HDAC inhibitors prevent the induction of the immediate-early gene FOSL1, but do not alter the nucleosome response

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ABSTRACT

Dynamic histone acetylation, catalyzed by lysine acetyltransferases and HDACs, is critical to IEG expression. Expression of IEGs, such as FOSL1, is induced by several signal transduction pathways resulting in activation of the protein kinase MSK and phosphorylation of histone H3 at serine 10 of nucleosomes (the nucleosome response) at the upstream promoter and regulatory region of target genes. HDAC inhibitors prevent FOSL1 gene induction and the association of HDAC1, 2 and 3 with the gene body. However, HDAC inhibitors did not prevent the nucleosome response. Thus HDAC inhibitors perturb events downstream of the nucleosome response required for FOSL1 transcription initiation.

1. Introduction

The nucleosomal response plays a major role in the expression of immediate early genes (IEGs), such as Fos, Jun, Fosl1 and Ptgs2 [1,2]. The nucleosomal response results from the stimulation of the RAS-mitogen activated protein kinase (MAPK) or stress activated pathways and the activation of ERK or p38, respectively. ERK and p38 phosphorylate and activate the mitogen- and stress-activated protein kinases (MSK) 1 and 2. Once activated, MSK is recruited to regulatory regions of IEGs to phosphorylate histone H3 at S10 or S28. The phosphorylated H3 recruits proteins 14-3-3 ε or ζ. The events that follow result in the remodeling of nucleosomes at the regulatory region, recruitment of transcription factors and the initiation of transcription [1,3].

Important in transcriptional induction of the IEGs is dynamic histone acetylation [4,5]. Lysine acetyltransferases (KAT) and histone deacetylases (HDAC) recruited to the regulatory regions of IEGs catalyze dynamic protein acetylation of histones and other proteins. These events contribute to the remodeling of nucleosomes located at the regulatory regions. HDAC1 and 2 in association with several other proteins are present in corepressor complexes Sin3A, NuRD and CoREST. HDAC3 is in corepressor complexes, SMRT and NCoR, with other HDACs such as HDAC4. The HDAC corepressor complexes are recruited to regulatory regions by transcription factors and other DNA-binding proteins. Dynamically acetylated histones are also associated with the coding regions of transcribed genes [1,6,7] and have a role in splicing of pre-mRNA [8,9]. In yeast, there is evidence that recruitment of HDACs to the gene body is mediated by RNA polymerase II and elongation [10,11]. Whether a similar mechanism is in play in recruiting HDACs to the coding region of IEGs in mammalian cells is currently not known.

Inhibition of HDAC activity impacts the expression of less than 10% of genes, with these genes either being up- or down-regulated. Further, there is emerging evidence that inhibition of HDACs impacts the splicing of many genes [8,9,12]. Among the genes that are up-regulated by HDAC inhibitors is CDKN1A (also known as p21, WAF1, CIP1). However, for induction to take place the mitogen- and stress-activated protein kinase (MSK)-induced nucleosomal response must be active [13].

In contrast to CDKN1A, the mitogen- or stress-induced expression of several IEGs is attenuated by the treatment of cells with HDAC inhibitors before the addition of the mitogen or stress agent [4]. Whether the nucleosomal response was compromised for these IEGs by HDAC inhibitors is currently not known.
In this study we determined which HDACs are recruited to the regulatory and coding regions of the induced IEG FOSL1 and the dependence of HDAC recruitment on transcription. Further we determined whether HDAC inhibitors altered the nucleosomal response required for the induced expression of the FOSL1 gene.

We demonstrate that HDAC1, 2 and 3 are recruited to the regulatory and coding regions of the induced FOSL1 gene, with on-going transcription being required for recruitment of HDAC1, 2 and 3 to the gene body. HDAC inhibitors did not impact the nucleosomal response, but disconnected the nucleosomal response from the events required for the initiation of transcription.

