Biochemical and Biophysical Research Communications 439 (2013) 481–486

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

CREB, another culprit for TIGAR promoter activity and expression

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ARTICLE INFO

Article history:
Received 26 August 2013
Available online 11 September 2013

Keywords:
TIGAR
Promoter
CREB
EMSA
ChIP

ABSTRACT

Cellular expression of the TP53-induced glycolysis and apoptosis regulator (TIGAR) protein results in the down-regulation of glycolysis, reduction of intracellular levels of reactive oxygen species, and protection from apoptosis. However, despite its biological importance, the mechanisms that regulate its expression remain obscure. The bioinformatic analysis performed in this study indicates that the TIGAR promoter region is highly conserved among species. Further analysis using 5′-deletion analysis and site-directed mutagenesis demonstrated that the region at −4/+13 contained a cAMP-response element (CRE). EMSA and chromatin immunoprecipitation showed that the site was recognized by CRE-binding protein (CREB). Furthermore, knockdown of CREB substantially reduced promoter activity and TIGAR expression in cells. In addition, over-expression of either CREB or forskolin enhanced promoter activity and TIGAR expression. These results provide evidence that CREB regulates TIGAR expression via a CRE-binding site at the TIGAR promoter.

1. Introduction

A high rate of glycolysis and low cAMP levels result in disequilibrium in energy and are hallmarks of tumour cells [1]. TIGAR (TP53-induced glycolysis and apoptosis regulator), a novel p53-inducible gene [2], functions as a fructose-2, 6-bisphosphatase that inhibits cellular glycolysis [3]. As a result, the reduction of glycolytic flux activates the pentose phosphate pathway and generates NADPH. Increased NADPH levels lower intracellular reactive oxygen species (ROS) and promote antioxidant function, thereby limiting ROS-associated apoptosis and autophagy [3–7]. However, the ability of TIGAR to enhance cell survival depends on the type of cell and the context in which it is expressed. In cells, survival highly depends on the maintenance of glycolytic flux, and thus, TIGAR expression results in decreased, rather than increased, survival [3,8]. However, over-expression of TIGAR reduces glucose utilization and increases apoptosis, thus emphasizing the importance of metabolic regulation in tumour suppression [8].

Cyclic adenosine 3′,5′-monophosphate (cAMP), a major regulator of cell proliferation in a wide variety of cells, regulates cell proliferation in a protein kinase A (PKA)-dependent manner [9]. Protein kinase A binds to cAMP and translocates to the cell nucleus, where it phosphorylates cAMP-response element (CRE)-binding protein (CREB) [10]. CREB-binding protein (CBP) then binds specifically to the PKA-phosphorylated form of CREB and functions as a link between CREB and the transcription pre-initiation complex to activate CRE-containing promoters [11]. In other words, activated CREB protein binds to a cAMP response element (CRE) region, and then binds to CBP, which co-activates it, allowing the switching off or on certain genes [11–13]. Previous studies have suggested that cAMP regulates a large number of transcription factors that control cell survival [14]. Further, CREB is a ubiquitous transcription factor that activates the transcriptional activity of various promoters through CRE-binding sites [15]. CREB has also been shown to play a major role in the survival and synaptic plasticity of neuronal cells [16–18]. Apart from this, studies have demonstrated the role of CREB activation in the proliferation of various tumours [19,20].

Because both cAMP and TIGAR have been implicated in cell survival, and on the other side CREB can regulate the transcription of survival-associated genes, it is possible that there may be a link between them. In addition, although TIGAR functions have been well studied, the regulatory mechanisms that control its activity remain unclear. Therefore, to gain more insight into the regulation of

Abbreviations: ChIP, chromatin immunoprecipitation; CRE, cAMP-response element; CREBP, CRE binding protein; EMSA, electrophoretic mobility shift assays; TF, transcription factor; TSS, transcription start site.

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0006-291X/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.08.098

TIGAR expression, we investigated whether CREB could bind to and activate the TIGAR promoter.

2. Materials and methods

2.1. In silico analysis

Upstream sequences of the human *TIGAR* (NM_020375) gene were obtained from the UCSC Genome Browser and aligned with TIGAR sequences from other species. The putative transcription factor binding sites within the 5′-upstream sequences of human TIGAR were determined and scored using publicly available web-based tools (TRANSFAC, MatInspector and PROSCAN).

