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Opposite Effects of Histone Deacetylase Inhibitors on Glucocorticoid and Estrogen Signaling in Human Endometrial Ishikawa Cells

Walter Rocha, Rocio Sanchez, Julie Deschênes, Anick Auger, Elise Hébert, John H. White, and Sylvie Mader

Department of Biochemistry (W.R., R.S., J.D., A.A., E.H., S.M.) and Institute of Research in Immunology and in Cancer (W.R., J.D., E.H., S.M.), Université de Montréal, Montréal, Canada; Montréal Center for Experimental Therapeutics in Cancer, Jewish General Hospital, Montréal, Canada (W.R., J.D., E.H., J.H.W., S.M.); and Departments of Physiology (J.H.W.) and Medicine (J.H.W., S.M.), McGill University, Montréal, Canada

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ABSTRACT

Histone deacetylase inhibitors (HDACi), which have emerged as a new class of anticancer agents, act by modulating expression of genes controlling apoptosis or cell proliferation. Here, we compared the effect of HDACi on transcriptional activation by estrogen or glucocorticoid receptors (ER and GR, respectively), two members of the steroid receptor family with cell growth regulatory properties. Like other transcription factors, steroid receptors modulate histone acetylation on target promoters. Using episomal reporter vectors containing minimal promoters to avoid promoter-specific effects, we observed that long-term (24-h) incubation with HDACi strongly stimulated GR-dependent but markedly repressed ER-dependent signaling in ER+/GR+ human endometrial carcinoma Ishikawa cells. These effects were reproduced on endogenous target genes and required incubation periods with HDACi substantially longer than necessary to increase global histone acetylation. Repression of estrogen signaling was due to direct inhibition of transcription from multiple ERα promoters and correlated with decreased histone acetylation of these promoters. In contrast, the strong HDACi stimulation of GR-dependent gene regulation was not accounted for by increased GR expression, but it was mimicked by overexpression of the histone acetyltransferase complex component transcriptional intermediary factor 2. Together, our results demonstrate striking and opposite effects of HDACi on ER and GR signaling that involve regulatory events independent of histone hyperacetylation on receptor target promoters.

Incorporation of DNA into chromatin plays a major role in regulating gene expression. Decondensed chromatin (euchromatin) is associated with transcriptional activity, whereas condensed heterochromatin is transcriptionally inactive. N-terminal tails of histones, which are subject to several post-translational modifications (Jenuwein and Allis, 2001), play an important role in regulation of chromatin structure. Positively charged histone tails of nucleosomes interact with DNA, other histones, and chromatin components. Acetylation of lysines by histone acetyl-transferases (HATs) neutralizes their positive charges, destabilizes nucleosomes, and can enhance or block other types of modifications, resulting in differential binding of many different chromatin proteins (Jenuwein and Allis, 2001).

Transcriptional activators recruit cofactors, including HATs. For example, nuclear receptors exhibit hormone-dependent recruitment of HAT complexes composed of p160 (SRC-1/TIF2/AIB1), CBP/p300, and pCAF families of coacti-

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ABBREVIATIONS: HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor(s); MMTV, mouse mammary tumor virus; ER, estrogen receptor; GR, glucocorticoid receptor; E2, 17β-estradiol; OHT, 4-hydroxytamoxifen; Dex, dexamethasone; SB, sodium butyrate; Act-D, actinomycin D; wt. wild-type; CAT, chloramphenicol acetyltransferase; EBV, Epstein Barr virus; ERE, estrogen response element; GRE, glucocorticoid response element; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TIF2, transcriptional intermediary factor 2; TAT, tyrosine aminotransferase; CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICI182,780, faslodex.

vators (Rosenfeld and Glass, 2001). Histone acetylation is remarkably dynamic on hormone-regulated promoters, because recruitment of HAT complexes alternates with that of histone deacetylases (HDACs) on the estrogen target promoter pS2 (Metivier et al., 2003). Nuclear receptor corepressors such as N-CoR and SMRT (Rosenfeld and Glass, 2001) or NRIP1/RIP140 and LCoR (White et al., 2004) recruit HDACs in the absence or presence of hormone, respectively. In addition, HATs and probably also HDACs are active with nonhistone protein substrates, including E2F, pRb, and p53 (McLaughlin and La Thangue, 2004).

HDACi have emerged as a new class of anticancer agents for treatment of both solid and hematological tumors (McLaughlin and La Thangue, 2004). The naturally occurring antifungal antibiotic trichostatin A has been invaluable in validating HDACs as potential anticancer targets. Structurally related inhibitors, including SAHA, PXD101, and LAQ-824, are currently in clinical trials (Kelly et al., 2003). Aliphatic acids valproate and butyrate function as less potent HDACi (McLaughlin and La Thangue, 2004). HDACi induce apoptosis or differentiation depending on the cell type (McLaughlin et al., 2003) and, notably, block proliferation of breast, endometrial, and ovarian cancer cells (Munster et al., 2001; Strait et al., 2002; Takai et al., 2004). Different HDACi alter transcription of a common set of genes that control pathways important for cell survival and proliferation (Glaser et al., 2003; Peart et al., 2005). It is noteworthy that both enhancement and repression of gene expression were observed in these studies, suggesting more complex mechanisms of action than enhancement of histone acetylation.

HDACi influence steroid receptor gene regulation in a cell-, promoter- and receptor-dependent manner. HDACi prevented activation of transiently transfected, episomal, or chromosomal MMTV promoters by glucocorticoids (Mulholland et al., 2003; Kinyamu and Archer, 2004). Although sodium butyrate inhibited glucocorticoid induction of the tyrosine aminotransferase gene in rat HTC cells (Plesko et al., 1983), it enhanced glucocorticoid induction of alkaline phosphatase in HeLa S3 cells (Littlefield and Cidlowski, 1984). Finally, trichostatin A induced estrogen-dependent transcription in MCF-7 cells (Ruh et al., 1999) and in stably transfected HepG2 cells (Mao and Shapiro, 2000).

Some of the effects of HDACi on estrogen target genes seem to be mediated by modulation of estrogen receptor (ER) expression. Inhibition of ERα expression by HDAC1 in MCF-7 breast cancer cells was reversed by trichostatin A (Kawai et al., 2003). Trichostatin A induced ERα expression in ER-negative breast cancer cells (Yang et al., 2001), whereas another study found that trichostatin A induced ERβ but not ERα expression in MDA-MB-231 cells (Jang et al., 2004). Finally, valproic acid induced ERα expression in endometrial carcinoma Ishikawa and in MCF-7 cells (Graziani et al., 2003). Conversely, others reported inhibition of ERα expression by HDACi, which may explain the increased sensitivity of ER+ breast cancer cell lines to HDACi (Alao et al., 2004; Margueron et al., 2004b; Reid et al., 2005). Finally, HDACi may induce hyperacetylation of nuclear receptors by associated HAT complexes, altering their function. Indeed, acetylation of ERα modulated sensitivity to hormone (Fu et al., 2004).

Variations in cell lines and/or target promoters, which can be regulated by steroid receptors through different mechanisms (Sanchez et al., 2002), probably account for the variability in the reported effects of HDACi on steroid-mediated transcription. Here, we compared the effects of HDACi on ERα and glucocorticoid receptor (GR)-dependent transcription on reporter vectors containing minimal estrogen- or glucocorticoid-responsive promoters propagated as episomes in human endometrial carcinoma Ishikawa cells, which express both receptors. Using this system, modulation by HDACi of receptor-dependent transcription can be monitored in the absence of a confounding influence of other transcription factors or of variable sites of chromosomal integration. Our results indicate striking and opposite effects of HDACi on estrogen and glucocorticoid signaling, leading us to explore the mechanisms underlying this differential regulation of two closely related steroid receptors in Ishikawa cells.

