Biochemical and Biophysical Research Communications 376 (2008) 15–20

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Epigenetic mechanisms and Sp1 regulate mitochondrial citrate carrier gene expression

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ARTICLE INFO

Article history:
Received 5 August 2008
Available online 13 August 2008

Keywords:
Citrate carrier
DNA methylation
Epigenetic mechanisms
Gene expression
Histone acetylation
Mitochondria
Promoter
Sp1

ABSTRACT

This study investigates the transcriptional role of the mitochondrial CIC proximal promoter. The wild-type (but not methylated) −335/−20 bp region of the CIC gene confers gene reporter activity, and the five wild-type (but not methylated) Sp1 binding elements in this region bind human recombinant Sp1. The DNA demethylating agent AzaC or the histone acetylating agent TSA increases CIC transcript and protein levels as well as the binding of Sp1 and of acetylated histones to the −335/−20 bp region of the CIC promoter in SK-N-SH cells but not in HepG2 cells; when untreated these cells exhibit low and high levels of gene expression, respectively. Finally, Sp1 silencing decreases proximal promoter-driven gene reporter activity as well as CIC mRNA and CIC protein in both untreated HepG2 cells and AzaC- and TSA-treated SK-N-SH cells. These results show that methylation, histone acetylation and Sp1 are important in the transcriptional regulation of the CIC proximal promoter.

The mitochondrial citrate carrier (CIC) is a nuclear-encoded protein located in the inner mitochondrial membrane (see [1,2] for reviews). It exports citrate from the mitochondria to the cytosol where citrate is cleaved by ATP-citrate lyase to acetyl-CoA and oxaloacetate. Acetyl-CoA is used for fatty acid and sterol biosynthesis and oxaloacetate is reduced to malate, which in turn is converted to pyruvate via malic enzyme with production of cytosolic NADPH (also necessary for fatty acid and sterol synthesis). CIC also participates in the isocitrate–oxoglutarate shuttle, in the citrate–malate shuttle, and in gluconeogenesis. Like all the members of the mitochondrial carrier family [2,3], it consists of three tandemly related domains of approximately 100 amino acids in length [4] and spans the membrane six times with both the N- and C-termini protruding towards the cytosol [5]. The human gene for CIC, named SLC25A1, is localized on chromosome 22q11.2 within a region implicated in DiGeorge syndrome [6]. Interestingly, the activity of CIC is high in liver, where active fatty acid synthesis occurs, and virtually absent in other tissues [7,8]. Moreover, CIC mRNA and/or CIC protein level is high in liver, pancreas, and kidney but low or absent in heart, skeletal muscle, placenta, brain, and lungs [9]. In early studies, decreased CIC activity was found in diabetic and unfed rats [10,11]. More recently, a reduction of both CIC activity and CIC messenger RNA was demonstrated in hypothyroid rats and in rats fed with a diet enriched with polyunsaturated fatty acids (PUFAs) [12,13].

In a previous study, we showed that the CIC gene promoter contains an active sterol regulatory element (SRE) and that insulin upregulates and PUFAs downregulate the CIC gene transcription through the SRE/SREBP-1 regulatory system [14]. Apart from this information, the mechanisms of transcriptional regulation of the CIC gene are yet to be uncovered. Since CIC plays an important role in intermediary metabolism their understanding might provide important clues in a variety of metabolic responses.

To shed more light on the transcriptional regulatory role of the CIC gene promoter, herein we investigated whether methylation and acetylation are regulatory events during CIC transcription. We show that demethylation of the CIC gene proximal promoter, from −335 to −20 bp, and histone acetylation activate CIC gene expression by promoting binding of the Sp1 transcription factor and of the acetylated histone H3 to the CIC proximal promoter. To our knowledge, this is the first study to investigate the role of Sp1 and epigenetic modifications in the transcriptional regulation of the CIC gene.

Materials and methods

Construction of plasmids. The region from −335 to −20 bp of the CIC gene promoter (chromosome 22q11.2, Cosmid Clone 79h12) was amplified by PCR and cloned into the pGL3 basic-LUC vector (Promega) upstream the LUC gene-coding sequence. The sequence

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doi:10.1016/j.bbrc.2008.08.015

of the insert was verified. Where indicated, the plasmid was methylated by incubating 40 μg with 100 U SssI CpG methylase, 50 mM NaCl, 10 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM dithioerythritol, and 160 μM *S*-adenosylmethionine, at 37 °C overnight, according to the manufacturer's instructions (New England Biolabs). Complete methylation was verified using the methylation-sensitive restriction enzyme HpaII.

