, this raised the possibility that these agents act as inhibitors of STAT1-dependent transcriptional activity. To test this hypothesis we utilized a construct containing eight GAS cloned upstream of the LUC gene and tested whether HDAC inhibitors interfere with IFN-γ-driven transcriptional activity. Hke-3 cells were transiently transfected with the 8×GAS-LUC plasmid. The cells were then either left untreated (CTRL) or treated with IFN-γ, butyrate, or the combination of IFN and butyrate for 7 h. The results are expressed as the relative promoter activity, calculated from the ratio between the LUC and Renilla activity (LUC/REN).

![Fig. 4. HDAC1, HDAC2, and HDAC3, but not HDAC4, support STAT1-dependent transcription. Hke-3 cells were transiently transfected with the 8×GAS-LUC reporter gene alone (CTRL) or together with increasing amounts of expression plasmids coding for HDAC1, HDAC2, HDAC3, or HDAC4 as indicated. Cells were left untreated (CTRL) or were treated with IFN-γ, butyrate, or the combination of IFN and butyrate for 7 h. The results are expressed as the relative promoter activity, calculated from the ratio between the LUC and Renilla activity (LUC/REN).](http://www.jbc.org/)

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The activity of the reporter construct was low in unstimulated cells, and treatment of cells with IFN-γ induced STAT1-dependent gene activation 150-fold (Fig. 3). Butyrate, SAHA, and TSA inhibited the basal transcriptional activity (not shown) and reduced IFN-γ-induced activation of the STAT1-driven reporter gene by more than 90% (Fig. 3). In contrast, inhibitors of HDAC activity have been shown to activate promoter activity of several genes, such as p21 (55) and gelsolin,2 demonstrating that HDAC inhibitors can both activate or repress gene activation.

These data suggested that deacetylase activity is required for optimal STAT1-dependent transcription. To test this hypothesis, we first examined the effect of elevated expression of HDACs on IFN-γ-induced STAT1-dependent transcription. Hke-3 cells were transiently transfected with the 8×GAS report gene together with an empty vector (Fig. 4, CTRL) or increasing concentrations of expression vectors that encode HDAC1, HDAC2, HDAC3, or HDAC4 (Fig. 4). 16 h posttransfection, cells either were left untreated (Fig. 4, CTRL) or were treated with IFN-γ (10 ng/ml), Na-Bu (3 mM), or the combination of IFN-γ and HDAC inhibitors as indicated in Fig. 3.

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and butyrate for 7 h. We demonstrated that exogenous expression of HDAC1, HDAC2, and HDAC3 enhanced IFNγ-induced stimulation of STAT1-dependent transcription (Fig. 4). In contrast, HDAC4 inhibited IFNγ-induced STAT1-dependent transcriptional activation in a dose-dependent manner. These data demonstrated that STAT1-dependent transcriptional activity is regulated positively by class I HDACs (HDAC1, HDAC2, and HDAC3) but negatively by a class II member, HDAC4.

However, despite their ability to positively regulate STAT1-driven transcription, HDAC1, HDAC2, and HDAC3 alone were not sufficient to rescue butyrate-mediated inhibition of STAT1-dependent transcription (Fig. 4). Butyrate is a broad inhibitor of HDAC activity (52); therefore overexpression of HDAC1, HDAC2, or HDAC3 alone may not be sufficient to relieve the inhibition of STAT1-dependent transcription by butyrate.

