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Coordinated transcriptional regulation of calmegin, a testis-specific molecular chaperon, by histone deacetylase and CpG methyltransferase

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Abbreviations: ChIP assay, chromatin immunoprecipitation assay; HDAC, histone deacetylase; RT-PCR, reverse transcriptase-PCR; TSA, trichostatin A; 5'Aza-dC, 5-Aza-2'-deoxycytidine

Abstract

Calmegin is a testis-specific molecular chaperon playing a key role in spermatogenesis. However, the transcriptional regulatory mechanisms for calmegin expression are entirely unknown. Herein, we revealed that calmegin is transcriptionally regulated by histone deacetylase (HDAC) and CpG methyltransferase. The cDNA microarray analysis of the human fibrosarcoma cells treated with trichostatin A (TSA) showed an increased level of calmegin mRNA. The induction of calmegin mRNA by TSA was added by the treatment with 5-aza-2'-deoxycytidine (5'Aza-dC), implying that epigenetic alterations are involved in the transcriptional repression of the gene. Moreover, chromatin immunoprecipitation assay using an anti-acetyl-histone H3 antibody exhibited that the proximal region (−152 ~ −31) of the calmegin promoter is responsible for HDAC-mediated transcriptional repression of the gene. These results demonstrate that calmegin expression is regulated by HDAC and CpG methyltransferase in a coordinative way.

Keywords: calmegin; DNA methylation; histone deacetylase; microarray analysis; spermatogenesis

Introduction

Spermatogenesis is an essential process for mammalian reproduction and requires the precise and well-coordinated program that regulates the constantly changing patterns of gene expression (Eddy, 2002; Park *et al*., 2003; Wang *et al*., 2004). Among several key players of the process, calmegin is known as a testis-specific molecular chaperon and is crucial for binding between eggs and sperms for fertilization. Accordingly, the expression of calmegin is developmentally regulated during spermatogenesis (Ikawa *et al*., 2001). Indeed, Watanabe *et al*., (1994) have shown that the promoter region of calmegin bears high GC contents and this region may be controlled by DNA methylation (Watanabe *et al*., 1994). However, the detail transcriptional regulatory mechanisms for calmegin expression have not been understood.

As the promoter of calmegin contains high GC contents, the gene appears to be regulated by the epigenetic events such as acetylation and/or methylation of histones or DNA (Baylin *et al*.,1991; Jones *et al*., 1998). Consistent with this idea, calmegin is identified as one of target genes regulated by histone deacetylase (HDAC) from our large-scale analysis of gene expression changes in human fibrosarcoma HT1080 cells treated with trichostatin A (TSA), a well-known specific inhibitor of HDAC. The expression of calmegin was transcriptionally regulated by HDAC and the inhibition of HDAC activity resulted in over-expression of calmegin in HT1080 as well as in F9 cells. Moreover, the induction of calmegin mRNA by the inhibition of HDAC activity was added by the treatment with 5-aza-2'-deoxycytidine (5'Aza-dC), implying that epigenetic alterations are involved in the transcriptional repression of the gene. Such an epigenetic alteration by HDAC and CpG methylation causes several malignant cellular properties in a variety of transformed cell lines (Marks *et al*., 2000). However, only a small number of genes having tumor suppressor activities such as gelsolin, $p21^{WAF1}$, p53, and plakoglobin have been identified as the target genes in response to HDAC inhibitors and there has been no report on the possible involvement of calmegin as a target gene of HDAC (Sowa *et al*., 1997; Yu *et al*., 2002; Shim *et al*., 2004).

The present study demonstrates that calmegin

expression is regulated by HDAC and CpG methyltransferase in a coordinative way. The validation of calmegin as a target gene of HDAC and identification of the putative HDAC binding site in the promoter of the calmegin gene are reported.

Materials and Methods

cDNA microarray and data analysis

Human cancer-focused 1024 genes-arrayed DNA chips (GenomicTree, Inc., Taejon, Korea) were used for cDNA microarray analysis. Microarray hybridization was performed as described previously (Kim *et al*., 2002; 2003; Shim *et al.,* 2005). A fluorescent image of the microarray was obtained using a GenePix 4000B scanner (Axon Instruments, Foster, CA). Data obtained from the scanner were further analyzed using GeneSight data analysis software, version 3.5 (BioDiscovery, Inc., Los Angeles, CA).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