Materials and Methods

Plasmids and Reagents. 17β-Estradiol (E2), 4-hydroxytamoxifen (OHT), dexamethasone (Dex), sodium butyrate (SB), cycloheximide, anisomycin, puromycin, and actinomycin D (Act-D) were purchased from Sigma Diagnostics (Oakville, ON, Canada), ICI182,780 (faslodex) was purchased from Tocris Cookson Inc. (Ellisville, MO), and trichostatin A was procured from Wako Pure Chemicals (Osaka, Japan). pSG5-hERα and pSG5-TIF2.1 were kind gifts from Prof. Pierre Chambon (Institut de Génétique et de Biologie Moleculaire et Cellulaire, Illkirch, France). pCDNA3.1-ERα and pCDNA3.1-ERα(K302A/K303A) were constructed as follows. cDNAs for the wt ERα cDNA and the ERα(K302A/K303A) mutant were released from pSG5-hERα and pCI-neo-ERα(K302A/K303A) (a kind gift from Dr. Richard G. Pestell, Georgetown University School of Medicine, Washington, DC), respectively, by EcoRI digest (MBI Fermentas, Burlington, ON, Canada), and ligated into the EcoRI site of pCDNA3.1 (Invitrogen Burlington, ON, Canada). Reporter vectors GRE5-TATA-CAT/EBV, ERE3-TATA-CAT/EBV, and ERE3-TATA-LUC have been described previously (Barsalou et al., 2002; Fernandes et al., 2003).

Cell Lines and Reporter Assays. MCF-7 breast carcinoma and endometrial carcinoma Ishikawa cells were maintained in α-minimal Eagle's medium (Wisent, St-Bruno, QC, Canada) supplemented with 10 and 5% fetal bovine serum, respectively (Sigma Diagnostics) supplemented with 1% penicillin/streptomycin (Wisent). Stable reporter cell lines Ishikawa-GRE5/EBV and Ishikawa-ERE3/EBV (Barsalou et al., 2002) were maintained in the same medium as the parental cells supplemented with 50 μg/ml hygromycin B.

Three days before experiments, Ishikawa cells were switched to phenol red-free Dulbecco's modified Eagle's medium containing 5% charcoal-stripped serum, 1% sodium pyruvate (Wisent), 1% penicillin/streptomycin, and 1% L-glutamine (Wisent). For CAT assays, cells were stimulated 24 h after seeding with 25 nM E2 or 25 nM Dex and either vehicle (ethanol), trichostatin A, or sodium butyrate (variable concentrations) for another 24 h. Whole cell extracts were prepared in 0.25 M Tris-HCl, pH 7.5, by three cycles of freeze-thawing and were standardized for protein amount. CAT assays were performed as described previously (Barsalou et al., 2002). Each assay included triplicates for each condition and was repeated at least three times. A typical experiment is shown.

For luciferase assays, Ishikawa cells were transfected with the calcium-phosphate method (Barsalou et al., 2002) in six-well plates (2×10^6 cells/well). Typically, a DNA mix contained 150 ng of expression vector, 350 ng of ERE3-TATA-Luc reporter vector, and 2 μg of pBlueScript as carrier; after 24 h, cells were washed with fresh medium and stimulated for another 24 h with 25 nM E2 and/or 300 nM trichostatin A or vehicle (ethanol). Cells were washed two times with 1× PBS and harvested in lysis buffer (100 mM Tris-HCl, pH 7.9, 0.5% Nonidet P-40, and 1 mM dithiothreitol). Luciferase activity

was measured in the presence of luciferin with a Fusion universal microplate analyser (PerkinElmer Life and Analytical Sciences, Woodbridge, ON, Canada). Each transfection was carried out in triplicate and repeated at least three times. Proteins were quantified by BioRad reagent (Bio-Rad, Mississauga, ON, Canada).

Alkaline Phosphatase Assays. Alkaline phosphatase assays were conducted as described previously (Barsalou et al., 2002). Treatments were performed in triplicates for 24 h, after which cells were washed in PBS twice, frozen at −80°C for 15 min, and incubated with 50 μl of reaction buffer (5 mM *p*-nitrophenyl phosphate, 0.24 mM $MgCl_2$, and 1 M diethanolamine, pH 9.8). Plates were incubated at room temperature until production of a yellow color, and levels of *p*-nitrophenyl were quantified by measuring absorption at 410 nm.

RNA Extraction and RT-PCR Assays. Ishikawa cells were seeded in six-well plates (2.5×10^5 cells/well) and treated with 300 nM trichostatin A or 5 mM sodium butyrate, with or without 25 nM E2 or 25 nM Dex for different times (as indicated in the figure legends). For treatments with 2 μg/ml actinomycin D, 10 μg/ml cycloheximide, 5 μM anisomycin, or 5 μM puromycin, incubation was initiated 1 h before HDACi addition and continued for 6 h thereafter. The medium was then removed, and total RNA was extracted in 1 ml of TRIzol reagent (Invitrogen) and quantified by UV absorption. RNAs (2 μg) were reverse transcribed using the RevertAid H first minus strand cDNA synthesis kit (MBI Fermentas) as recommended by the manufacturer. Sequences of oligonucleotides used for polymerase chain reaction amplification are available upon request. Primers used for alternative ERα 5′ exons were designed according to published GenBank references (Kos et al., 2001). Polymerase chain reaction was performed using TAQ polymerase (MBI Fermentas). Amplified cDNA fragments were resolved on 2% agarose gels and stained with ethidium bromide. Each assay was reproduced at least three times. A typical experiment is shown.