**HDAC1, HDAC2, and HDAC3 Are Required for STAT1-dependent Gene Activation**—To confirm the requirement of HDAC1, HDAC2, and HDAC3 for the optimal responsiveness to IFNs, we silenced HDAC1, HDAC2, and HDAC3 through RNA interference. We transiently transfected Hke-3 cells with 50 nM siRNAs directed against HDAC1, HDAC2, or HDAC3 using the calcium phosphate method as described under "Materials and Methods." Control cells were transfected with 50 nM nonspecific, scrambled siRNA. We first examined the efficiency of silencing of HDAC1, HDAC2, and HDAC3 by immunoblotting. As shown in Fig. 5A, silencing of HDAC1 occurred only in cells transfected with HDAC1 siRNA, silencing of HDAC2 in cells transfected with HDAC2 siRNA, and silencing of HDAC3 only in cells transfected with HDAC3 siRNA, establishing a specificity of siRNA. Furthermore, we demonstrated that silencing of HDAC2 resulted in increased expression of HDAC1, and silencing of HDAC3 resulted in increased expression of HDAC2. It is noteworthy that HDAC1-deficient embryonic stem cells also display increased expression of both HDAC2 and HDAC3 (56). Treatment of cells with IFNγ did not modulate the expression of HDAC1, HDAC2, or HDAC3 (Fig. 5A).

Next we examined whether silencing of HDAC1, HDAC2, or HDAC3 interferes with the responsiveness of the STAT1-dependent reporter gene to IFNγ. Hke-3 cells were transfected with the 5×GAS-LUC reporter gene in the presence of 50 nM nonspecific siRNA or siRNAs directed against HDAC1, HDAC2, and HDAC3. Cells were transfected for 24 or 48 h and treated with IFNγ for 6 h. As shown in Fig. 5B, the inducibility of the STAT1-dependent reporter gene by IFNγ was markedly reduced in cells with silenced HDAC1, HDAC2, and HDAC3 compared with cells transfected with nonspecific siRNA. Silencing of HDAC1 and HDAC3 affected the responsiveness to IFNγ more than silencing of HDAC2, suggesting a predominant role of HDAC1 and HDAC3 in STAT1-dependent transcription.

These results firmly established the requirement for deacetylase activity in IFN signaling and strongly support our hypothesis that Bu, SAHA, and TSA interfere with IFNγ-induced transcriptional activation through their ability to inhibit the activity of HDACs.

**HDAC Inhibitors Prevent IFNγ-induced Expression of IRF-1 but Do Not Interfere with IFNγ-induced Expression of STAT1 and STAT2**—Intact STAT1 signaling has been shown to be required for the expression of the majority of IFN target genes, suggesting that HDAC inhibitors, through the inhibition of STAT1 signaling, might interfere with the expression of IFN target genes. First we examined the effect of HDAC inhibitors on the expression of IRF-1, an IFN-inducible transcription factor that has been shown to be induced by IFNγ in a STAT1-dependent manner (14). Hke-3 cells either were left untreated or were treated with IFNγ for 24 h alone or in the presence of HDAC inhibitors as indicated in Fig. 6. We demonstrated that IFNγ-induced expression of IRF-1 was markedly reduced by butyrate, TSA, and SAHA in a concentration-dependent manner (Fig. 6, A and C), consistent with the requirement of STAT1 activation for the induction of IRF-1 by IFNγ (14).

Next we examined the effect of HDAC inhibitors on the expression of STAT1 and STAT2, transcription factors inducible by IFNγ in Hke-3 cells. Despite the fact that all three HDAC inhibitors efficiently inhibited STAT1 phosphorylation (Fig. 1), STAT1 nuclear translocation (Fig. 2), and STAT1-dependent transcriptional activity (Fig. 3), they did not prevent IFNγ-induced expression of STAT1. These data therefore established that STAT1 phosphorylation and nuclear translocation are not required for IFNγ to induce STAT1 expression. Similarly, HDAC inhibitors did not prevent IFNγ-induced expression of STAT2, demonstrating that like STAT1, STAT2 is induced by IFNγ in the absence of functional STAT1 signaling (Fig. 6, B and C). This experiment was repeated at least three times. Consistently, HDAC inhibitors significantly reduced IFNγ-induced IRF-1 expression but did not interfere with the inducible expression of STAT1 and STAT2. We also confirmed...
this observation in another colorectal cancer cell line, HT29 (Supplemental Fig. 5).