HT1080 (human fibrosarcoma) and F9 (mouse teratocarcinoma) cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and maintained in an humidified incubator adjusted with 5% CO_2. HT1080 or F9 cells were treated with either TSA (200 ng/ml) or 5'Aza-dC (10 μM), and total RNA was isolated by the RNeasy kit (Qiagen, Valencia, CA). The synthesis of cDNA and a standard PCR were performed as described previously (Kim *et al*., 2002). Primer pairs used for RT-PCR are as the following: 5'-ATTTCCAAGCCTTTTGGCTA-3' and 5'-CTCCACATTTATCTGGTCC-3' for calmegin, 5'-ATGGAGGTGATGAACCTG-3' and 5'-GATGGCATAGAACAGGAC-3' for plakoglobin, 5'-AGATCTGGCGTGTGGAGAAGTTCGA-3 and 5'-CTTTGACCTGGAAGAGTCTCTGCAC-3' for gelsolin, and 5'-CCTGACCCTGAAGTACCCCA-3' and 5'-CGTCATGCAGCTCATAGCTC-3' for actin. PCR amplification conditions of each gene are as followings; 58°C of annealing T and 30 cycles for plakoglobin, 54°C of annealing T and 35 cycles for calmegin (in HT1080 cells), 54°C of annealing T and 40 cycles for calmegin (in F9 cells), 57°C of annealing T and 26 cycles for actin, and 60°C of annealing T and 30 cycles for gelsolin. The PCR products were resolved by 1 % agarose gel electrophoresis.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously (Shim *et al*., 2004). Briefly, cells grown in 100 mm dishes were treated with or without HDAC inhibitor for indicated time points. Before harvesting, the cells were treated with 1% formaldehyde for 10 min at 37°C to crosslink histones to DNA. ChIP assay was performed using ChIP Assay Kit (Upstate Biotechnology, Lake Placid, NY), according to the manufacturer's instructions. The antibody used in ChIP assay commercially obtained from Upstate Biotechnology (catalog # 06-599). Specific promoter sequences from no antibody control and immunoprecipitated samples were detected by PCR analysis using the following primer sets: 5'-GGTCTGAAGAGACGCGG-3' and 5'-AACATCATCATCCATAAATTC-3' for P1, and 5'-GAGCTCCAGAGAGGGACTGA-3' and 5'-AGGATGCAGGATCGAGTGGC-3' for P2. PCR amplification conditions for each primer set are as followings: 56°C of annealing T and 40 cycles for P1, and 46°C of annealing T and 40 cycles for P2.

Results and Discussion

In the course of our screening of new HDAC target genes using cDNA microarray analysis of transcriptional changes in HT1080 cells treated with TSA, calmegin was identified as one of significant genes that were responded by TSA (Table 1). The ex-

Table 1. Representative genes that were changed by the inhibition of HDAC in HT1080 cells.

	Gene ID	Log_2 of Cy5/Cy3 ratio	Name of genes
Up-regulated genes	AA778675	3.51	Calmegin
	AA454094	2.67	Cullin 2
	H54417	1.95	Non-metastatic cells 4
Down-regulated genes	R46653	-2.91	Plexin B3
	AA668681	-2.71	Cell division cycle 42 (GTP-binding protein)
	H07920	-2.48	Mitogen-activated protein kinase kinase
	R00884	-2.43	Dihydrofolate reductase

