Biochemical and Biophysical Research Communications 404 (2011) 268–272

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Histone deacetylation directs DNA methylation in *survivin* gene silencing

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ARTICLE INFO

Article history:
Received 29 September 2010
Available online 2 December 2010

Keywords:
Gene silencing
DNA methylation
Histone acetylation/deacetylation
Bisulfite sequencing
ChIP assay

ABSTRACT

DNA methylation and histone acetylation are major epigenetic modifications in gene silencing. In our previous research, we found that the methylated oligonucleotide (SurKex) complementary to a region of promoter of *survivin* could induce DNA methylation in a site-specific manner leading to *survivin* silencing. Here, we further studied the role of histone acetylation in *survivin* silencing and the relationship between histone acetylation and DNA methylation.

First we observed the levels of histone H4 and H4K16 acetylation that were decreased after SurKex treatment by using the chromatin immunoprecipitation (ChIP) assay. Next, we investigated the roles of histone acetylation and DNA methylation in *survivin* silencing after blockade of histone deacetylation with Trichostatin A (TSA). We assessed *survivin* mRNA expression by RT-PCR, measured *survivin* promoter methylation by bisulfite sequencing and examined the level of histone acetylation by the ChIP assay. The results showed that histone deacetylation blocked by TSA reversed the effects of SurKex on inhibiting the expression of *survivin* mRNA, inducing a site-specific methylation on *survivin* promoter and decreasing the level of histone acetylation. Finally, we examined the role of histone acetylation in the expression of DNA methyltransferase 1 (DNMT1) mRNA. The results showed that histone deacetylation blocked by TSA reversed the increasing effect of histone deacetylation on the expression of *survivin* mRNA. This study suggests that histone deacetylation guides SurKex-induced DNA methylation in *survivin* silencing possibly through increasing the expression of DNMT1 mRNA.

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1. Introduction

Epigenetic information includes direct modifications on DNA or chromatin histones. DNA methylation and histone acetylation are two main reversible epigenetic modifications that may affect the structure and function of chromatin [1]. DNA methylation normally occurs on cytosine at CpG dinucleotides across the human genome and is associated with gene silencing. For example, DNA methylation is involved in X chromosome inactivation in females, DNA imprinting, and events involved in monoallelic gene expression. It also contributes to genome stability by preventing translocations of repetitive and transposable sequences, and likely plays a dynamic role in development [2,3].

Histone hyperacetylation is generally associated with chromatin decondensation, which increases the accessibility of DNA to binding proteins as well as transcriptional activity, whereas histone hypoacetylation contributes to chromatin condensation and transcriptional repression [4,5]. The N-terminal tails of the core histones are essential for condensation of chromatin and are involved in the genesis of chromatin structural states. Of the histone tails, the histone H4 tails have a prominent role in compacting the 30 nm fiber. An *in vitro* study demonstrated that K16 acetylation of H4 prevented the formation of the 30 nm chromatin fiber [6,7]. Acetylation of histone H4K16 and loss or reduction of linker histones result in a decondensed chromatin fiber [8].

It is well established that both DNA methylation and histone modifications are strongly implicated in the process of gene silencing. However, there is no consensus on which epigenetic mechanism initiates and steers this communication. There is a heated debate about which event would be a dominant event in gene silencing. Some experiments supported that DNA methylation guide histone modifications [9–12], however, other studies show contradictory evidence. Ou et al. [13] demonstrated that increase in histone acetylation by Trichostatin A (TSA) was associated with a significant decrease in global methylation. Januchowski et al. [14] confirmed that histone deacetylation affected DNA methylation by down regulation of DNA methyltransferase 1 (DNMT1) in Jurkat T cells. Smallwood et al. [15] considered histone methylation as initiated epigenetic modifications in *survivin* silencing. They found that the methylation of histone H3K9 created a binding platform for HP1, allowing DNMT1 to interact with HP1, resulting in increased DNA methylation on DNA and chromatin templates. Wu et al. [16] found that blockade of histone deacetylation by a depsipeptide actively silenced genes such as p16, SALL3 and GATA4 in different human cancer cell lines through decreasing methylation of both CpG and H3K9 on their promoters. These experiments

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doi:10.1016/j.bbrc.2010.11.105

support the theory that histone modifications guide DNA methylation.