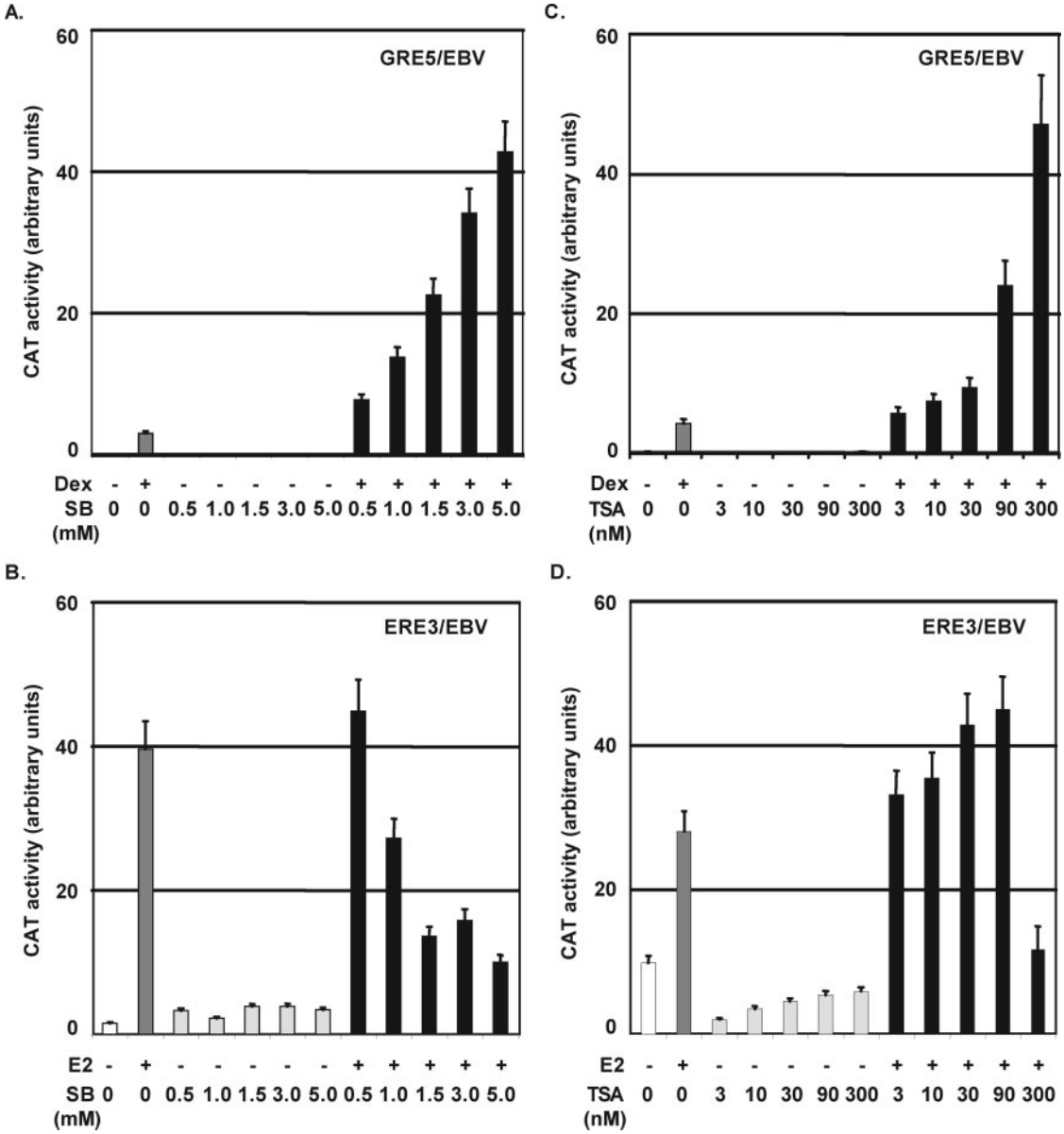

Fig. 1. HDAC inhibitors trichostatin A and sodium butyrate have opposite effects on GR and ER transcriptional activity with episomal reporter vectors containing minimal steroid-responsive promoters. Ishikawa-GRE5/EBV (A and C) or Ishikawa-ERE3/EBV (B and D) cells, which propagate the GRE_5-TATA-CAT/EBV or ERE_3-TATA-CAT/EBV episomal reporter vectors, respectively, were treated for 24 h with or without 25 nM Dex (A and C) or 25 nM E2 (B and D) and either SB (A and B) or TSA (C and D) at the indicated concentrations. CAT activity was assayed in whole cell extracts and normalized on protein concentration.

Western Analysis. Ishikawa cells were treated with 25 nM E2, 25 nM Dex, 100 nM OHT, 100 nM ICI182,780, or vehicle for 24 h with or without HDACi (300 nM trichostatin A or 5 mM sodium butyrate. Cells were harvested in ice-cold PBS, and whole cell extracts were prepared by three freeze-thaw cycles in high salt buffer (25 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, pH 8.0, 400 mM NaCl, 10% glycerol, 1 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors). After electrophoresis on an SDS-polyacrylamide gel (7.5% acrylamide), proteins were transferred onto polyvinylidene difluoride membranes (Hybond P; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Blots were incubated with anti-ERα mouse monoclonal or anti-TIF2 mouse monoclonal antibodies (B10 and 3Ti-3F1, respectively; both kind gifts from Prof. P. Chambon), anti-GR rabbit polyclonal antibody (PA1–511; ABR Affinity BioReagents, Golden, CO), anti-acetylated-H3, anti-acetylated-H4 (Upstate Biotechnology, Lake Placid, NY), or anti-β-actin mouse monoclonal antibody (Sigma Diagnostics). Immunodetection was performed using enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences, Boston, MA) as recommended by the manufacturer. Each result was reproduced at least three times. A typical experiment is shown.

Chromatin Immunoprecipitation Assays. Ishikawa cells were treated with 1.5% formaldehyde for 10 min at room temperature and fragmented by sonication as reported previously (Bourdeau et al., 2004), yielding fragments of approximately 350 base pairs, average size. Antibodies against acetylated H3 and acetylated H4 were purchased from Upstate Biotechnology. The sequences of the primers used in chromatin immunoprecipitation assays are available upon request. Chromatin immunoprecipitation experiments were performed twice with similar results. A representative set of results is shown.

Results

To investigate the effect of HDACi on steroid receptor-mediated transcription, we used stably transfected Ishikawa cell lines carrying Epstein Barr virus episomal reporter vectors sensitive to either glucocorticoids or estrogens. The reporter vectors contain a CAT reporter gene under control of minimal promoters composed of a TATA box placed downstream of either five glucocorticoid response elements (GRE5-TATA-CAT/EBV; Mader and White, 1993) or three estrogen response elements (ERE3-TATA-CAT/EBV; Barsalou et al., 2002). The use of minimal promoters and the absence of integration into the host cell chromosomes enable monitoring the effects of HDACi on transcriptional activation by GR or ER without confounding cooperative effects of transcription factors. Surprisingly, in contrast to the reported repressive effects of HDACi on glucocorticoid stimulation of the MMTV promoter (Mulholland et al., 2003; Kinyamu and Archer, 2004; and references therein), a marked and dose-dependent increase in the GRE5-TATA reporter activity was observed with increasing concentrations of the HDACi SB (0.5–5 mM) in the presence of 25 nM Dex but not in its absence, in the Ishikawa-GRE5/EBV cell line. The maximal stimulation by sodium butyrate, obtained at the highest concentration tested, was ~10-fold (Fig. 1A).

The effects of sodium butyrate on reporter expression from Ishikawa-ERE3/EBV cells were opposite to those observed from GRE5/EBV in that a dose-dependent decrease in reporter activity was noted in the presence of 25 nM E2, reaching more than 4-fold repression at 5 mM (Fig. 1B). The differential effects observed here with the two reporter cell lines suggest that sodium butyrate has a differential functional impact on estrogen and glucocorticoid signaling pathways rather than a general effect on global transcription, or on the stability of the CAT enzyme.

To verify that the effects of sodium butyrate on both signaling pathways are related to its HDAC inhibitory properties, we incubated the two reporter cell populations with trichostatin A, a structurally unrelated HDACi. Similar to results described above, glucocorticoid-stimulated reporter gene expression was markedly enhanced by increasing concentrations of trichostatin A (Fig. 1C), and estrogen-induced expression was repressed at the highest dose assayed, 300 nM (Fig. 1D). Note that the apparent increase in E2-regulated expression at lower trichostatin A concentrations was not statistically significant. The comparable actions of sodium butyrate and trichostatin A on estrogen- and glucocorticoid-driven reporter gene expression suggest that they are acting through a common mechanism (i.e., the inhibition of one or several of the HDACs expressed in Ishikawa cells) (HDACs 1–10; Fig. 2A).

To confirm that HDACi treatment increases global histone acetylation in the two episomal cell populations, we performed Western analysis of Ishikawa cell nuclear extracts using antibodies specific for acetylated H3 and H4 (Fig. 2B). Marked increases in acetylation were observed in both cell populations at 1 h after treatment using 300 nM trichostatin A (Fig. 2B). Furthermore, experiments using extracts from cells harvested at different times after trichostatin A treatment indicate that elevated histone acetylation was detectable for at least 16 h after incubation with 300 nM trichostatin A, but it was much more transient after treatment with a 30 nM dose (Fig. 2C). The high levels of trichostatin A necessary to obtain maximum alteration of dexamethasone- and estradiol-mediated expression at 24 h (Fig. 1) suggest that prolonged exposure to trichostatin A is necessary to induce the observed changes in gene expression.