2.2. Cell culture

All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal bovine serum, 100 U penicillin and 0.1 mg/ml streptomycin and cultured at 37 °C in an atmosphere of 5% CO_2. Cell culture reagents were obtained from Invitrogen unless otherwise specified.

2.3. Construction of luciferase expression plasmids

Insert DNA containing sequences from the human TIGAR promoter were generated by PCR using extracted genomic DNA from the HepG2 cell line as a template. Primer sequences containing *Mlu*I or *Xho*I restriction enzyme sites were used in the PCR and are listed in Table 1. After enzymatic digestion with *Mlu*I and *Xho*I, the amplicon was subsequently sub-cloned into a pGL3-basic vector (Promega). For site-directed mutagenesis of the putative CRE-binding motif, PCR was performed using a MutantBEST kit (TaKaRa) according to the manufacturer's instructions. Sequencing was performed to verify all constructs prior to transfection.

2.4. Transient transfection and luciferase assays

Cells were seeded onto plates and grown overnight prior to treatment. For promoter activity assessment, each well was transfected with 900 ng of the reporter constructs and 100 ng of a phRL-TK plasmid vector encoding *Renilla* luciferase (Promega) as an internal control for transfection efficiency. 24 h later, cells were harvested and luciferase activities were measured using a Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's protocol. DNA, RNA, or protein was extracted from transfected cells as described below. Protein and mRNA levels were then assessed according to methods described below. Each experiment was repeated at least 3 times, and each sample was studied in triplicate.

2.5. Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSAs)

Electrophoretic mobility shift assays (EMSAs) were performed as described previously [21]. Briefly, nuclear extracts were obtained using a nuclear extraction kit (Active Motif) according to the manufacturer's instructions unless otherwise noted. Synthetic complementary oligonucleotides (Table 1) were first annealed at 95 °C and then cooled to room temperature to generate double-stranded probes. Probes were then labeled using T4 polynucleotide kinase (TaKaRa) and [γ-32P] ATP (PerkinElmer). EMSAs were then conducted using a Gel Shift Assay System (Promega). Binding reaction mixtures were prepared for 20 min at room temperature. For the competition experiments, an excess of unlabeled oligonucleotides was added. Complexes were resolved on 8% non-denaturing polyacrylamide gels in 0.5× tris-buffered EDTA (TBE) buffer. Gels were run at 100 V for 2 h at 4 °C, dried on Whatman paper, and exposed to an X-ray film overnight at −80 °C.

2.6. Chromatin immunoprecipitation assay

The assay was carried out using a ChIP–IT Express kit (Active Motif) according to the protocol provided by the manufacturer. Protein-DNA complexes were immunoprecipitated with antibodies (Santa Cruz). After recovery, the immunoprecipitated DNA was subjected to PCR analysis using TIGAR promoter-specific primers (indicated in Table 1). The soluble chromatin prior to immunoprecipitation was used as an input control. The PCR products were electrophoresed in a 3% agarose gel. Samples from three independent immunoprecipitations were then analysed.

Table 1
Oligonucleotides applied in this work.

Name	Sequence (5′–3′)	Purpose
pGL3-114F[a]	ATCACGCGTCCAATCACAGGCCGGCGCGCAGGGG	Promoter sub-cloning
pGL3-54F[a]	ATAACGCGTGCTCCCTCGTCTCCCCCGCCCCGTC	Promoter sub-cloning
pGL3-4F[a]	ATCACGCGTCGGGGATGACGTGCGAGGCCG CCTC	Promoter sub-cloning
CREBmt1[b]	CGGGGActgaGTGCGAGGCCGCCTC	Site-directed mutagenesis
CREBmt2[b]	CGGGGATGgatcGCGAGGCCGCCTC	Site-directed mutagenesis
CREBmt3[b]	CGGGGATGACtcatGAGGCCGCCTC	Site-directed mutagenesis
pGL3+13F[a]	ATAACGCGTGCCGCCTCGGCCTATGGCGGCGGAG	Promoter sub-cloning
pGL3+190R[c]	ATACTCGAGGACATCCTGCCACAGCCATACTCAC	Promoter sub-cloning
CRE native	CGGGGATGACGTGCGAGGCCG CCTC	EMSA Probe
CRE (mt)	CGGGGATGACaattGAGGCCG CCTC	EMSA competitor Probe
TIGAR frw	GGCTTCGGGAAAGGAAATACG	RT-PCR
TIGAR rev	AACCTGGAATAC CGCTGTCTG	RT-PCR
β-Actin frw	TGAAGGTGACAGCAGTCGGTTGGA	RT-PCR and realtime RT-PCR
β-Actin rev	GGGACTTCCTGTAACAACGCATC	RT-PCR and realtime RT-PCR
TIGAR frw	ATACGGGGTTGTAGAAGGCAAAGCG	Realtime RT-PCR
TIGAR rev	ATTTTCACCTGGTCCAGCGTCTC	Realtime RT-PCR
ChIP frw	CTATCGAGGGAAGGAATCCTACCGC	ChIP
ChIP rev	TCATCCCCGAAGGCCACTCAG	ChIP