These data confirmed the existence of STAT1-independent signaling by IFNγ and demonstrated that STAT1 and STAT2, transcription factors required for signaling by both type I and type II interferons, are induced by IFN in a STAT1-independent manner. These results also suggest that HDAC inhibitors may act as selective inhibitors of expression of STAT1-dependent IFN target genes. However, global analyses of gene expression will be necessary to test this hypothesis.

**Inhibition of STAT1 Expression by siRNA**—Our data demonstrated that inhibitors of HDAC activity prevent IFNγ-induced STAT1 phosphorylation and its nuclear translocation (Figs. 1 and 2) but do not interfere with the ability of IFNγ to induce accumulation of STAT1 in cells (Fig. 6). Because unphosphorylated STAT1 has been shown to support the constitutive expression of several genes (10, 54), we examined whether the lack of STAT1 expression had the same impact on the expression of IFNγ target genes as the inhibition of STAT1 signaling. We compared the expression of STAT1, STAT2, and IRF-1 in parental 293T cells and in cells that had STAT1 expression silenced by siRNA. We transfected cells with 100 nM siRNA representing a mixture of four RNA duplexes directed against the coding region of STAT1 (positions 766, 983, 1638, and 2438) (Dharmacon). 16 h after transfection, cells were treated with IFNγ for 24 h, and cell lysates were examined for the expression of IRF-1, STAT1, and STAT2. Results obtained from two independent experiments were used for densitometric analysis.
pointing to an efficient silencing of both the basal and inducible expression of STAT1 (Fig. 7A). We demonstrated that IFNγ failed to induce the expression of IRF-1 in 293T cells transfected with STAT1 siRNA, confirming that the expression of IRF-1 strictly depends on functional STAT1. These data are consistent with a report that IFNγ failed to induce IRF-1 in STAT1−/− cells (14) and with our data, which demonstrated that HDAC inhibitors prevented IFNγ induction of IRF-1 through inhibition of STAT1 signaling (Fig. 6). In agreement with our data that STAT1 signaling is not required for STAT2 expression (Fig. 6), the basal expression and the inducible expression of STAT2 were not significantly affected by STAT1 siRNA in 293T cells (Fig. 7A).

To exclude the possibility that our observation is restricted to a single cell line, we also silenced STAT1 expression in Hke-3 cells and examined its effect on the expression of STAT2, IRF-1, and caspase-7, another IFNγ target gene. Cells were transfected with increasing concentrations of siRNA (from 5 to 100 nM). As shown in Fig. 7B, transfection of cells with 5 nM siRNA was sufficient to efficiently silence the expression of STAT1. Likewise, the inducible expression of IRF-1 was abolished in the transfected cells. In contrast, transfection of cells with 100 nM STAT1 siRNA did not significantly impair the inducibility of STAT2 and caspase-7 by IFNγ, confirming that IFNγ induces STAT2 and caspase-7 expression through a pathway that bypasses STAT1 signaling. The densitometric analysis of Fig. 7B is provided in the supplemental figures (Supplemental Fig. 6).

**HDAC Inhibitors Promote IFNγ-induced Apoptosis**—How does the inhibition of STAT1 signaling by the HDAC inhibitors affect the biological activity of IFNγ? We first examined the effect of HDAC inhibitors on IFNγ-induced apoptosis. HT29 colon carcinoma cells either were left untreated or were treated for 16 h with 3 mM butyrate, 5 μM TSA, or 2 μM SAHA, concentrations that did not affect the viability of the cells (Fig. 8A; see also Fig. 2). The medium containing butyrate, TSA, or SAHA was then removed, and the cells were treated with IFNγ (50 or 100 ng/ml) for 24, 48, or 72 h. Although IFNγ alone was a relatively poor inducer of apoptosis, Bu, TSA, and SAHA significantly enhanced IFNγ-induced apoptosis (Fig. 8A).