Time-course experiments of treatment with dexamethasone or estradiol indicate that increases in levels of the CAT

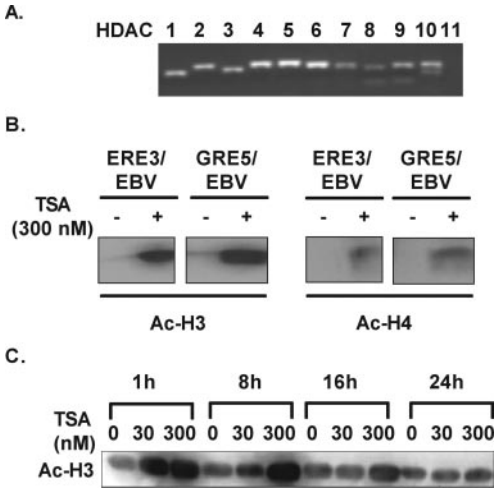

Fig. 2. Expression of HDACs and dose- and time-dependent effects of trichostatin A on histone acetylation in Ishikawa cells. A, expression of HDAC mRNAs in Ishikawa cells was monitored by RT-PCR from total RNAs using primers specific for each HDAC. B, TSA treatment (300 nM; 1 h) increases global acetylation levels of histone H3 and H4 in Ishikawa-ERE3/EBV and Ishikawa-GRE5/EBV cells. Levels of acetyl-H3 (Ac-H3) and Acetyl-H4 (Ac-H4) were detected by Western analysis as described under *Materials and Methods*. C, hyperacetylation of histone H3 by 30 or 300 nM TSA is reversible in a time- and concentration-dependent manner.

enzyme were gradual, being detectable at 6 to 8 h and rising through 24 h (Fig. 3, A and B). Trichostatin A (300 nM) had little effect at 8 h on reporter gene expression, whereas its effects became pronounced at 24 h. Trichostatin A only affected minimally dexamethasone- or estradiol-dependent expression if added during the last 8 h of a 24-h exposure to either hormone (Fig. 3, C and D). Finally, addition of an 18-h pretreatment with trichostatin A before treatment with dexamethasone and trichostatin A boosted the stimulatory effect of trichostatin A on GR-dependent expression (from 20- to 30-fold; Fig. 3E) and its repressive effect on ER-dependent expression (from 2.5- to 10-fold; Fig. 3F). Together, these results indicate that the effects of trichostatin A on steroid-induced expression from our minimal promoters require higher concentrations and are much slower than its effects on global histone acetylation levels.

To verify that HDACi have global effects on ER- and GR-mediated pathways as suggested by experiments using minimal reporter vectors, we examined the effect of HDACi on expression of endogenous estrogen and glucocorticoid target genes. The ALPPL2 alkaline phosphatase gene is strongly induced by estrogen at the transcriptional level in Ishikawa cells, and to a lesser extent by the partial antiestrogen OHT, but not by the full antiestrogen ICI182,780 (Fig. 4A). Alkaline phosphatase activity is also markedly induced by estrogen at 24 h (Fig. 4B), whereas induction by 4-hydroxytamox-

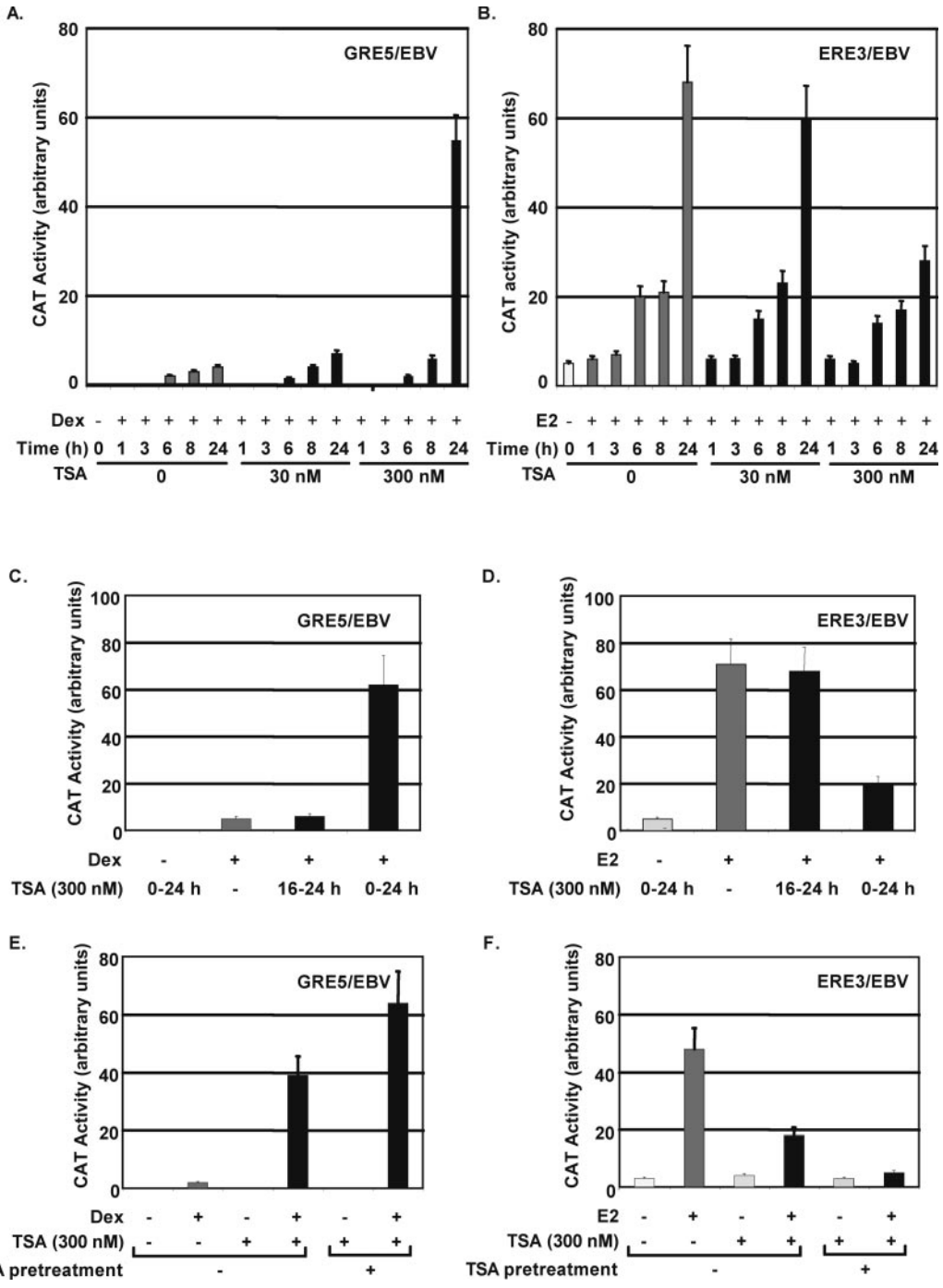

Fig. 3. Long-term coincubation with trichostatin A is necessary for effects on GR- and ER-dependent expression in Ishikawa reporter cell lines. Ishikawa-GRE5/EBV (A, C, and E) or Ishikawa-ERE3/EBV (B, D, and F) cells were treated for different times with or without 25 nM Dex (A, C, and E) or 25 nM E2 (B, D, and F) and TSA at the indicated concentrations and times. A time course of cotreatment with hormones and trichostatin A indicates that a long-term incubation (24 h) is required for marked effects (A and B). Addition of trichostatin A during the last 8 h of hormone treatment (16 to 24 h after hormone addition) does not lead to significant effects (C and D). In contrast, pretreatment with trichostatin A for 18 h (+) before hormone addition further increased the magnitude of these effects (E and F).

ifen is detectable only at later times. Treatment with trichostatin A had a slight stimulatory effect on basal levels of ALPPL2 activity, an effect that was independent of ER function because it was not repressed by treatment with the full antiestrogen ICI182,780. However, the stimulatory effects of estrogen on transcription of ALPPL2 (Fig. 4A, arrows) and on alkaline phosphatase activity (Fig. 4B) were both lost upon HDACi treatment. The weak stimulation of alkaline phosphatase activity by 4-hydroxytamoxifen in the presence of trichostatin A, although consistently observed in three experiments, was not statistically significant in a Student's *t* test analysis.