[a] A *Xho*I restriction enzyme site is indicated by underlined.
[b] Mutant nucleotides were indicated by small letters.
[c] A *Mlu*I restriction enzyme site is indicated by underlined.

2.7. CREB siRNA and over-expression studies

siRNAs against CREB were purchased from GenePharma. Over-expression plasmids of CREB were kindly provided by Dr. Jiayi Wang. Co-transfection of expression plasmids or siRNAs along with the TIGAR core promoter was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Following transfection, cells were treated with forskolin (50 nM) for 6 h. Promoter activity was then measured as mentioned above. TIGAR gene expression was monitored by RT-PCR and real-time PCR as described below. TIGAR protein levels were detected by Western blotting. All assays were performed at least 3 times in triplicate.

2.8. RT-PCR and quantitative real-time RT-PCR

Total RNA was extracted from cells using Trizol (Life Technology). The concentration and quality of the total RNA was examined by measuring its absorbance at 260 nm. First-strand cDNA was synthesised from 1 μg of total RNA using the Reverse Transcription system from Promega. An aliquot of each cDNA product was used to amplify the human TIGAR and β-actin transcript by PCR. The amplicons were analysed on a 3% agarose gel. β-Actin expression was used as an endogenous control. Quantitative real-time PCR was then performed with the Mx3000P real-time PCR system (Stratagene) using SYBR Premix Ex Taq (TaKaRa). The primers are listed in Table 1. All tests were executed thrice.

2.9. Western blotting analysis

Briefly, cells were lysed in RIPA lysis buffer (Beyotime) and protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Thermo). 30 μg of protein was separated by 10% SDS–PAGE, and the proteins were transferred onto PVDF membranes (Bio-Rad). Then, membranes were incubated with primary antibodies purchased from Santa Cruz, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signal Technology). Immobilized membranes were detected using an ECL Plus Detection kit (Beyotime) on an X-ray film (Kodak).

2.10. Statistical analyses

Statistical differences between groups were determined using ANOVA and unpaired t-tests. All results are shown as mean ± SD. P values less than 0.05 were considered significant.

3. Results

3.1. The human TIGAR promoter is highly conserved between species

Bioinformatics analysis of the TIGAR 5′ region using CpG Island Searcher revealed the presence of a large CpG island spanning −766 to −35 relative to the TIGAR translation initiation site, suggesting that TIGAR expression is driven by a TATA-less promoter. Additional analysis using the MatInspector software program revealed a CCAAT box at position −247 and did not reveal any canonical consensus TATA box (Supplement Fig. 1). Putative consensus-binding sites for transcription factors were identified, including a CREB box, a negative element, and 3 GC boxes. We have previously described GC boxes for SP1 binding [21]. The 5′-flanking region of human TIGAR was aligned with that of TIGAR of other primates by using the UCSC database. The results show a particularly high region of conservation existing upstream of the translation initiation site that contains a number of putative DNA-binding motifs.

3.2. Deletion of CRE compromises TIGAR promoter activity

To better define the TIGAR gene promoter and identify putative binding elements, we gained a series of deletion amplicons of the 5′-flanking region and fused them to a luciferase reporter measurement. The constructs were then transfected into cell lines for promoter activity. Deletion of the regions from −54 bp to −4 bp lowered the promoter activity substantially (Fig. 1), consistent with the presence of an SP1-binding site located in this region. This