SurKex is a methylated oligonucleotide complementary to a region of human *survivin* promoter (sequence from 2766 to 2787, genbank accession no. NC_000017), designed according to the method from Yao et al. [17]. In our previous study, we demonstrated that SurKex inhibited not only *survivin* expression *in vitro*, but also the growth of NSCLC tumors in nude mice as well. Moreover we confirmed that SurKex led to site- and sequence-specific methylation of the *survivin* promoter by 50% at 5 μM [18]. In this study, we attempted to reveal the relationship between DNA methylation and histone acetylation in SurKex-induced *survivin* by using the histone deacetylase inhibitor TSA.

2. Materials and methods

2.1. Materials

SurKex sequences were designed as follows: A^{m}CGGGTC CCG^{m}CG ATTCAAATCT, which is complementary to a region of the human *survivin* promoter (sequence from 2766 to 2787, genbank accession no. NC_000017). Random is a control cognate of the SurKex oligonucleotide, consisting of a methylated 22-mer oligonucleotide with a random sequence (ATGCT^{m}CGGAACCTTT^{m}CG CAGGA), which is not related to *survivin*, but it contains the same number of ^{m5}C residues as SurKex. TSA (Trichostatin A), a histone deacetylase inhibitor, was purchased from Sigma–Aldrich Co. Ltd., USA. Antibodies against acetylated histone H3, H4, and H4 at lysine16 were purchased from Upstate Biotechnology, USA. Human non-small cell lung cancer (NSCLC) cell line, NCI-H460, was purchased from Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences, Shanghai, China.

2.2. Cell culture

NCI-H460 cells were maintained in RPMI 1640 medium (Gibco, USA) supplemented with 10% FBS, at 37 °C in a humidified 5% CO_2 incubator. On the day before transfection, cells were seeded at a density of 1×10^5 per well in a 24-well culture plate or in 100×20 mm cell dishes.

2.3. RT-PCR

Total RNA was extracted and purified using Trizol (Invitrogen, USA). M-MLV (RNase H$^-$) (TaKaRa, Japan) was used for reverse transcription reaction. The primers used were: *survivin* sense primer: 5′-ctaca ttcaa gaact ggcct-3′ and anti-sense primer: 5′-aagaa gacga agttc ctcga c-3′; *DNMT1* sense primer: 5′-ctt ctt cag cac aac cgt ca-3′ and anti-sense primer: 5′-gaaga gccgg taggt gtcag-3′; *β-actin* sense primer: 5′-gggaa ttcaa aactg gaacg gtgaa gg-3′ and anti-sense primer: 5′-acacc ggctc ctgaa actat tcgaa gg-3′, at an annealing temperature of 60 °C. A triplicate PCR was performed for each sample and the expression of *β-actin* was as an internal control.

2.4. Measurement of DNA methylation by sodium bisulfite sequencing

Genomic DNA was extracted and methylation of *survivin* promoter region was examined by bisulfite-sequencing method according to the protocol described by Frommer et al. [29].

2.5. Chromatin immunoprecipitation assay (ChIP)

The ChIP assays for histone acetylation were performed as described by EZ ChIP™ Chromatin Immunoprecipitation Kit (Upstate Biotechnology, USA) according to the protocol of manufacture. Individual ChIP assays were repeated at least twice to confirm the reproducibility of the PCR based experiment. PCR amplification was performed using either immunoprecipitated DNA, a control without antibody, or an input that had not been immunoprecipitated. To evaluate the level of histone acetylation in each immunoprecipitation, the ratio was determined by quantifying the intensities of the PCR product in immunoprecipitated DNA versus 1% input DNA (total chromatin) amplified by PCR in a linear range.

3. Results

3.1. Effects of SurKex on histone acetylation

Histone acetylation is tightly related to gene expression. Histone hyperacetylation is generally associated with chromatin decondensation, allowing DNA to be accessible to binding proteins and thus increased transcriptional activity, whereas histone hypoacetylation contributes to chromatin condensation and transcriptional repression. In order to determine the changes of histone acetylation of the *survivin* promoter after SurKex treatment, the ChIP assay was used. Compared with lipofectamin 2000 control and Random control, the acetylation levels of H4 and H4K16 were significantly ($P < 0.05$, by ANOVA followed by two-tailed Student's *t*-test) decreased where the level of H4 acetylation was decreased by 94.1% and 93.9% and the level of H4K16 acetylation was decreased by 38.9% and 30.3%, respectively, whereas the level of H3 acetylation was not affected by SurKex treatment (Fig. 1).