The human tyrosine aminotransferase (TAT) gene is a strongly induced glucocorticoid target gene in fetal liver (Nagao et al., 1987). Dexamethasone treatment was found to stimulate the expression of TAT transcripts in Ishikawa cells (Fig. 4C). Trichostatin A treatment alone did not affect TAT expression, but cotreatment with dexamethasone and trichostatin A markedly augmented the effect of dexamethasone alone. Thus, the effects of HDACi on expression of endogenous estrogen and glucocorticoid target genes in parental Ishikawa cells were similar to those observed with our reporter cell lines, supporting the notion that components essential to ER and GR signaling are regulated by HDACi, with opposite effects on the activities of these two pathways.

HDACi could potentially affect the ER signaling pathway at several levels. Because the delayed kinetics of HDACi effects on ER-dependent transcription are compatible with modulation of receptor expression, we assessed mRNA levels of ERα and ERβ in Ishikawa cells treated with sodium butyrate or trichostatin A. Although no significant effects were observed on ERβ expression (data not shown), ERα expression was strongly repressed by 5 mM sodium butyrate and by 300 nM trichostatin A at 16 h, irrespective of ligand treatment (Fig. 5A). At 24 h, receptor levels were returned to near-untreated levels in the presence of trichostatin A but not of sodium butyrate (Fig. 5A), consistent with the stronger repression of estrogen reporter gene expression observed with sodium butyrate (Fig. 1). Sodium butyrate also repressed ERα protein levels to a greater extent than trichostatin A over a 24-h treatment period (Fig. 5B).

If inhibition of ERα expression by HDACi is the main basis for their repressive effects on ER target genes, then expression of exogenous ERα should reverse this repression. Indeed, although estradiol-induced expression from a transfected ERE-TATA-Luc reporter vector was repressed by trichostatin A in Ishikawa cells, cotransfection of the pCDNA3.1-ERα expression vector led to a marked synergism between trichostatin A and estradiol for reporter gene expression (Fig. 5C). This synergism was due in part to a stimulatory effect of trichostatin A on ERα expression from the pCDNA3.1 vector (Fig. 5D) and was colinear with the concentration of exogenous expression vector cotransfected (data not shown). Finally, similar results were obtained when an expression vector for ERα (K302A/K303A) was cotransfected instead of the vector expressing wild-type ERα. K302 and K303 are tandem lysine residues that are acetylated by p300 (Wang et al., 2001). This suggests that acetylation of the receptor does not play a major role in the effects of HDACi under our experimental conditions (Fig. 5, C and D). Effects of HDACi on exogenous ERα expression are probably due to a stimulation of the CMV promoter of the expression vector, as expression from a CMV-βGal reporter vector was also markedly stimulated (data not shown).

Expression of ERα is driven from several promoters that function in a tissue-specific manner (Kos et al., 2001). In Ishikawa cells, we detected ERα transcripts expressed from promoters A, B, and C (Fig. 6A). Expression from promoter F was detectable only in MCF-7 cells (Fig. 6A). In Ishikawa cells, levels of transcripts originating from promoters A, B, and C were reduced by trichostatin A or sodium butyrate, whereas expression of GAPDH was not affected (Fig. 6B). In MCF7 cells, trichostatin A also reduced levels of transcripts originating from promoters A, B, F, and to a lower extent C, whereas expression of GAPDH was not affected (Fig. 6C). Western blot analysis confirmed that both the 66-kDa form of ERα, originating from promoters A, B, and C, and the 46-kDa form originating from promoter F were less abundant in MCF7 after treatment with trichostatin A (Fig. 6D).

Trichostatin A could exert its effects through regulation of transcript stability by a mechanism involving regulatory sequences common to all repressed RNA isoforms. Therefore, we assessed whether repression by HDACi would be observed in the presence of the transcriptional inhibitor Act-D. Although basal ERα transcript levels were repressed by actinomycin D treatment at 6 h, as expected, no further repression by sodium butyrate was observed (Fig. 6E). This suggests that HDACi repress transcription from the ERα promoters rather than mRNA stability. We then investigated

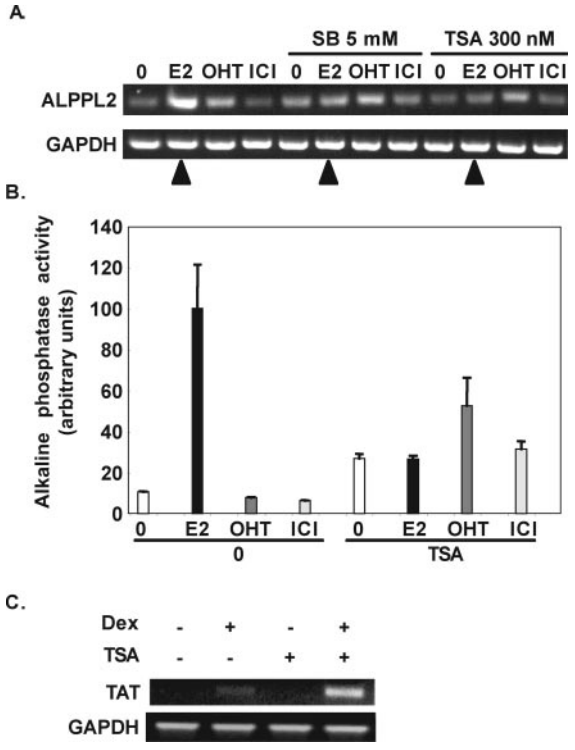

Fig. 4. HDACi repress estradiol-stimulated expression of ALPPL2 and stimulate induction of the TAT gene by dexamethasone. A and B, Ishikawa cells were treated with 25 nM E2 or with antiestrogens OHT (100 nM) or ICI182,780 (ICI; 100 nM) in the absence or presence of 5 mM SB or of 300 nM TSA for 24 h. mRNA levels of the ALPPL2 gene were assessed by RT-PCR (A), and alkaline phosphatase activity was assayed by *p*-nitrophenyl hydrolysis (B). C, Ishikawa cells were treated with 25 nM Dex or 300 nM trichostatin A or both for 24 h. mRNA levels of the human TAT gene and of the control housekeeping GAPDH gene were monitored by RT-PCR.

the levels of acetylated histones H3 and H4 on ERα promoters A, B, and C in Ishikawa cells in the presence or absence of HDACi. Treatment with trichostatin A for 6 h led to a reduction in the levels of acetylated H3 or H4 associated with these promoters (Fig. 6F), despite the large increase in overall acetylated histone levels in the cell at this time (Fig. 2C). These results suggest that these promoters are in a transcriptionally less active state in the presence of HDACi. Finally, we investigated whether the transcriptional repression of ERα by HDACi is independent of protein synthesis. The repressive effects of trichostatin A on promoters A, B, and C were maintained, although attenuated, in the presence of protein synthesis inhibitors cycloheximide (10 μg/ml), anisomycin (5 μM), or puromycin (5 μM), indicating that de novo protein synthesis was not required for at least part of the repressive effect (Fig. 6G). Likewise, repression by sodium butyrate was also still observed in the presence of cycloheximide (data not shown).