2. Materials and methods

2.1. Cell culture and treatments

The human colon carcinoma cell line, HCT116 was cultured at 37 °C in a humidified atmosphere containing 5% CO₂ in McCoy’s 5A medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B. To induce the RAS-MAPK signaling pathway, 80–90% confluent cells were serum starved for 48 h and then treated with a phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) (100 nM, Sigma) for various time periods (0, 30, or 60 min). When mentioned, serum starved HCT116 cells were treated with HDAC inhibitor, trichostatin A (TSA, 250 nM, Sigma) or apicidin (150 nM, Sigma) for 30 min prior to TPA treatment. In transcriptional inhibition studies, serum starved HCT116 cells were treated with TPA (60 min) or pre-treated with DRB (5,6-dichlorobenzimidazole riboside, Sigma) (25 µg/ml) or actinomycin D (Sigma) (20 µg/ml) for 1 hr followed by TPA treatment for 60 min.

2.2. RNA isolation and real time RT-PCR

Total RNA was isolated from either serum starved HCT116 cells treated with TPA (0, 30, or 60 min) alone or cells pretreated with TSA or apicidin for 30 min followed by TPA treatment (0, 30, or...
60 min), using RNeasy Mini Kit (QIAGEN) according to manufacturer’s instructions. Total RNA (400 ng) was used for cDNA synthesis using M-MLV reverse transcriptase (Invitrogen). Real-time PCR reactions were performed on iCycler IQ5 (BioRad).

2.3. Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) experiments were done as previously described [1], with an additional protein–protein cross-linking step. Cells were incubated with 1.0 mM DSP (dithiobis[succinimidylpropionate]) (Thermo Fisher Scientific) for 30 min at room temperature according to manufacturer’s instruction, prior to formaldehyde cross-linking. Dual cross-linked chromatin was processed to mononucleosomes and chromatin immunoprecipitations were done with antibodies against HDAC1 (Affinity Bioreagents), HDAC2 (Affinity Bioreagents), HDAC3 (Abcam), acetyl histone-H3 (Millipore), acetyl histone-H4 (Millipore), RNA polymerase II (RNAPII) (Millipore), RNAPIIS2ph (Abcam), H3S10ph (Santa Cruz Biotechnology), 14-3-3ε (Santa Cruz Biotechnology) and 14-3-3ζ (Santa Cruz Biotechnology). Equal amounts of input and ChIP DNA (1.0 ng) were used for real time PCR on iCycler IQ5 (BioRad). The fold enrichment was calculated as previously described [1]. The following primers were used:

- **FOSL1-promoter-F:** 5’-GTGCTATTTTGTGGGAGCAG-3’
- **FOSL1-promoter-R:** 5’-TGGTGTTAACTTCCTCGCCG-3’
- **FOSL1-Ex1-F:** 5’-GCATTCCGCCGAGACTCCGGG-3’
- **FOSL1-Ex1-R:** 5’-TGCTGCTACTCTTGCGATGA-3’
- **FOSL1-Ex4-F:** 5’-CACACACCCTCCCTAACTCTTT-3’
- **FOSL1-Ex4-R:** 5’-TGCCTGACTCTTGCCGATGA-3’

2.4. Preparation of cellular extract, histone isolation and immunoblotting

Cellular extracts were prepared in IP buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1.0 mM EDTA, 0.5% NP-40) containing phosphatase and protease inhibitors (Roche). Total cellular protein extracts (20 µg) were resolved by 10% SDS–PAGE and immunoblotting was performed with anti-ERK1/2 (Invitrogen) and anti-phospho-p44/42 MAPK (ERK1/2) Thr202/Tyr204 (Cell Signaling Technology), anti-p38 MAPK (Cell Signaling Technology), and anti phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology) antibodies. Acid extraction of histones was done as described previously [14] and were resolved (5.0 µg of histones) by 15% SDS–PAGE and stained immunocytochemically with antibodies against anti-H3S10ph (Santa Cruz Biotechnology), anti-H3S28ph (Abcam), and anti-H3 (Millipore).
3. Results