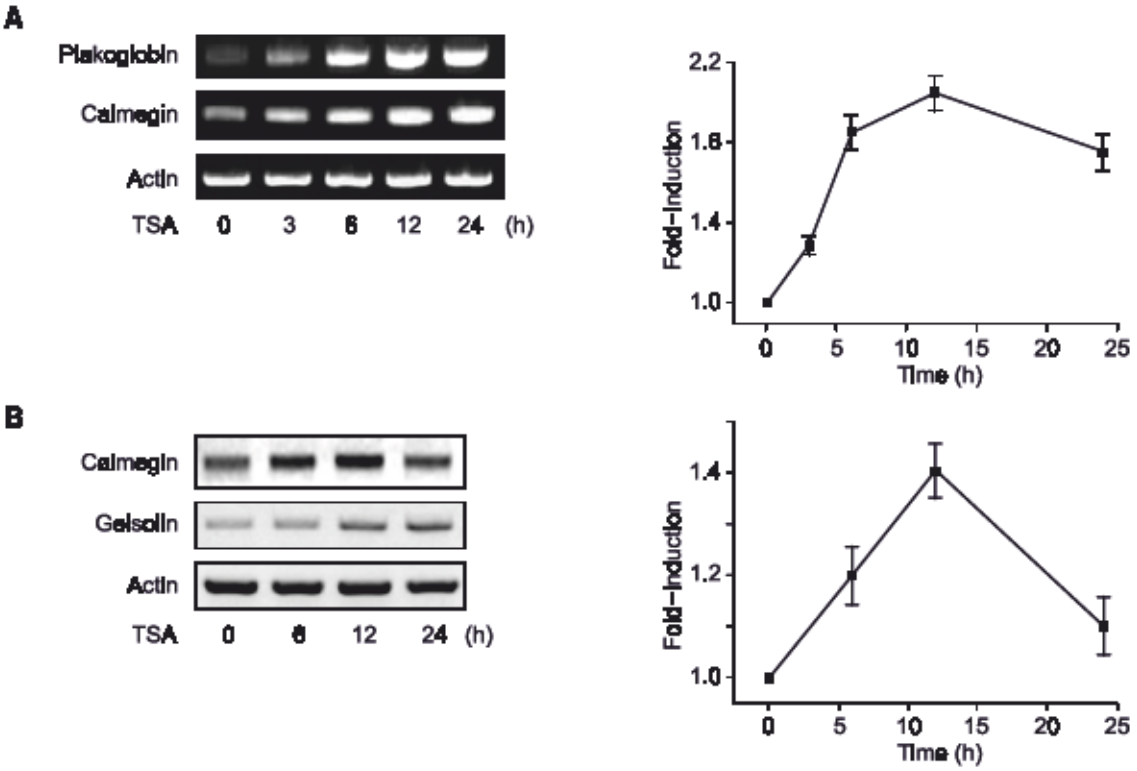

Figure 1. HDAC is involved in the transcriptional regulation of calmegin. (A) Expression level of calmegin in HT1080 cells treated with TSA (200 ng/ml) was estimated in a time-course using RT-PCR analysis. Plakoglobin was used as a positive reference. In *right panel*, expressional pattern of calmegin in time dependent manner was presented using densitometry. (B) Expression level of calmegin in F9 cells treated with TSA (200 ng/ml) was estimated as described above. Gelsolin was used as a positive reference for the inhibition of HDAC. *Right panel* shows quantitative analysis of calmegin expression from three independent experiments.

pression of calmegin was up-regulated more than 3-fold by the HDAC inhibitor, suggesting that the expression of gene is epigenetically regulated. To validate the data, we conducted the RT-PCR analysis of calmegin expression in HT1080 cells with or without TSA treatment. As shown in Figure 1A, the mRNA level of calmegin was increased by TSA treatment in a time-dependent manner. The expression of plakoglobin mRNA, another HDAC target gene, was also increased in the same way to that of calmegin. We, next, examined the effect of HDAC inhibitor on calmegin expression in a testis cell line F9. Likewise, the mRNA level of calmegin was time-dependently induced in response to TSA in F9 cells (Figure 1B). Together, these data demonstrate that calmegin is transcriptionally regulated by HDAC in mammalian cells.

Transcriptional repression of genes often involves epigenetic alterations of the gene promoter such as CpG methylation (Jones *et al*., 1998). Once the promoter is CpG-methylated, methyl-CpG-binding protein (MeCP2) can recruit HDAC complex to the target element in the promoter, leading to the coordinate transcriptional repression of the gene. To examine whether MeCP2 is involved in the transcriptional repression of calmegin, RT-PCR analysis was performed using HT1080 cells treated with either TSA or 5'Aza-dC, an inhibitor of CpG methyltransferase (Cameron *et al*., 1999). Treatment with 5'Aza-dC in HT1080 cells induced a gradual increase of the expression of calmegin in a time-dependent manner (Figure 2). However, the expression patterns of calmegin by 5'Aza-dC showed a delayed up-regulation starting from 12 h whereas those of TSA did an early response indicating that time and spatial dependent regulation may be involved in the transcriptional regulation of calmegin. Interestingly, co-treatment with TSA and 5'Aza-dC increased the expression level of calmegin in an additive way from 6 h. These results demonstrate that an epigenetic alteration is involved in the transcriptional repression of calmegin gene. Consistent with these observations, Watanabe *et al*., (1994) have shown that the promoter region of calmegin contains high GC contents and this region may be controlled by DNA methylation (Watanabe *et al*., 1994).