This result suggested that HDAC inhibitors sensitized cells to IFNγ-induced apoptosis. We first demonstrated that the number of cells with subG1 content of DNA increased upon treatment of cells with both Bu and IFNγ (Supplemental Fig. 4) and confirmed the apoptotic nature of IFNγ-treated cells by annexin V staining (Fig. 8B). HT29 cells treated with butyrate (3 mM), IFNγ (50 ng/ml), or the combination of butyrate and IFNγ were stained with annexin V-fluorescein isothiocyanate for 15 min at room temperature and analyzed by flow cytometry. As shown in Fig. 8B, the number of annexin V-positive cells and the intensity of staining increased significantly in cells treated with both butyrate and IFNγ. Finally, we demonstrated the cleavage of poly(ADP-ribose) polymerase in cells treated with both butyrate and IFNγ (Fig. 9A), pointing to an activation of a caspase pathway only in cells treated with the combination of IFNγ and HDAC inhibitor. We demonstrated that IFNγ increased the expression of caspase-7; however, butyrate did not further increase its expression or result in the activation of caspase-7, suggesting that caspase-7 is most likely not responsible for increased apoptosis in cells treated with Bu and IFNγ (Fig. 9A). Therefore, the identity of the caspase that mediates increased apoptosis in these cells remains to be determined. The levels of gelsolin, a butyrate target gene with an antiapoptotic activity (57, 58), were similar in cells treated with butyrate alone or together with IFNγ (Fig. 9A).

To further define the mechanism whereby HDAC inhibitors promote IFNγ-induced apoptosis, we examined the ability of IFNγ and butyrate to regulate the expression of two antiapoptotic proteins, Bcl-2 and Bcl-x, both transcriptional targets of IFNγ and STAT1 (59). HT29 cells either were left untreated or were treated with IFNγ alone, butyrate alone, or the combination of butyrate and interferon for 48 h. The levels of Bcl-2 in HT29 cells were low and were not regulated by HDAC inhibitors and IFNγ (data not shown). Similarly, the levels of Bcl-x were not changed in cells treated with IFNγ or Bu alone (Fig. 9B). However, the expression of Bcl-x was markedly reduced in cells treated with both butyrate and interferon for 48 h, indicating that in HT29 cells IFNγ negatively regulates Bcl-x expression in the absence but not in the presence of functional STAT1 signaling (Fig. 9B). Bcl-x is a protein with a strong antiapoptotic activity; therefore, its inhibition is likely to underlie the accelerated death of cells treated with inhibitors of HDAC activity and IFNγ.

**DISCUSSION**

In this study we demonstrated that the silencing of HDAC1, HDAC2, and HDAC3 interferes with STAT1-dependent transcription and that inhibitors of HDAC activity curtail the expression of STAT1-dependent genes, thereby altering the biological activity of interferon γ. We also present evidence that the inducible expression of STAT1, STAT2, and caspase-7 was not repressed by HDAC inhibitors, which demonstrates that IFNγ induces the expression of STAT1, STAT2, and caspase-7 independently of functional STAT1 signaling.
Signaling by IFNγ is deregulated in several inflammatory diseases, including IBD (21–24). Elevated levels of STAT1 or activation of STAT1 has been demonstrated in Crohn's disease, in ulcerative colitis, and in pouchitis (26, 27). In addition, several transformed cells display constitutive activation of STATs, including STAT1, STAT3, and STAT5 (31, 60). Therefore, pharmacological inhibitors of IFN/STAT signaling could be used to alleviate mucosal inflammation and to potentially restrict proliferation of tumor cells. There is only a limited number of agents that are known to interfere with signaling by STATs. Curcumin, an anti-inflammatory and a chemopreventive agent, negatively regulates JAK/STAT1 signaling through activation of SHP-2 (61). Sulindac, a chemopreventive agent used to prevent polyp formation in familial adenomatous polyposis patients, inhibits constitutive STAT3 activation in human oral squamous cell carcinoma (62), suggesting that sulindac induces cell death and inhibits cell proliferation at least in part through the inhibition of constitutive activation of STAT3 (63–67). Agonists of PPARγ, 15d-PGJ2, and the antidiabetic rosiglitazone have been shown to suppress JAK/STAT signal-