Cell culture, RNA interference, and transient transfection. SK-N-SH cells (ICLC, Interlab Cell Line Collection) were maintained in RPMI 1640 medium (Roswell Park Memorial Institute); HepG2 and HEK293 cells (Sigma) in high glucose DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2; and human hepatocytes (Lonza) in hepatocyte culture medium (Lonza) following the manufacturer's instructions. Where indicated, cells were incubated with TSA (Sigma) or AzaC (Sigma), either separately or together, for 24 h. Subsequently, the TSA-treated cells were lysed, whereas the cells treated with both AzaC and TSA were suspended in fresh medium with the addition of AzaC for further 24 h. For the RNA interference experiments, the specific pre-designed small interfering RNA (siRNA) targeting human Sp1 (ID 143158, Ambion) was transfected in HepG2 and SK-N-SH cells using siPORT™ NeoFX™ Transfection Agent (Ambion). A siRNA (Cat. No. C6A-0126, Ambion) with no significant similarity to human, mouse, or rat gene sequences was used as negative control. Transient transfection was performed as reported [15] using 0.5 μg of pGL3 basic-LUC vector containing the −335/−20 bp region of the CIC gene promoter and 10 ng of pRL-CMV (Promega) to normalize the extent of transfection [14]. Transfected cells were assayed for LUC activity using the Dual-Luciferase® Reporter Assay System (Promega). In some experiments, HepG2 and SK-N-SH cells were transfected first with siRNA targeting human Sp1 for 24 h and then with the pGL3 basic-LUC vector containing the −335/−20 bp region of the CIC gene promotor and the pRL-CMV, as described above, for additional 24 h.

Chromatin immunoprecipitation. ChIP experiments were performed as previously reported [16]. Briefly, 2×10^7 of HepG2 or SK-N-SH cells, treated with or without AzaC, TSA or both, were fixed by 1% formaldehyde at 37 °C for 10 min; afterwards, the cells were lysed and sheared by sonication in a 1% SDS lysis buffer to generate cellular chromatin fragments of 300–400 bp. The chromatin was immunoprecipitated for 14–16 h at 4 °C using specific antibodies to AcH3 (Upstate, Cat. No. 06-599) and to Sp1 (Santa Cruz Biotechnology, Cat. No. sc-59X). After reverse cross-linking, chromatin immunoprecipitates were purified by ethanol precipitation, then 2 μl of each sample were analyzed by PCR (35 cycles) using a forward primer (5′-ACTGCTCGGAGCTGGGCCAC-3′) and a reverse primer (5′-GCCCTGTGGCGGCTTCGGGTCC-3′) suitable to amplify the −335/−20 bp region of the CIC gene promoter.

Other methods. EMSA experiments were performed as described in Sambrook et al. [17] using human Sp1 recombinant protein. The double-stranded oligonucleotide probes, named Sp1A (−303/−286), Sp1B (−239/−222), Sp1C (−210/−188), Sp1D (−185/−164), Sp1E (−163/−143), Sp1F (−142/−121), and Sp1G (−112/−95), were 5′-end labeled using T4 polynucleotide kinase and [γ^{32}P]ATP at 37 °C for 30 min. Methylated probes were prepared by incubating 20 μg of each oligonucleotide with 80 U of SssI CpG methylase under the same conditions reported above. The gels were dried and images acquired by phosphoimager (Bio-Rad). Total RNA was extracted from 1×10^6 HepG2, SK-N-SH and HEK293 cells, or hepatocytes treated as indicated, and reverse transcription was performed as reported [15]. Real-time PCR was performed as previously described [18]. Assay-on-demand for human CIC (Cat. No. Hs00761590_m1), human Sp1 (Cat. No. Hs00293689_s1), and human β-actin (Cat. No. Hs99999903_m1) were purchased from Applied Biosystems. The CIC transcript levels were normalized against the expression levels of actin. For Western blot analysis, proteins were electroblotted onto PVDF membranes (Roche) and subsequently treated with anti-CIC (specific for the C-terminus of the human mitochondrial CIC [5]) or anti-β-actin (BioLegend) antibodies. The immunoreaction was detected by the ECL plus system (Amersham).