3.1. Transcription dependent recruitment of class I HDACs to FOSL1

We have previously reported that TPA stimulation of the RAS-MAPK pathway in mouse fibroblasts results in the induced expression of the Fosl1 and Ptgs2 genes [1]. TPA treatment of serum starved human colorectal carcinoma HCT116 cells also resulted in the increased expression of the FOSL1 and PTGS2 genes (Fig. 1A and Fig. S1A). Histone acetylation turnover is important for activation of inducible genes [5]. To determine the loading of class I HDACs along the FOSL1 gene, we applied a dual cross-linking (DSP followed by formaldehyde) chromatin immunoprecipitation (ChIP) assay [15]. In response to the TPA-induction of the FOSL1 gene, there was an accumulation of class I HDACs (HDAC1, 2 and 3) at the upstream promoter region and gene body (exons 1 and 4), increasing in parallel with the increased expression of the FOSL1 gene (Fig. 1B and C).

The requirement of on-going transcription in the recruitment of HDACs to the upstream promoter region and FOSL1 gene body was determined. Serum starved HCT116 cells were incubated for 60 min with or without the transcription inhibitors, DRB or actinomycin D, followed by an incubation with TPA for 60 min to induce FOSL1 gene expression. The results of the dual cross-linking ChIP assays show that both transcription inhibitors prevented the recruitment of class I HDAC to the FOSL1 upstream promoter region and gene body (Fig. 2). These results suggest that TPA-induced transcription of the FOSL1 is required to recruit class I HDACs to the gene.

3.2. HDAC inhibitors attenuate the recruitment of HDACs to the FOSL1 gene body

Previous reports have shown that the HDAC inhibitor, TSA (a pan HDAC inhibitor) attenuated the TPA-induced expression of IEGs in mouse fibroblast [4]. Incubation of TSA or apicidin (a class I HDAC inhibitor) 30 min before the addition of TPA to serum starved HCT116 cells, prevented the induction of the FOSL1 and PTGS2 genes (Fig. 3 and Fig. S1B and C). However, TSA or apicidin alone did not induce these genes (Fig. S2). Dual cross-linking ChIP assays were applied to determine whether the HDAC inhibitors were affecting the recruitment of RNA polymerase II (RNAPII) to the FOSL1 promoter. TPA induction of the FOSL1 gene resulted in the accumulation of RNAPII at the promoter at 30 min, followed by a decrease of RNAPII at 60 min (Fig. 4A). The initiation-competent form of RNAPII (RNAPII S5ph) also increased at 30 min followed by a decline at 60 min. TSA or apicidin incubation of HCT116 cells before TPA addition prevented the accumulation of RNAPII at the FOSL1 promoter and the initiation of transcription as indicated by the lack of elevated levels of RNAPII S5ph following TPA induction (Fig. 4B and C).

The effect of the HDAC inhibitor on the loading of class I HDACs on the FOSL1 gene was evaluated by dual cross-linking ChIP assays. Fig. 5 shows that neither TSA nor apicidin treatment prevented the recruitment of HDAC1, 2 and 3 to the gene body, but had minimal impact on the accumulation of class I HDACs at the FOSL1 upstream promoter region.