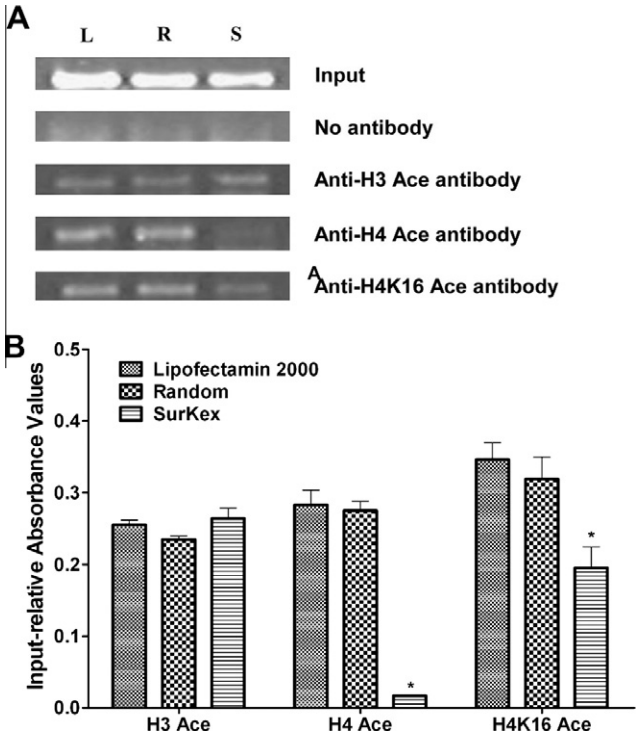

Fig. 1. Analysis of chromatin proteins associated with SurKex-silenced *survivin* gene using the ChIP assay. L, R and S denote transfection reagent Lipofectamin 2000 control, Random oligonucleotide control and SurKex, respectively. The ChIP assay was performed with anti-H3-acetylation, anti-H4-acetylation and anti-H4K16-acetylation antibodies. Input DNA fractions were amplified by PCR to adjust for chromatin loading as a positive control, and no antibody was performed as a negative control. A representative gel is shown in (A). One percent input-relative absorbance values are quantified in (B). Data are presented as means ± SEM, $n = 3$ in each treatment. ∗Denotes statistically significant difference from Lipofectamin 2000 control or Random control ($P < 0.05$ by ANOVA followed by post hoc two-tailed Student's *t*-test).

3.2. Blocking effect of TSA on SurKex-mediated histone deacetylation

Our previous experiment showed that *survivin* promoter was site-specifically methylated [18], and histone H4 and H4K16 of the *survivin* promoter were hypoacetylated after SurKex treatment (Fig. 1). In order to clarify whether DNA methylation guided histone acetylation or vice versa, the histone deacetylase inhibitor TSA was used to block histone deacetylation to examine roles of DNA methylation and histone acetylation in *survivin* silencing. *Survivin* mRNA expression was assessed by RT-PCR. The results showed that hyperacetylation by TSA reversed SurKex-induced inhibition of *survivin* mRNA expression (Fig. 2A). Methylation of the *survivin* promoter was also determined by bisulfite sequencing and the results showed that SurKex-induced site-specific methylation at No. 11 CpG was downregulated by TSA treatment (Fig. 2B), suggesting that hyperacetylation induces DNA demethylation. We further analyzed the acetylation of H4 and H4K16 after TSA treatment by the ChIP assay. The results showed that TSA treatment also improved SurKex-reduced levels of H4 and H4K16 acetylation (Fig. 2C). The above evidence suggests that histone deacetylation occurs prior to DNA methylation in *survivin* silencing, thus histone deacetylation may guide DNA methylation.

3.3. Effects of histone deacetylation on DNMT1 mRNA expression

It was reported that DNMT1 plays a key role in DNA methylation [19,20], and it combines with HDAC to form an enzyme complex through MeCP2 in gene silencing [21–23]. In order to determine whether histone deacetylation guides DNA methylation via affecting the expression of DNMT1, we assessed the level of DNMT1 mRNA expression under deacetylation (induced by SurKex) and acetylation (via blockade of histone deacetylation by TSA) conditions. As shown in Fig. 3, mRNA expression of DNMT1 measured by RT-PCR was significantly ($P < 0.05$, by ANOVA followed by two-tailed Student's *t*-test) decreased when histone deacetylation was blocked by TSA.

4. Discussion

Methylation of the cytosine in CpG dinucleotides has emerged as an important epigenetic modification that regulates gene transcription [19]. In general, promoter DNA methylation is correlated with gene repression. When a gene is hypermethylated, especially in its promoter region, transcription is usually diminished [20]. In view of this, oncogenes could be silenced by inducing methylation of their promoter regions.