**Fig. 8.** HDAC inhibitors enhance IFNγ-induced apoptosis. A, HT29 cells were left untreated (CTRL) or were pretreated with butyrate (3 mM), TSA (1 μM), or SAHA (2 μM) for 16 h. Medium containing HDAC inhibitors was then removed, and the cells were left untreated (0) or were treated with IFNγ (50 or 100 ng/ml) for 72 h. B, the extent of apoptosis in cells treated in Bu, IFN, or the combination of agents was determined by annexin V staining 48 h after treatment. MF, mean fluorescence; FITC, fluorescein isothiocyanate.
With IFN-STAT1 phosphorylation and nuclear translocation requires demonstration that the inhibitory effect of HDAC inhibitors on and subsequently prevent STAT1 nuclear translocation. We induced JAK1 activation and STAT1 tyrosine phosphorylation in a mouse model of IBD (68, 69) and to inhibit Ras-

We showed that inhibitors of HDAC activity, including butyrate, SAHA, and TSA, inhibit IFN-γ-induced JAK1 activation and STAT1 tyrosine phosphorylation and subsequently prevent STAT1 nuclear translocation. We demonstrated that the inhibitory effect of HDAC inhibitors on STAT1 phosphorylation and nuclear translocation requires preincubation of cells with HDAC inhibitors prior to treatment with IFN-γ for at least 4 h (Supplemental Fig. 2). Nusinzon and Horvath (44) recently reported that TSA blocks IFNα stimulated gene induction without affecting JAK activation or the nuclear translocation and DNA binding of ISGF3 (44). In contrast to our studies, the authors assessed the effect of TSA on JAK/STAT1 signaling by simultaneously treating cells with TSA and IFN. Our data therefore do not support the hypothesis that inhibition of HDAC activity directly inhibits JAK activation; rather, our data suggest that a factor required for JAK1 activation is negatively regulated by HDAC inhibitors or, alternatively, that the JAK1 inhibitor is up-regulated by HDAC inhibition. We are currently testing whether inhibitors of HDAC activity negatively regulate STAT1 signaling through up-regulation of negative regulators of JAK activation, such as SOCS1 and SOCS3 (71-73), or activation of phosphatases responsible for dephosphorylation of JAK1/JAK2, such as SHP-1 and SHP-2 (19, 74, 75).

In addition, we present evidence that IFN-driven, STAT1-dependent transcription requires deacetylase activity (Figs. 3, 4, and 5). We determined that inhibitors of HDAC activity curtail the expression of STAT1-dependent genes, such as IRF-1, but do not interfere with the inducible expression of STAT1, STAT2, and caspase-7, suggesting that activation of expression of STAT1, STAT2, and caspase-7 by IFN-γ does not require STAT1 signaling. The inducible expression of STAT2 and caspase-7 was also intact in cells with silenced STAT1 expression (Fig. 7), confirming STAT1-independent induction of STAT2 and caspase-7 by IFN-γ. These data therefore suggest that HDAC inhibitors function as selective inhibitors of STAT1-dependent signaling by IFN-γ and that the expression of STAT1-independent IFN target genes does not require HDAC activity. However, a genome-wide analysis of IFN target genes in STAT1-deficient cells and HDAC1/23-deficient cells will be required to define a subset of IFN target genes that depend on HDAC activity.