We then examined whether sodium butyrate or trichostatin A increased endogenous GR mRNA levels in Ishikawa cells, which would provide a mechanism for the observed stimulation of GR-dependent expression. Trichostatin A did not alter GR mRNA expression at 8 or 24 h (Fig. 7A) or GR protein levels at 1, 8, or 24 h (Fig. 7B). Treatment with sodium butyrate also did not change GR mRNA or protein levels at 24 h (data not shown). To test whether the effects of HDACi on glucocorticoid signaling can be mimicked by increased HAT activity, we transiently transfected a truncated form of the p160 coactivator TIF2/SRC2, TIF2.1, which contains the receptor interaction domain and activation domains (Voegel et al., 1998). TIF2.1 increased GR-dependent expression by 10-fold, but it attenuated the effects of HDAC inhibitors from ~10- to ~2-fold (Fig. 8). Thus, increased expression of TIF2.1, which can recruit HAT activities such as CBP/p300 and PCAF, has the same effect as global suppression of HDAC activity. This suggests that a substrate com-

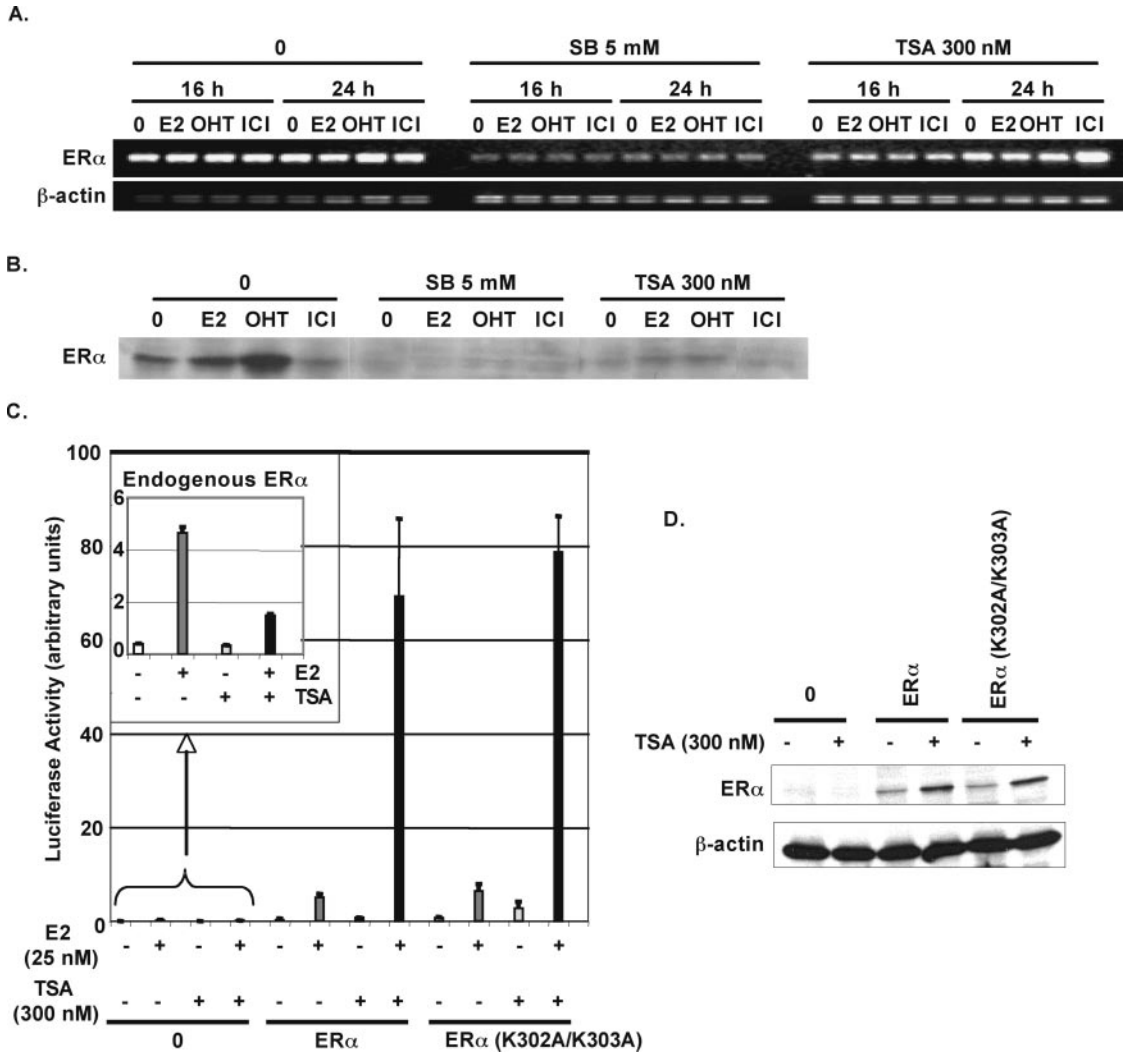

Fig. 5. Repressive effects of HDACi on estrogen signaling are due to repression of ERα expression in parental Ishikawa cells. A and B, ERα expression is inhibited by HDACi at the mRNA and protein levels. Ishikawa cells were treated with 25 nM E2, 100 nM 4-hydroxytamoxifen, or 100 nM ICI182,780 in the absence or presence of 5 mM SB or of 300 nM trichostatin A for 16 or 24 h. mRNA levels of the human ERα and of the control β-actin gene were monitored by RT-PCR. Primers for ERα were chosen in the coding region common to all transcripts. C and D, reexpression of ERα by transient transfection prevents the repressive effects of trichostatin A independently from acetylation of the receptor. Ishikawa cells were transiently transfected with an ERE3-TATA-Luc reporter vector with expression vectors for wt ERα or for the ERα(K302A/K303A) mutant affected in the acetylation sites or with the parental pCDNA3.1 expression vector. Cells were treated with 25 nM E2 and/or 300 nM TSA as indicated for 24 h (C). Western analysis of ERα expression levels was performed in parallel and indicates that trichostatin A increases expression directed by the CMV promoter in the pCDNA3.1 vector (D).

mon to the type I/II HDACs expressed in Ishikawa cells and to the HAT activities in the p160-CBP/p300-PCAF complex stimulates GR signaling in these cells in an acetylation-dependent manner.