Yao et al. [17] established a novel method, by which expression of a given gene in mammalian cells can be specifically blocked by synthesized methylated sense oligonucleotide that was complementary to the sequence of the P4 promoter region of human IGF2 gene. This oligonucleotide was shown to induce site-specific hypermethylation of the promoter region of IGF2 and thus inhibit IGF2 transcription. Ishii et al. [24] also found that the methylated oligonucleotides complementary to the GSTP1 promoter induced methylation of the GSTP1 promoter and suppressed expression of GSTP1 protein in A549 lung adenocarcinoma cell. From these reports, we synthesized a 22-mer anti-sense methylated oligonucleotide SurKex that was complementary to a region of the human *survivin* promoter (sequence from 2766 to 2787, genbank accession no. NC_000017). Our previous results showed that SurKex induced site-specific methylation of the *survivin* promoter and reduced expression of *survivin* mRNA and protein *in vitro*, as well as inhibited the growth of NSCLC tumors in nude mice [18].

Epigenetic modifications fall into two main categories: DNA methylation and histone modifications. The fact that SurKex-induced DNA methylation prompted us to investigate whether changes of histone acetylation were occurring during SurKex-induced *survivin* silencing. We first examined the level of histone

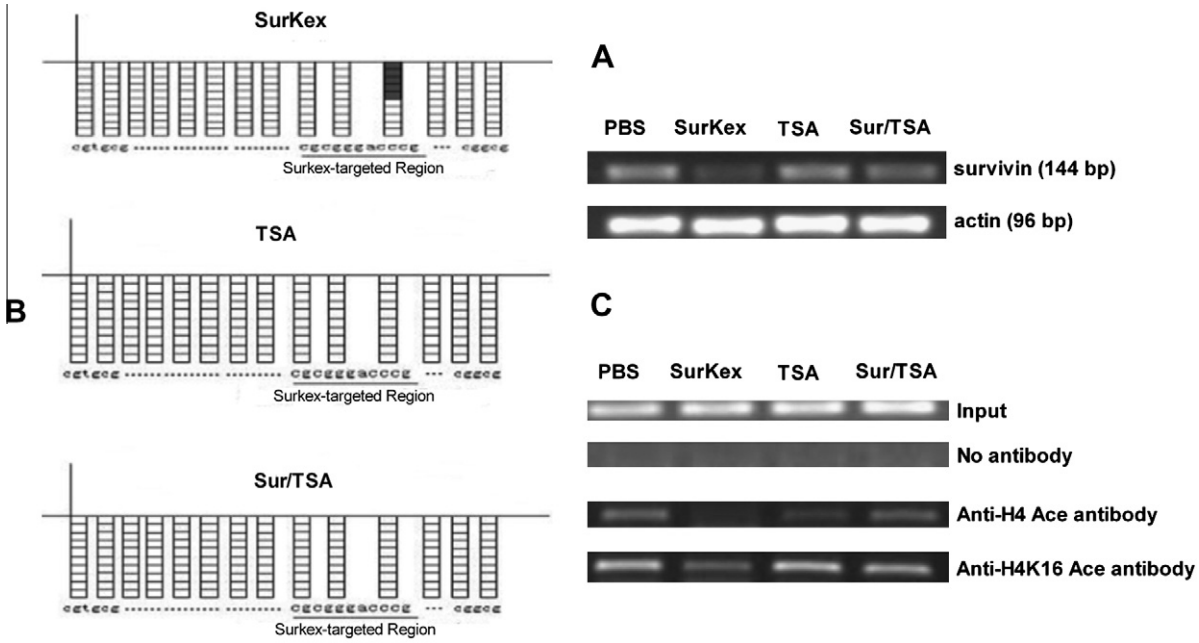

Fig. 2. Reversal by the histone deacetylase inhibitor Trichostatin A (TSA) of SurKex-induced decreased *survivin* mRNA expression (A), induced site-specific methylation of the *survivin* promoter (B) and decreased histone acetylation (C) in NCI-H460 cells derived from the human non-small cell lung cancer (NSCLC) cell line. NCI-H460 cells were treated with PBS, 5 μM SurKex, 100 nM TSA and 5 μM SurKex + 100 nM TSA. In the gene expression study (A), mRNA levels were assessed by RT-PCR in cells collected 60 h following treatment. β-actin mRNA expression was used as the internal control. In the *survivin* promoter methylation study (B), the efficiency of DNA methylation was determined by bisulfite sequencing. The region of methylated cytosines (No. 11 CpG) is indicated by grey squares. The efficiency of methylated cytosines (out of 10) was induced (50%) by SurKex. TSA not only failed to induce methylation, but also inhibited SurKex-induced methylation. In the histone acetylation study (C), the ChIP assay was performed with anti-H4-acetylation and anti-H4K16-acetylation antibodies. Input DNA fractions were amplified by PCR to adjust for chromatin loading as a positive control, and no antibody treatment was performed as a negative control. A representative gel is shown but similar results were observed in three replicate experiments.