To analyze the changes in histone acetylation levels at the upstream promoter region of FOSL1, dual cross-linking ChIP assays were performed with antibodies against acetylated H3 (H3ac9/14) and acetylated H4 (H4ac5/8/12/16) in TPA-induced cells pretreated with or without TSA or apicidin. A temporal increase in histone H3 and H4 acetylation was observed in promoter region of FOSL1 gene, with a greater level of H3 and H4 acetylation being attained in the HDAC inhibitor treated cells (Fig. 6). These observations provide evidence that the increased KAT activity at the FOSL1 upstream promoter region following TPA induction is not prevented by inhibiting the activity of the HDACs residing at the upstream promoter regions.
resulting in the phosphorylation of histone H3 (the nucleosome response) at the regulatory regions of IEGs [4]. As the nucleosome response is required for the induction of CDKN1A gene, we determined whether HDAC inhibitors affected the nucleosome response pathway. As ERK activation is a key step in the pathway, we determined the levels of phosphorylated ERK in TPA and TSA/TPA or apicidin/TPA treated serum starved HCT116 cells. Fig. 7A shows that neither TSA nor apicidin affected the phosphorylation and activation of ERK. MSK can be activated by p38 MAP kinase pathway as well [2]; we therefore analyzed the activation and phosphorylation of p38 kinase in our experimental settings. As a control, we treated HCT116 cells with UV to activate p38 kinase. Fig. 7B shows that UV treatment resulted in the phosphorylation of p38. In contrast, separately or in combination TPA and the HDAC inhibitors (TSA or apicidin) did not activate the p38 kinase pathway. Next we determined the phosphorylation of H3S10 along the regulatory and coding region of FOSL1 gene by ChIP assay and whether this phosphorylation event was altered by HDAC inhibitors. Fig. 8A shows that TPA-induced H3 S10 phosphorylation occurred at the FOSL1 upstream promoter region but not within the gene body (exons 1 and 4). The TPA-induced H3 S10 phosphorylation and localization to the upstream promoter region was not prevented by the HDAC inhibitors TSA and apicidin. TSA induced phosphorylation of H3S28, but not of H3S10, has been reported in mouse epidermal JB6 cells [16]. In serum starved HCT116 cells, neither of the HDAC inhibitors induced phosphorylation of H3S10 or H3S28. Further neither of the HDAC inhibitors prevented TPA induction of H3S10 and S28 phosphorylation (Fig. 8B).

Following phosphorylation of H3, a critical event in the induction of IEGs is the recruitment of 14-3-3e and 14-3-3f [17]. We investigated whether HDAC inhibitors prevented the recruitment of the 14-3-3 proteins to the upstream promoter region of the FOSL1 gene following TPA induction. Following TPA induction, the 14-3-3 proteins were recruited to the upstream promoter, but not to the coding region of the FOSL1 gene (Fig. 8C). Neither TSA nor apicidin prevented the recruitment of the 14-3-3 proteins to the FOSL1 upstream promoter region, indicating that the phospho mark (H3S10ph) was effectively ‘read’ by 14-3-3 proteins in cells treated with HDAC inhibitors. Together these results show that the HDAC inhibitors do not interfere with the nucleosome response pathway.

4. Discussion

Dynamic histone acetylation plays a critical role in the expression of IEGs [5]. Further dynamic acetylation occurs independently of MSK-catalyzed H3 phosphorylation [18].
Fig. 6. Effect of TSA or apicidin on acetylation of histone H3 and H4 at the FOSL1 upstream promoter region. HCT116 cells were serum-starved and then incubated with or without HDAC inhibitors, TSA (250 nM) (A) or apicidin (150 nM) (B) for 30 min before being stimulated with TPA (100 nM) for 0, 30 or 60 min. Cells were double cross-linked, processed to mononucleosomes for ChIP assays with acetylated Histone-H3 and H4 antibodies and analyzed by real time PCR at the upstream promoter region of FOSL1 gene. Enrichment values are relative to time 0 values and are the average of three independent experiments. The error bars represent standard deviation.

acetylation is catalyzed by KATs (CBP/p300/PCAF) and TSA-sensitive HDACs [5]. TPA-induction of the IEG genes results in an increased acetylation of histones at the upstream promoter regions of these genes [1,5]. For nucleosomes positioned at the FOSL1 upstream promoter region, HDAC inhibitors (TSA and apicidin which are structurally unrelated) did not prevent but enhanced the TPA-induced histone acetylation. However, the HDAC inhibitors prevented initiation of FOSL1 transcription induced by TPA. Previous reports have shown that HDAC inhibitors alter gene expression, ranging from 10% to 40% of genes within the genome, with the responsive genes being either up- or down-regulated [19–21]. Thus FOSL1 is with the group of genes which are down-regulated by HDAC inhibitors.