Results

The unmethylated −335/−20 bp region of the CIC gene promoter confers gene reporter expression activity

Analysis of the CIC promoter from −5000 to −1 bp revealed a CpG island of 760 bp (with 74% GC content and 0.89 CpG dinucleotide frequency) immediately upstream the translation start codon. This promoter region contains seven putative Sp1 binding

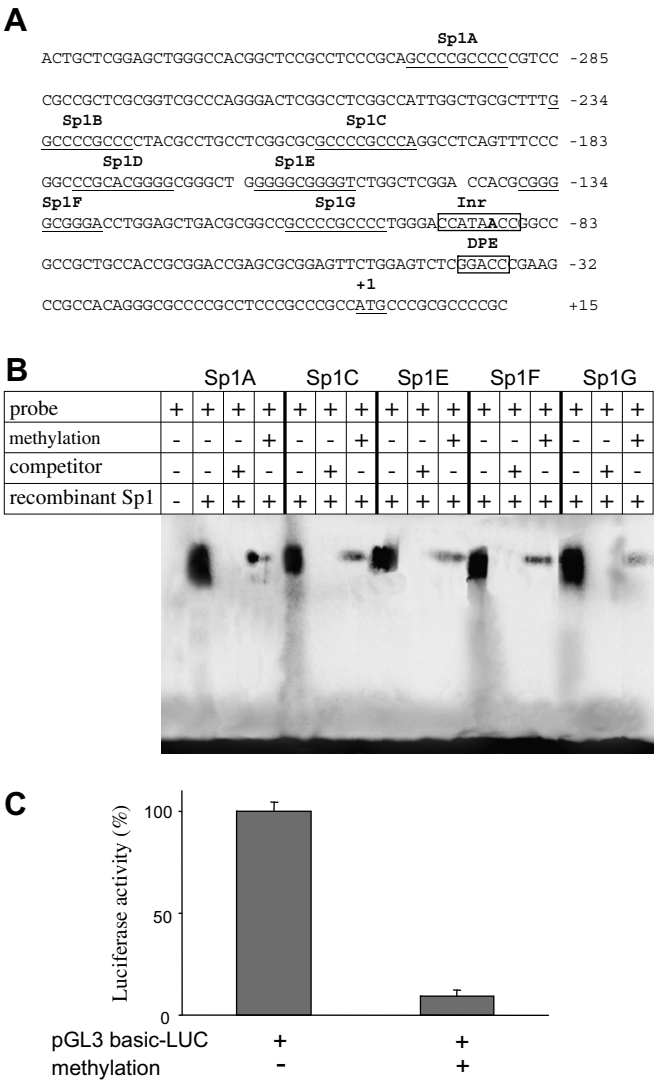

Fig. 1. Effect of methylating the CIC gene proximal promoter on Sp1 binding and on gene reporter expression activity. (A) The nucleotides preceding the translation start codon ATG of the CIC gene are indicated by negative numbers. Underlined and boxed nucleotide sequences indicate putative Sp1 binding (Sp1A-G), initiator (Inr), and downstream core promoter (DPE) elements. The adenine (in bold) at −89 within Inr represents the putative transcriptional start site. (B) EMSA were performed by incubating 2 μg of recombinant human Sp1 with methylated and unmethylated 5′-end labeled oligonucleotide probes, specified in Materials and methods. Where indicated, unlabeled probe was added in 100-fold molar excess. (C) HepG2 cells co-transfected with the pGL3 basic-LUC vector, containing the methylated or unmethylated −335/−20 bp region of the CIC gene, and with the pRL-CMV vector were assayed for LUC expression activity. Means ± SD of three duplicate independent experiments are shown.

elements located between −299 and −100 bp (Fig. 1A). Because Sp1 regulates the expression of a large number of genes [19], we focused our analysis on this region.

To test whether Sp1 can actually bind to the putative Sp1 binding elements, EMSA experiments were performed. Seven probes encompassing the Sp1 binding sites were labeled and incubated with human recombinant Sp1. Five (Sp1A, Sp1C, Sp1E, Sp1F, and Sp1G) exhibited Sp1 binding activity that was abolished by an excess of unlabeled probe (Fig. 1B). These data suggest that multiple GC-rich Sp1 binding elements can potentially contribute to CIC gene proximal promoter activity.