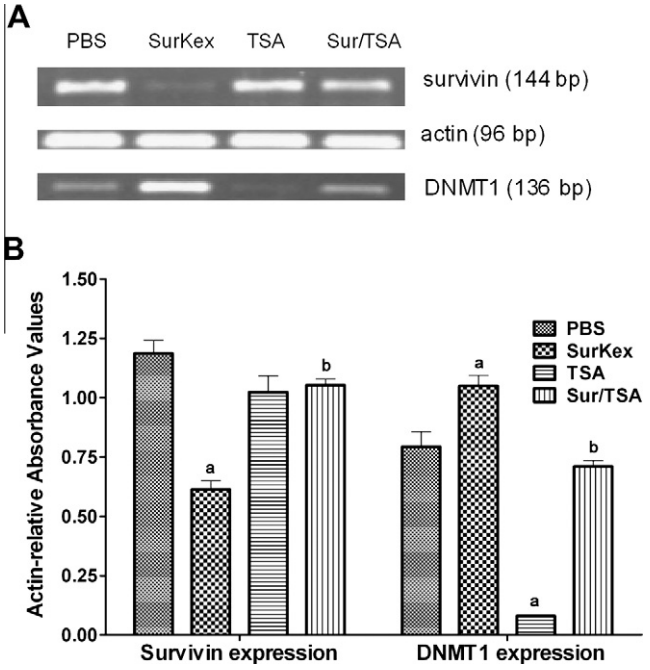

Fig. 3. Effects of SurKex-induced deacetylation and Trichostatin A (TSA)-induced acetylation (via blockade of deacetylation) on DNMT1 mRNA expression. DNMT1 mRNA expression in the human non-small cell lung cancer (NSCLC) cell line, NCI-H460, treated with 5.0 μM of SurKex and 100 nM of TSA, as assessed by RT-PCR. A representative gel is shown in (A). Actin-relative absorbance values are quantified in (B). Data are presented as means ± SEM, $n = 3$ in each treatment, [a]denotes statistically significant difference from PBS control ($P < 0.05$ by ANOVA followed by post hoc two-tailed Student's *t*-test); [b]denotes statistically significant difference from SurKex treatment or TSA treatment ($P < 0.05$ by ANOVA followed by post hoc two-tailed Student's *t*-test).

acetylation on H3, H4 and H4K16 after SurKex transfection by ChIP. Our results showed that the acetylation levels of H4 and H4-K16 but not H3 were significantly reduced after SurKex transfection. These results demonstrated that both *survivin* promoter methylation and H4 deacetylation were involved in *survivin* silencing.

The relationship of these two modifications in *survivin* silencing was further determined in the current study. We blocked histone deacetylation by using the histone deacetylase inhibitor TSA. The results showed that once histone deacetylation of H4 and H4K16 was blocked, both DNA methylation of the *survivin* promoter and inhibition of *survivin* mRNA expression induced by SurKex treatment were abolished accordingly, which revealed that histone deacetylation guided DNA methylation in *survivin* silencing.

The DNMT1 promoter of human cell line is also regulated by E2F/pRb/HDAC protein complex during cell replication [25]. HDAC could decrease histone hyperacetylation and increase activation of the DNMT1 promoter [25,26]. Januchowski [14] reported that TSA down-regulated DNMT1 in Jurkat T cells, suggesting a link between histone hyperacetylation and inactivation of the DNMT1 promoter. In our study, SurKex as a hemi-methylated oligonucleotide appears to be the optimal substrate of DNMT1 [27,28] and we proposed that histone deacetylation affects the DNA methylation via its influence on DNMT1. Indeed, we showed that SurKex-increased mRNA expression of DNMT1 was reduced after blockade of histone deacetylation by TSA, suggesting that histone deacetylation guiding DNA methylation may be via its increasing effect on DNMT1 expression.

Acknowledgments

This work was supported by Grants (03JC14049 and 034319209) from Shanghai Science and Technology Administration and a Grant (2009CB521900 PI: Dr. Ding-Feng Su) from National Basic Research "973" Program, Ministry of Science and Technology, China.

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