Discussion

In this study, we have used minimal reporter vectors to assess the overall effects of HDACi on two steroid receptor genomic pathways. Estrogen and glucocorticoid receptors are closely related and share similar functional properties, but they have distinct DNA binding specificities. Synthetic promoters composed of their respective binding sites inserted upstream of a TATA box thus allow easy monitoring of the activity of the corresponding signaling pathways, with minimal influence from other transcription factors. Our reporter vectors are propagated as episomes, which are stably maintained at moderate copy number in the form of chromatin, circumventing variations in promoter activity caused by different sites of chromosomal integration (Mader and White, 1993). Because both receptors recruit coactivators with HAT activity to remodel chromatin at target promoters, it might be expected that HDACi treatment would enhance both ER- and GR-mediated transcription. Acetylation of steroid receptors themselves has also been shown to occur in a dynamic

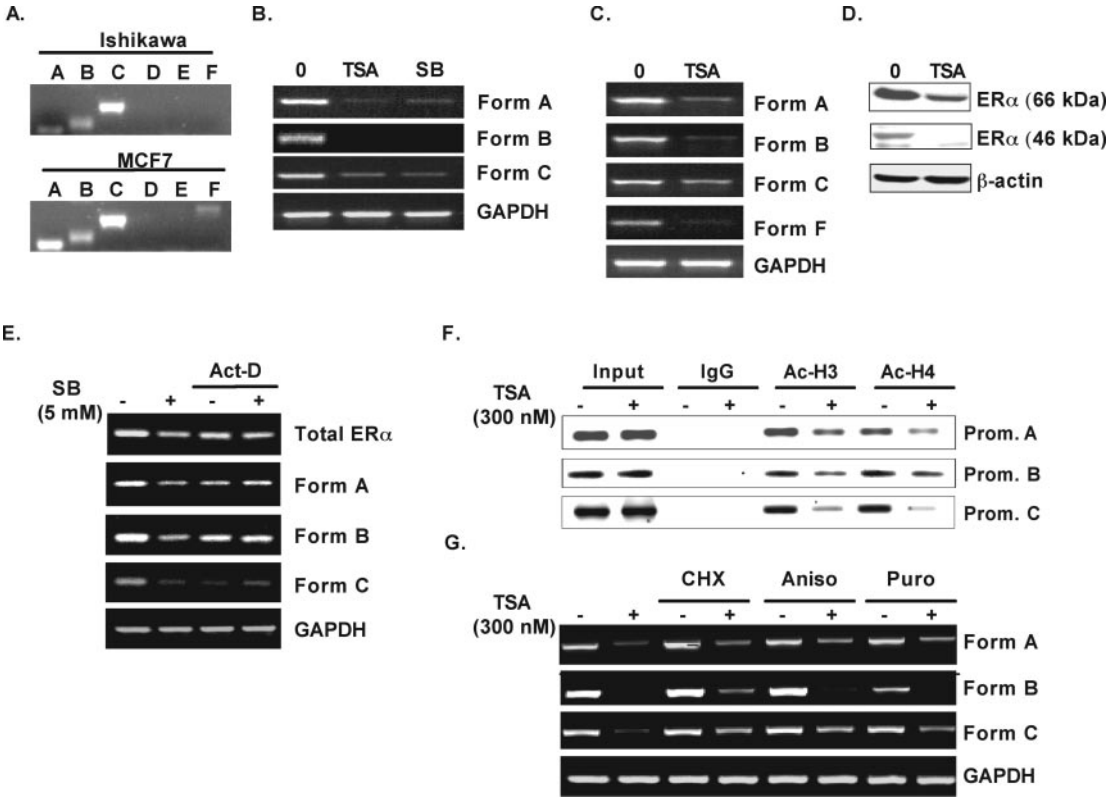

Fig. 6. HDACi decrease ERα transcription from promoters A, B, and C in Ishikawa cells in the absence of de novo translation. A, promoters A, B, and C drive expression of ERα in Ishikawa cells, as demonstrated by detection of the corresponding transcripts with alternative 5′ exons. Note that promoter A and promoter F are more active in MCF-7 cells. B, treatment with 300 nM TSA or 5 mM SB for 6 h represses expression from all active promoters (A, B, and C) in Ishikawa cells, whereas GAPDH expression is not affected. C, treatment with 300 nM TSA for 6 h represses expression from all active promoters (A, B, C, and F) in MCF7 cells. D, expression of both the 66- and 46-kDa isoforms of ERα is inhibited by TSA treatment (300 nM; 6 h) in MCF7 cells. E, Act-D treatment (2 μg/ml) prevents repression of ERα expression by sodium butyrate (5 mM; 6 h). F, chromatin immunoprecipitation experiments indicate that treatment of Ishikawa cells with TSA (300 nM; 6 h) leads to hypoacetylation of histones H3 and H4 on promoters A, B, and C. G, treatment with translation inhibitors cycloheximide (CHX; 10 μg/ml), puromycin (Puro; 5 μM), and anisomycin (Aniso; 5 μM) does not prevent repression of ERα transcription by 300 nM trichostatin A (6 h).

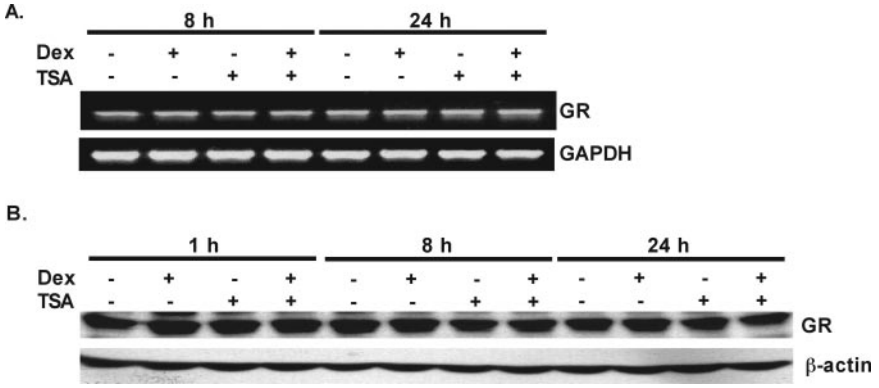

Fig. 7. HDACi do not increase glucocorticoid receptor expression in Ishikawa cells. A, treatment of Ishikawa cells with 5 mM SB or 300 nM TSA does not lead to increases in GR mRNA in the absence or presence of 25 nM Dex at 8 or 24 h. Expression of the GAPDH mRNA is shown as a control. B, GR protein levels, detected by Western analysis using the polyclonal rabbit PA1–511 antibody, were not up-regulated at 1, 8, or 24 h. Expression of β-actin is shown as a control.

manner and may impact their transcriptional activation properties (Fu et al., 2004).

Remarkably, our results indicate that HDACi had opposite effects on estrogen and glucocorticoid genomic signaling in Ishikawa cells. Effects on endogenous target genes were similar to those obtained with our reporter vectors. Dose-dependent stimulation of glucocorticoid signaling by HDACi was unexpected because studies of integrated or episomal MMTV reporter vectors in different cell lines have reported repressive effects of HDACi on glucocorticoid-mediated transcription at the concentrations used in this study. Also unexpected was the requirement for high doses of HDACi and long incubation periods to observe these effects. Both factors are in fact intricately linked, because our observations indicate that trichostatin A has a relatively transient effect on histone acetylation in Ishikawa cells, which can be prolonged by use of higher doses of this inhibitor. These requirements suggest that the observed effects of HDACi may involve long-term effects on components of the receptor signaling pathways rather than immediate modulation of target promoter histone acetylation.