Finally, we conducted chromatin immunoprecipi-

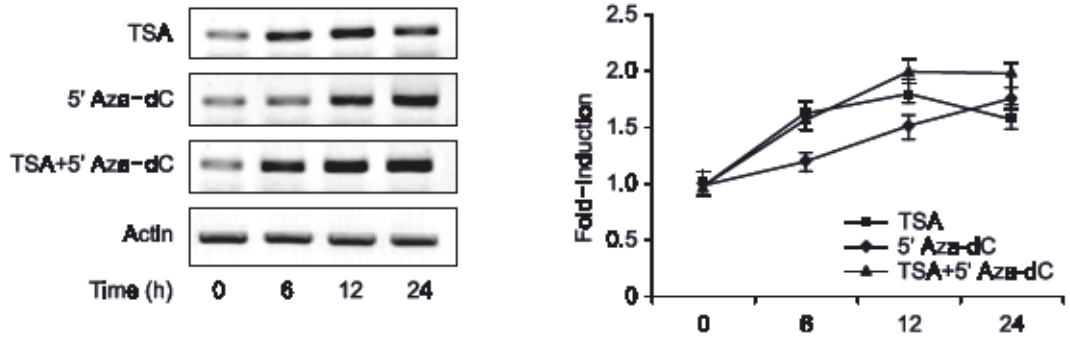

Figure 2. Coordinative transcriptional regulation of calmegin by HDAC and CpG methyltransferase. RT-PCR analysis was conducted using RNAs from HT1080 cells treated with TSA (200 ng/ml) and/or 5'Aza-dC (10 μM). The transcription level of calmegin is presented using densitometry. Right panel shows quantitative analysis of the RT-PCR data from three independent experiments.

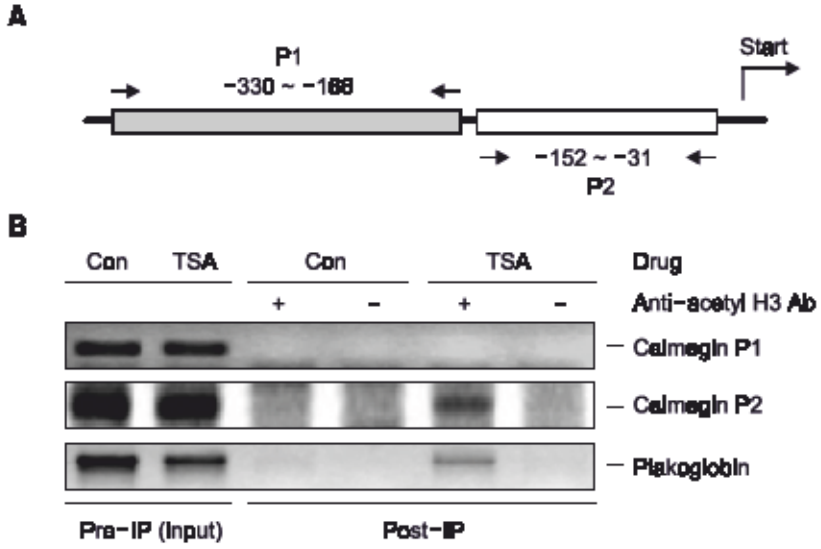

Figure 3. Identification of the putative HDAC binding region in the promoter of calmegin. (A) Schematic representation of the calmegin promoter and primer sets (P1 and P2) used in this study. (B) ChIP analysis of calmegin promoter with the chromatin mixture prepared from the prior (Pre-IP) or after (Post-IP) immunoprecipitation by anti-acetylated histone H3 antibody. Anti-acetylated histone H3 antibody was used in immunoprecipitation.

tation (ChIP) assay using anti-acetyl H3 antibody to determine the binding site of HDAC in the promoter of calmegin. It is well known that the inhibition of HDAC activity causes the hyperacetylation of local histones and the immunoprecipitation of these acetylated histones co-precipitates DNA sequences bound to the acetylated histones (Ashburner *et al*., 2001; Shim *et al*., 2004). The co-immunoprecipitated DNA sequences can be analyzed by PCR using specific primer pairs corresponding to the specific promoter region. We generated two primer sets (P1 and P2), each corresponding to the distal region (−166 ~ −330) and proximal region (−31 ~ −152) of the calmegin promoter, respectively (Figure 3A). As a result, input DNA for P1 and P2 were amplified in DNA samples prepared from both control and TSA-treated HT1080 cells before immunoprecipitation by anti-acetyl-H3 antibody. However, P1 DNA was not amplified in both control and TSA-treated cells after immunoprecipitation by the antibody (Figure 3B). In contrast, P2 DNA was amplified only in the DNA sample prepared from TSA-treated cells after immunoprecipitation. These data suggest that the proximal region (P2, −31 ~ −152) of the calmegin promoter is responsible for the HDAC-mediated transcriptional repression of the gene.

Many of testis-specific genes in male germ cells are controlled transcriptionally or post-translationally during spermatogenesis (Erickson, 1990). Interestingly, both mRNA and protein of calmegin were detected simultaneously from pachytene spermatocyte, indicating that the calmegin gene is transcriptionally controlled and is not significantly affected at the translational level during male germ cell development. In this respect, the data present here strongly suggest the possible role of HDAC in spermatogenesis since both MeCP2 and HDAC appear to exert coordinate regulation of the ex-

pression of calmegin at the transcriptional level. The biological significance of the up-regulation of calmegin after HDAC inhibition in a testis cells is currently under investigation.

In conclusion, the present study demonstrates for the first time that calmegin is a new target gene of HDAC and coordinately regulated by both HDAC and CpG methyltransferase.

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