In this report, we demonstrate the transcription dependent recruitment of HDAC1, 2 and 3 to the gene body of the FOSL1 gene in HCT116 cells. Throughout the study, we applied a dual cross-linking ChIP assay. We previously reported that the use of DSP before formaldehyde was more efficient in monitoring HDACs along genes than using formaldehyde alone or other cross-linkers (such as EGS/formaldehyde) [15,22]. We have also observed the transcription dependent recruitment of HDAC1 and 2 to the induced (TPA or estradiol) trefoil factor 1 gene body in human breast cancer cell line MCF7 (data not shown). Thus the transcription dependent recruitment of class I HDACs in mammalian cells to the coding region of transcribed genes appears to be by a similar mechanism as that reported in yeast [10,23]. Although the recruitment of HDACs to the FOSL1 upstream promoter region and gene body are dependent on transcription, the mechanisms by which these enzymes are recruited to these two gene regions are different and can be distinguished with HDAC inhibitors. The mechanism of HDAC recruitment to the gene body is thought to be via RNAPII which is required to transfer HDAC to the coding region of the gene. As the HDAC inhibitors prevent the initiation of transcription, RNAPII is not present to do this task. For the upstream promoter region, the nucleosome response pathway results in nucleosome remodeling of the FOSL1 upstream promoter, allowing the recruitment of transcription factors which in turn recruit HDACs which are in multiprotein complexes such as Sin3A. HDAC inhibitors do not inhibit these events and thus HDAC recruitment to the upstream promoter region is not affected by the HDAC inhibitors.

The activation of MSK and the nucleosome response is required for the HDAC inhibitor-induced expression of CDKN1A [13]. For several IEGs, HDAC inhibitors attenuate the TPA-induced expression of IEGs [4]. Our results show that the HDAC inhibitor, TSA or apicidin did not impact the signal transduction pathways resulting in the activation and phosphorylation of p38 and ERK kinases or the MSK catalyzed nucleosome response events, including H3S10 phosphorylation and 14-3-3 recruitment to the FOSL1 upstream promoter region. The HDAC inhibitors prevented subsequent events leading to the productive initiation of FOSL1 transcription. Consistent with our results, others have shown that HDAC inhibitors can attenuate transcriptional initiation by abrogating the binding of RNAPII and basal transcription factors to promoter regions [24,25]. These results demonstrate that inhibition of class I HDACs and dynamic protein acetylation can either promote or hinder transcription and likely depends on promoter/upstream promoter region context.

Fig. 7. Effect of HDAC inhibitors on TPA induced activation of MAP kinase pathways. Serum starved HCT116 cells were pretreated or not with 250 nM TSA (left panel) or with 150 nM apicidin (right panel) for 30 min followed by TPA treatment (100 nM) for indicated time points. Total cellular extracts (20 μg) were analyzed for the induction of phospho-ERK (A) and phospho-p38 (B) by phospho-ERK1/2 and phospho-p38 antibodies. Total ERK1/2 and p38 were used as loading controls and UV treated cellular lysates were analyzed as a positive control for phospho-p38.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.03.029.

References


Fig. 8. TSA or apicidin does not alter the TPA-induced nucleosomal response or the recruitment of chromatin modifiers to the regulatory region of FOSL1 gene. (A) HCT116 cells were cultured in serum-depleted conditions and were treated or not with 250 nM TSA (left panel) or with 150 nM apicidin (right panel) for 30 min prior to 100 nM of TPA stimulation for 0, 30 or 60 min. Cells were dual cross-linked, processed to mononucleosomes and ChIP assays were performed with anti-H3S10ph antibody and analyzed by real time PCR at the promoter and coding region of FOSL1 gene. Enrichment values are relative to time 0 values and are the average of three independent experiments. The error bars represent standard deviation. (B) Serum starved HCT116 cells were treated or not with TSA (250 nM) (left panel) or with apicidin (150 nM) (right panel) for 30 min before the stimulation with TPA (100 nM) for 0, 30 or 60 min. Acid-soluble nuclear histones (5 μg) were resolved on a 15%-SDS–PAGE and immunoblotting were done with anti-H3S10ph, anti-H3S28ph and anti-total H3 (loading control) antibodies. (C) ChIP assays were done with or without TSA (left panel) or apicidin (right panel) pretreatment followed by TPA induced conditions with14-3-3 ε and 14-3-3 ζ antibodies as described in A.