Modulatory effects of HDAC inhibitors on ER expression have been described in the literature, although with variable end results. Although ERα expression was found to be repressed in breast cancer cells in several studies (Alao et al., 2004; Margueron et al., 2004a; Reid et al., 2005), induction of ERα expression has also been reported in breast cancer cells by HDACi (Keen et al., 2003; Yang et al., 2001) and in Ishikawa cells by the HDACi valproate (Graziani et al., 2003). Induction of ERβ by trichostatin A was also observed in MDA-MB-231 cells (Jang et al., 2004). We did not observe significant effects on ERβ expression in Ishikawa cells, but we detected a strong reduction in ERα transcription. It is unclear whether the difference between these repressive effects of trichostatin A or butyrate and the previously reported induction of ERα by valproate in Ishikawa cells results from use of different HDACi or from different isolates of the Ishikawa cell line. Note, however, that valproate stimulated growth of Ishikawa cells (Graziani et al., 2003), whereas sodium butyrate and trichostatin A inhibited proliferation under our experimental conditions (data not shown). Further analysis confirmed that reduction in ERα mRNA levels requires transcription; i.e., mRNA destabilization by HDACi is not involved. Several alternative promoters control ERα expression in Ishikawa and in MCF7 cells. The various transcript isoforms encode the same 66-kDa protein, except for transcripts originating from promoter F. In MCF7 cells, 10% of these transcripts give rise through alternative splicing to a truncated 46-kDa form (Kos et al., 2001). Interestingly, repression of transcripts originating from all active promoters was observed both in Ishikawa and in MCF7 cell types. Note that promoter F is located ~115-kilobase upstream of promoter C, indicating either long-range or multiple sites of transcriptional shut-off. It is unlikely that induced expression of a repressor is involved, because the effects of HDACi were also observed in the presence of three different protein synthesis inhibitors. Our results differ in this respect from those of Reid et al. (2005), who reported that the repressive effects of valproate or trichostatin A on ERα expression in MCF7 cells are abolished by cycloheximide, but are compatible with the lack of effect of cycloheximide on repression of ERα expression observed with trichostatin A by Alao et al. (2004). Potential mechanisms may be activation of a transcriptional repressor or loss/repression of a transcriptional activator by acetylation, both being compatible with the observed decrease in histone acetylation on the repressed promoters. Of note, Reid et al. (2005) reported recruitment of the methyl binding protein MeCP2 on the ERα A promoter in the presence of valproate, suggesting induction of promoter methylation by this HDACi, an event often associated with decreased histone acetylation.

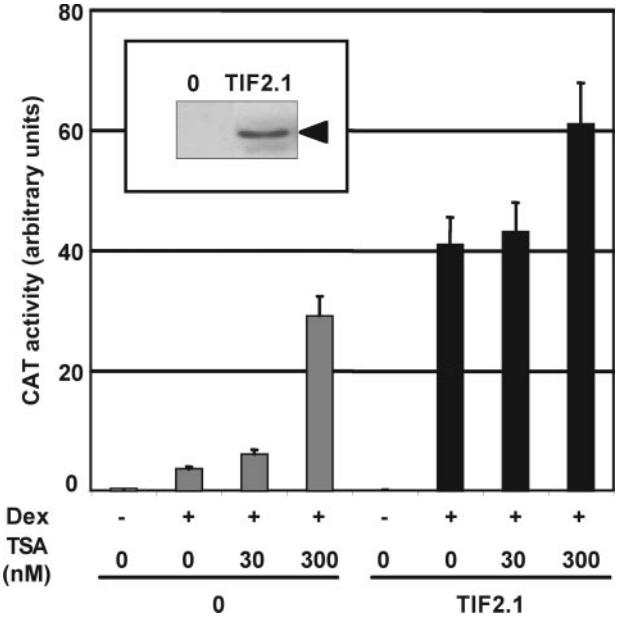

Fig. 8. Overexpression of the p160 coactivator derivative TIF2.1 attenuates the trichostatin A stimulation of GR-dependent transcription. A, Ishikawa cells were transiently transfected with 2 μg of GRE_5-TATA-CAT/EBV and 2 μg of pSG5-TIF2.1 by the calcium phosphate method. After 18 h, cells were treated with 25 nM Dex in the presence of 30 or 300 nM TSA for 24 h. Expression of the transfected TIF2.1 is confirmed by Western blot analysis using the mouse monoclonal antibody 3Ti-3F1 (inset).

The strong dose-dependent stimulatory effects of HDACi on GRE5-TATA-CAT and endogenous TAT gene expression differ markedly from previously reported results demonstrating down-regulation of the stimulatory effect of glucocorticoids on the MMTV promoter in various cell types (Mulholland et al., 2003; Kinyamu and Archer, 2004) or on the TAT gene in rat hepatoma cells (Plesko et al., 1983). Our results, in contrast, are compatible with earlier observations that sodium butyrate enhances dexamethasone responsiveness of the alkaline phosphatase gene in HeLa S3 cells (Littlefield and Cidlowski, 1984). Although the long time course of induction of glucocorticoid reporter vectors in Ishikawa cells may suggest indirect effects mediated by the altered expression of a component of the glucocorticoid signaling pathway, our assays for GR mRNA and protein levels are not consistent with an induction in GR expression. Interestingly, transient expression of the p160 coactivator derivative TIF2.1, which is highly expressed and contains all domains of TIF2 required for coactivation of nuclear receptors (Voegel et al., 1998), mimicked the effect of HDACi. TIF2, like other p160 members, is a component of HAT complexes containing cofactors CBP/p300 and PCAF (Rosenfeld and Glass, 2001). Although overexpression of a HAT coactivator is thus a plausible hypothesis, no increases in the mRNA levels of the HAT

coactivators of steroid receptors were detected by RT-PCR in the presence of HDACi (data not shown). It remains possible that expression of a HAT coactivator may be affected at the post-transcriptional level or alternatively that HAT/HDAC activities may affect the expression of a common substrate that plays an important role in glucocorticoid signaling.

We have also considered two other potential mechanisms by which HDACi could synergize with glucocorticoids for GR-mediated transcription. Decreased expression/activity of an enzyme involved in degradation of glucocorticoids might in theory explain the observed effects of HDACi on increased GR activity, but this is unlikely to be the case in our experimental system because dose-response curves of dexamethasone stimulation at 24 h did not reveal a shift in the exogenous hormone concentrations required for the response (data not shown). In addition, RT-PCR amplification of the 11β-HSD type II enzyme, which is responsible for limiting the antiproliferating activity of glucocorticoids in breast cancer cells (Lipka et al., 2004) did not reveal differences in expression in the absence or presence of HDACi (data not shown). Another potential mechanism may be effects of HDACi on the cell cycle, because most HDACi induce a block at the G_1/S transition in different cell lines. The GR has been reported to have differential transcription activity in G_1 and S phases (permissive) and in G_2/M phases (nonpermissive) (Hsu and DeFranco, 1995; King and Cidlowski, 1998). Long-term (3 day) effects of sodium butyrate on GR activation of the alkaline phosphatase gene in HeLa S3 cells were attributed to synchronization of the cells in the permissive G_1 phase (Littlefield and Cidlowski, 1984). Note, however, that a recent study reported that treatment with 300 nM trichostatin A for 3 days is accompanied by a decrease in the proportion of cells in both the G_0/G_1 and S phases and an increase in cells in G_2/M (Takai et al., 2004). Thus, effects on the cell cycle seem unlikely to explain the synergism observed in Ishikawa cells between glucocorticoids and HDACi at the level of GR transcription.

Although additional experiments will be needed to further pinpoint the exact mechanisms of action of HDACi in Ishikawa cells, including an assessment of whether distinct subsets of the HDAC expressed in Ishikawa cells are involved in the effects of HDAC on estrogen or glucocorticoid signaling, it is of interest that signaling pathways involving different nuclear receptors can be modulated differentially by HDACi, whose use in cancer treatment seems promising. Inhibition of ERα expression would be of benefit in the treatment of ERα positive breast tumors if it entails repression of growth-stimulatory ERα target genes, although the reversible character of this inhibition may require repeated administration of high doses of HDAC. In addition, glucocorticoid receptors have been reported to have growth inhibitory properties in several hematological and solid tumor cells, including in Ishikawa cells (King and Cidlowski, 1998). It will be of interest in the future to assess whether HDACi also have a stimulatory effect on the glucocorticoid target genes that mediate these antiproliferative activities.

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Address correspondence to: Dr. Sylvie Mader, Département de Biochimie, Université de Montréal, CP 6128 Succursale Centre-Ville, Montréal, Québec H3C 3J7, Canada. E-mail: sylvie.mader@umontreal.ca