Since cytosine methylation may prevent binding of transcription factors, we analyzed the effect of methylating the five functionally active Sp1 binding sites on their binding to the human recombinant Sp1 protein. Methylation of the oligonucleotides corresponding to each Sp1 binding site nearly abolished their ability to bind recombinant Sp1 (Fig. 1B). To further analyze the effect of DNA methylation on CIC gene proximal promoter activity, the −335/−20 bp DNA fragment was cloned into pGL3 basic-LUC vector, which was methylated and then used to transfect HepG2 cells. LUC activity of HepG2 cells transfected with the methylated construct was markedly diminished (to 9%) as compared to the activity of cells transfected with the unmethylated construct (Fig. 1C), despite the fact that the extent of transfection was similar in both cases.

Sp1 binds to the CIC gene promoter in HepG2 cells

In another set of experiments, we performed ChIP analysis to demonstrate that Sp1 is bound to the −335/−20 bp GC-rich region of the CIC proximal promoter in living cells. HepG2 and SK-N-SH cell lines were chosen because they exhibited high and low levels, respectively, of CIC transcript (Fig. 2A) and CIC protein (Fig. 2B), whereas the HEK293 cell line showed intermediate levels (Figs. 2A and B). As can be seen, an Sp1-specific antibody immunoprecipitated the CIC proximal promoter DNA from HepG2 but not from SK-N-SH cells (Fig. 2C, lane Sp1). No PCR product was found without addition of the antibody (Fig. 2C, lane NoAb). By contrast, a product of the expected molecular weight was obtained in both cell lines when amplification was performed using total chromatin before immunoprecipitation (Fig. 2C, input, indicated as lane I). These results show that Sp1 binds to the CIC proximal promoter in HepG2 cells containing high levels of CIC mRNA and protein, whereas no Sp1 binding was observed in SK-N-SH cells containing low levels of CIC mRNA and protein.

Effect of AzaC and TSA on CIC gene expression

Subsequently, we investigated the effect of AzaC, an inhibitor of DNA methyltransferase [20], on CIC gene expression in HepG2 and SK-N-SH cells. After incubation with AzaC, total mRNA from both cell lines was extracted and used to determine CIC transcript levels. The CIC mRNA level was virtually unaffected by 50 μM AzaC in HepG2 and increased by 73% in SK-N-SH cells (Fig. 3A). In other experiments, 5–50 μM AzaC were found to increase CIC transcript in a dose-dependent manner in SK-N-SH cells without affecting it in HepG2 cells (data not shown). From these results we inferred that the CIC gene promoter is largely demethylated in HepG2 and largely methylated in SK-N-SH cells.

Because CIC mRNA in AzaC-treated SK-N-SH cells is 44% of that present in untreated HepG2 cells, we hypothesized that histone acetylation/deacetylation is also involved in the regulation of CIC gene transcription. We therefore investigated the effect of TSA, an inhibitor of histone deacetylase [21], on CIC gene expression in HepG2 and SK-N-SH cells. In these experiments, a 500-ng/ml