The mechanisms whereby IFN-γ regulates the expression of its target genes in the absence of functional STAT1 signaling and the physiological consequences of STAT1-independent signaling by IFN-γ remain to be determined. Our preliminary data demonstrated that IFN-γ does not activate NFκB or STAT3 in Hke-3 cells (data not shown), excluding the involvement of these transcription factors in the expression of STAT1-independent genes. A point mutant of STAT1 (V710F), which cannot be phosphorylated on Tyr-701 and thereby cannot dimerize, has been shown to bind DNA and to down-regulate the constitutive expression of several genes, such as cyclin A, Hsp70, and Bcl-x, and also to up-regulate several genes, e.g. LMP2, β2M, and caveolin 2 (10). Thus, STAT1 has an essential function regulating the expression of genes as a monomer. It is therefore possible that unphosphorylated STAT1 participates not only in constitutive activation but also in inducible activation of genes, such as STAT1 and STAT2, and in ligand-mediated inhibition of gene expression, such as Bcl-x. It remains to be determined how STAT1 regulates gene expression in the absence of tyrosine phosphorylation, but it may involve cooperation with other transcription factors or sequestration of transcription factors in the cytoplasm that physically associate with STAT1, such as Sp1 (76) and HSF-1 (77).

We present evidence that the interruption of the balance between STAT1-dependent and STAT1-independent signaling has important consequences for the biological activity of IFN-γ. We showed that inhibitors of HDAC activity sensitize cells to IFN-γ-induced apoptosis, demonstrating that STAT1-dependent signaling protects cells from IFN-γ-induced apoptosis. This is consistent with reports that STAT1 can protect against cytotoxic effects of multiple cytokines, including IFN-γ and IFNα (78, 79). Enhanced apoptosis of cells treated with IFN-γ and butyrate suggests that IFN-γ in the absence of STAT1 signaling induces the expression of genes that promote apoptosis or inhibits the expression of antiapoptotic genes. We found that IFN-γ in the absence of STAT1 signaling inhibits the expression of Bcl-x, a protein with a potent antiapoptotic activity. Because these data contrast with a published report that IFN-γ inhibits Bcl-x transcription in a STAT1-dependent manner (59), experiments to elucidate the mechanism of the negative regulation of Bcl-x expression by IFN-γ and HDAC inhibitors are underway in our laboratory.

Treatment of cells with butyrate, TSA, and SAHA induces histone acetylation through inhibition of HDAC activity. The fact that these agents, or the silencing of HDAC1, HDAC2, and HDAC3 expression, negatively regulate STAT1-dependent transcriptional activation suggests that hyperacetylation of histones negatively regulates STAT1-dependent gene activation. Although HDAC inhibitors play a fundamental role in regulating gene expression through remodeling of chromatin, they are also known to interfere with the deacetylation of several non-histone proteins, such as p53 (80), E2F (81), and MyoD (82). We therefore cannot exclude the possibility that SAHA, TSA, and butyrate inhibit STAT1-dependent transcriptional activity.
through modification of non-histone proteins, such as coactivators that are involved in the STAT1 transcriptional response or potentially STAT1 itself.

Our data demonstrate that class I HDACs, HDAC1, HDAC2, and HDAC3, support STAT1-dependent transcription and that a member of the class II HDACs, HDAC4, inhibits STAT1-dependent gene activation. The diversity among HDACs, such as their different subcellular localization and their differential ability to form multiprotein complexes with repressors such as NuRD and Sin3 (83), may underlie both their distinct and overlapping roles in STAT1-dependent transcription. It is noteworthy that STAT1 has been shown to physically associate with HDAC1 and HDAC2 but not with HDAC4 (44), suggesting that physical association may be required for the positive regulation of STAT1-dependent transcription.

In conclusion, we demonstrated that deacetylase activity is required for STAT1-dependent gene activation and that HDAC inhibitors interrupt the balance between STAT1-dependent and STAT1-independent signaling, thereby altering the biological activity of IFNγ. This suggests that HDAC inhibitors represent a new class of drugs for the treatment of inflammatory conditions associated with enhanced STAT1 signaling and that these inhibitors may also interfere with constitutive activation of STAGs found in a number of malignancies.

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