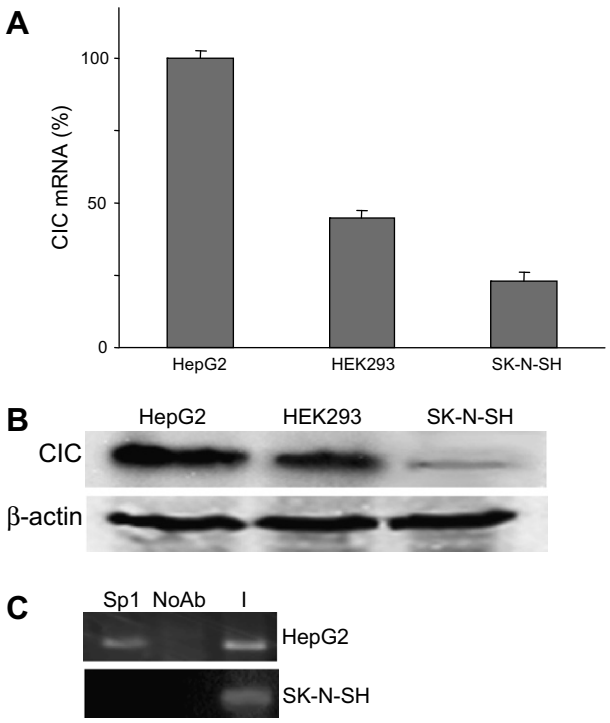

Fig. 2. CIC expression and ChIP analysis of Sp1 in HepG2 and SK-N-SH cells. (A) Total RNA from HepG2, HEK293, and SK-N-SH cell lines was used to quantify CIC mRNA by real-time PCR. Means ± SD of three duplicate independent experiments are shown. (B) CIC and β-actin of each cell line were immunodecorated with specific antibodies. (C) Chromatin of lysed HepG2 and SK-N-SH cells (lane Sp1) was immunoprecipitated by an anti-Sp1 antibody. PCR was performed using forward and reverse primers encompassing the CIC gene promoter from −335 to −20 bp. Lane NoAb, PCR of the precipitate without antibody; lane I, PCR of input DNA (1/10 dilution).

concentration of TSA was used since higher doses (as well as doses >50 μM of AzaC) cause apoptosis of both cell lines. CIC mRNA was unaffected by TSA in HepG2 cells expressing high levels of CIC transcript when untreated (Fig. 3A). In contrast, CIC mRNA was increased 2.6-fold by the same inhibitor in SK-N-SH cells expressing low levels when untreated. When SK-N-SH cells were incubated with the combination AzaC + TSA, an additional increase in CIC transcript level was observed (∼3.4-fold vs untreated cells; Fig. 3A). In agreement with these results, the amount of CIC protein in SK-N-SH cells was increased by adding AzaC and TSA individually and even more by the combination of the two inhibitors, whereas in HepG2 cells it remained virtually unchanged (Fig. 3B). In other experiments, we tested the effect of AzaC and TSA on CIC expression in human hepatocytes. These exhibited the same CIC transcript and protein levels as HepG2 cells, and were unaffected by treating hepatocytes with AzaC or with TSA, both alone or in combination (data not shown).

The binding of Sp1 and of acetylated histones to the CIC gene proximal promoter was assessed in HepG2 and SK-N-SH cells in the presence or absence of TSA and AzaC by ChIP using anti-Sp1 and anti-acetylated histone H3 antibodies. In HepG2 cells, both Sp1 and acetylated histone were bound to the CIC gene proximal promoter even in the absence of AzaC and TSA (Fig. 3C). By contrast, in SK-N-SH cells Sp1 was bound upon addition of AzaC, acetylated histone upon addition of TSA, and both Sp1 and acetylated histone were bound after treatment with the combination AzaC + TSA (Fig. 3C). Without the addition of antibodies (Fig. 3C, lanes N) and in SK-N-SH untreated cells (Fig. 3, lanes S and H) no immunoprecipitation was observed.

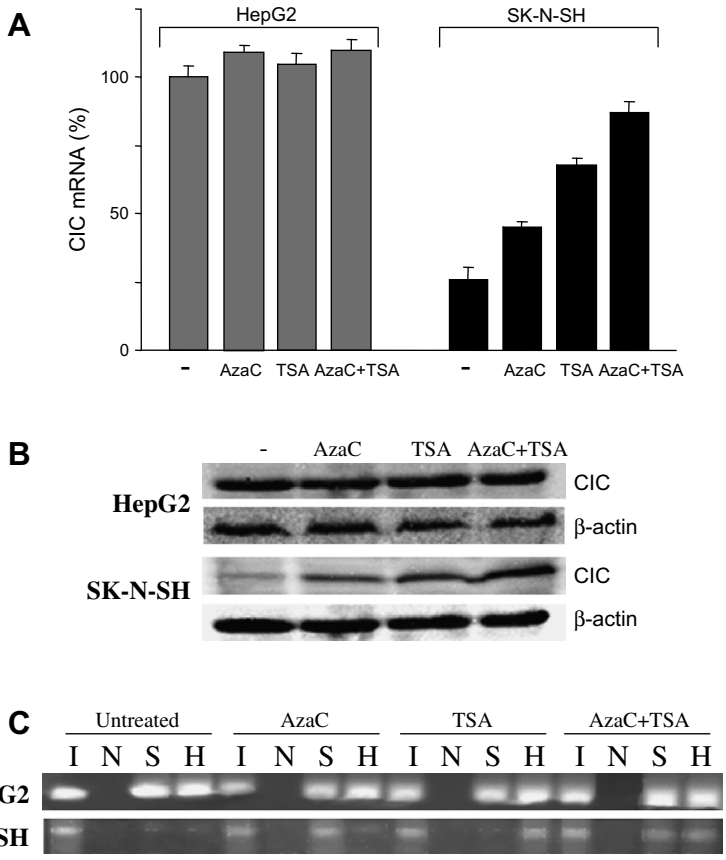

Fig. 3. Effect of TSA and AzaC on CIC expression and on Sp1 and acetylated histone H3 binding to the CIC gene proximal promoter. (A) Total RNA from HepG2 and SK-N-SH cells treated with AzaC, TSA, or both was used to quantify CIC mRNA by real-time PCR. Means ± SD of three duplicate independent experiments are shown. (B) CIC and β-actin of HepG2 and SK-N-SH cells treated with AzaC, TSA, or both, were immunodecorated with specific antibodies. (C) Chromatin of lysed HepG2 and SK-N-SH cells, treated with or without AzaC, TSA or both, was immunoprecipitated by anti-Sp1 (lanes S) and anti-acetylated histone H3 (lanes H) antibodies. PCR was performed using forward and reverse primers encompassing the CIC gene promoter from −335 to −20 bp. Lanes N, PCR of the precipitate without antibody; lanes I, PCR of input DNA (1/10 dilution).

Effect of Sp1 silencing on CIC gene expression

To confirm the role of Sp1 in the regulation of CIC gene expression, HepG2 and SK-N-SH cells were transfected with siRNA targeting human Sp1. After 24 hours, AzaC and TSA were added to the transfected SK-N-SH cells to increase CIC gene expression to a level comparable to that of HepG2 cells. At 48 h from the beginning of the transfection, Sp1 mRNA was 25% in HepG2 transfected cells and 22% in SK-N-SH transfected cells as compared to controls (data not shown). At the same time period, the Sp1 protein was also decreased in both cell lines as revealed by an anti-Sp1 antibody (Fig. 4A). Parallel to Sp1 silencing, CIC at transcript and protein level (Figs. 4A and B) was markedly diminished in both cell lines as compared to untransfected cells. Furthermore, the gene reporter expression activity of both HepG2 and SK-N-SH cells, transfected with the pGL3 basic-LUC vector containing the −335/−20 bp DNA fragment of the CIC gene promoter, was strongly decreased in Sp1-silenced cells with respect to control cells (Fig. 4C).

Discussion

DNA methylation and histone acetylation/deacetylation, followed by changes in chromatin structure, play a critical role in regulating gene expression in eukaryotes [22].

The experimental data presented in this study show that CIC gene transcription is regulated by epigenetic mechanisms, since DNA demethylation and histone acetylation enhance CIC gene expression as estimated by measuring CIC mRNA content by real-time PCR and the amount of CIC protein by a specific antibody. Major evidence supporting this conclusion is the increased expression of CIC in SK-N-SH cells by the DNA demethylating agent AzaC or by the histone acetylating agent TSA. The fact that treatment with both drugs in combination results in an additional increase of CIC expression, i.e., they act in a synergistic fashion, indicates that DNA methylation and histone deacetylation repress CIC gene expression by largely independent mechanisms. However, our ChIP experiments show that both events prevent access of the Sp1 transcription factor and of the acetylated histone H3 to the −335/−20 bp region of the CIC gene promoter. The role of methylation in repressing CIC expression by preventing Sp1 binding to DNA is directly supported by the finding that the CIC promoter Sp1 binding sites lose their ability to bind recombinant Sp1 when methylated and by the observation that methylation of the entire −335/−20 bp region strongly decreases gene reporter expression activity. It is noteworthy that Sp1 binding elements are present in the promoters of all mitochondrial CIC genes, sequenced in mammals, within a CpG island located immediately upstream the translocation start codon.

Our data also provide evidence that the CIC gene is de-repressed in HepG2 cells and repressed in SK-N-SH cells, as revealed by an approximately 4-fold higher level of both CIC transcript and protein in the former with respect to the latter. This is likely due to the fact that the −335/−20 bp region of the CIC promoter is largely demethylated and associated to Sp1 and acetylated histones in HepG2 cells but not in SK-N-SH cells. The following evidence strongly supports this interpretation. SP1 and acetylated histones

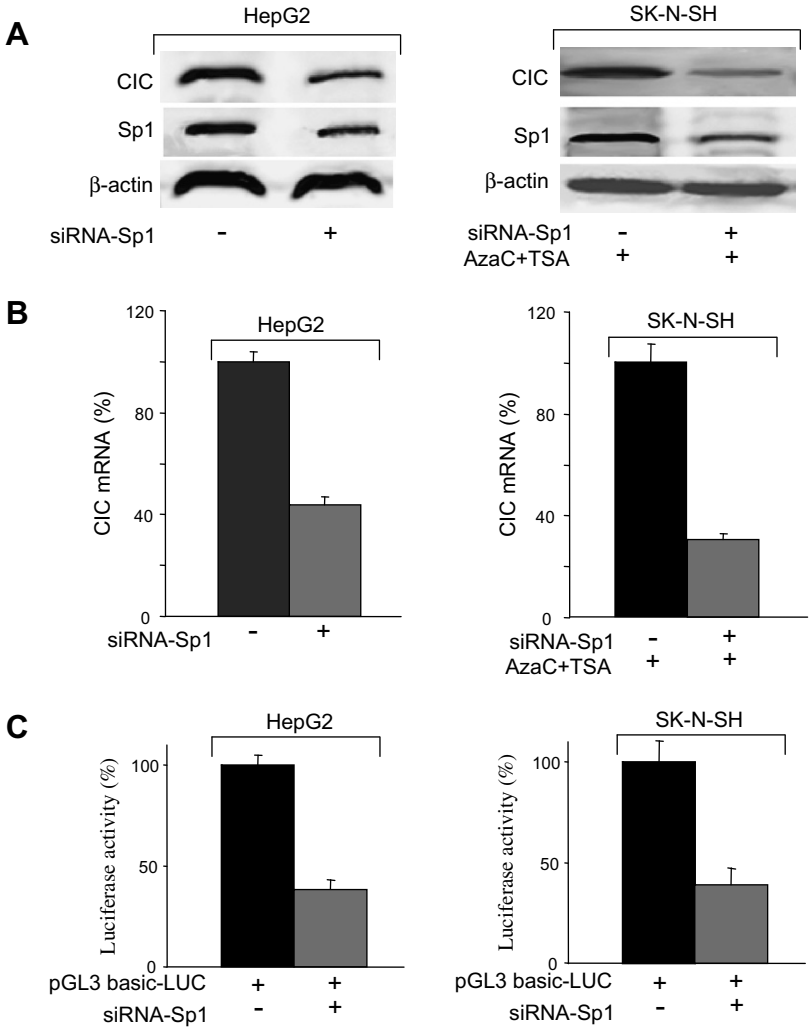

Fig. 4. Effect of Sp1 silencing on CIC gene expression. (A) CIC, Sp1, and β-actin of HepG2 and SK-N-SH cells transfected with or without siRNA targeting human Sp1 and (where indicated) treated with the combination AzaC + TSA were immunodecorated with specific antibodies. (B) Left panel, CIC mRNA of HepG2 cells, transfected with or without siRNA targeting human Sp1, was quantified by real-time PCR. Right panel, CIC mRNA of SK-N-SH cells, transfected with or without siRNA targeting human Sp1 and treated with AzaC + TSA, was quantified by real-time PCR. Means ± SD of three duplicate independent experiments are shown. (C) HepG2 and SK-N-SH cells, transfected with or without siRNA targeting human Sp1 and with pGL3 basic-LUC vector containing the CIC promoter −335/−20 bp region, were assayed for LUC expression activity. Means ± SD of three duplicate independent experiments are shown.

are found to be associated to the CIC proximal promoter of HepG2 cells even when untreated with AzaC and TSA; by contrast, Sp1 is associated to the CIC proximal promoter of SK-N-SH cells after treatment with AzaC, and acetylated histone H3 after treatment with TSA. AzaC, and TSA added alone or in combination do not affect CIC gene expression in HepG2 cells contrary to the stimulating effect in SK-N-SH cells. Moreover, CIC mRNA and protein levels were markedly decreased by silencing Sp1 in untreated HepG2 cells and in SK-N-SH cells when treated with AzaC + TSA. Therefore, our results explain at the molecular level, at least in part, the differences in CIC activity and/or CIC levels observed previously among tissues and cell lines [7–9,23,24].

Acknowledgments

This work was supported by MIUR, Apulia Region, the Center of Excellence in Genomics and the University of Bari.

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