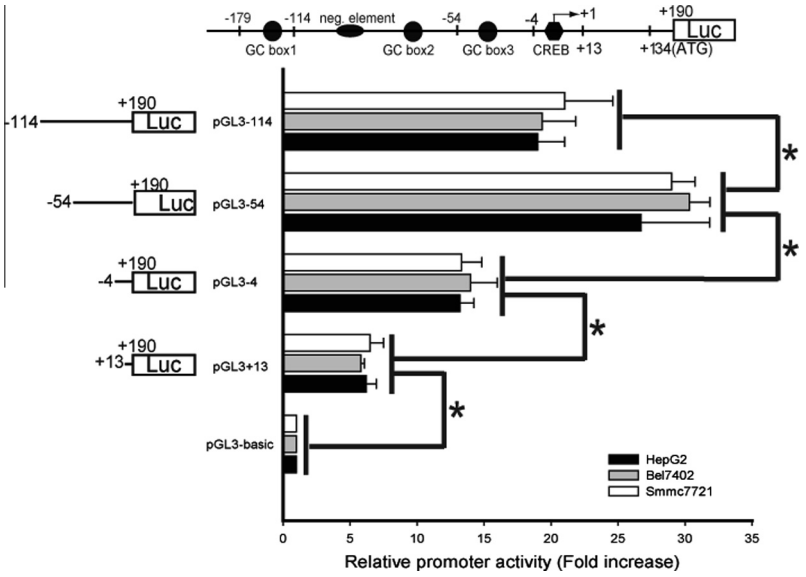

Fig. 1. Deletion analysis of the TIGAR promoter. The top figure shows a schematic diagram of the genomic 5′-flanking region and the putative motif locations. Filled circles represent GC boxes for sp1 binding, ellipses represent negative elements, and hexagons indicate the *creb* motif. Both arrow and +1 indicate the transcriptional start site. The various progressive deletion fragments were sub-cloned into luciferase reporter vector pGL3-basic. The left side of the diagram shows the individual promoter-luciferase reporter constructs, and the right side shows the promoter activities of constructs after transfection into cell lines. Specific luciferase activity was measured 24 h after transfection. Luciferase activity was normalized by co-transfection with a pRL-TK reporter plasmid that contained the *Renilla* luciferase gene. Results are shown as fold change from the empty pGL3-basic vector. The columns and bars in the graph represent the mean and the standard deviation, respectively. Each activity value represents the average of three independent experiments. The asterisks indicate statistically significant results (*$P < 0.05$).

motif has been found to positively regulate TIGAR expression [21]. Furthermore, deletion of an additional 17 bp from pGL3-4 to generate pGL3+13 resulted in a significant decrease in luciferase activity. Constructs containing site mutations of putative motif were also created and were used to characterize nucleotides that are indispensable for pGL3-4 promoter activity. CREB mt2 and CREBmt3 were found to attenuate the promoter activity of pGL3-4 by nearly 30% and 33%, respectively. However, CREB mt1 had no effect on pGL3-4 promoter activity (Fig. 2A).

3.3. Modulation of CREB function alters promoter activity and TIGAR expression significantly

siRNAs or over-expression plasmids of CREB were co-transfected with pGL3-4 in order to determine if CREB can influence TIGAR promoter activity or expression. CREB siRNA lowered the promoter activity of pGL3-4 by approximately 30% (Fig. 2B) and decreased the level of TIGAR mRNA by 25% (Fig. 2C). Conversely, the CREB over-expression plasmid enhanced TIGAR promoter activity by nearly 23% (Fig. 2D) and increased TIGAR mRNA expression by almost 20% (Fig. 2E). CREB siRNA was also found to down-regulate TIGAR protein levels, whereas CREB over-expression increased TIGAR protein expression (Fig. 2F).

3.4. CREB physically interacts with the CRE motif in the TIGAR promoter

To determine whether CREB can physically interact with the CRE motif within the TIGAR promoter region, EMSA and ChIP assays were performed. The specific DNA-protein complexes were detected from all cell line extracts (Fig. 3A–C). The presence of the DNA-protein complex was gradually abolished in the presence of an increasing amount of cold and self-probe competitors. Nevertheless, the complex still persisted even in the presence of 200-fold concentrations of mutant probes. ChIP assays indicated that the anti-CREB antibody could immunoprecipitate the TIGAR promoter, as demonstrated by the presence of a positive signal that was similar in size to the amplicon generated with Input DNA (Fig. 3D). Taken together, the results indicate that CREB can physically interact with the CRE-binding motif in the TIGAR promoter.

3.5. Forskolin exerts obvious effects on TIGAR promoter activity and TIGAR expression

Forskolin (Fsk) was used to provide further evidence to corroborate the hypothesis that CREB activates the TIGAR promoter resulting in increased TIGAR expression. Treatment with 50 nM Fsk was found to increase pGL3-4 activity by nearly 23%

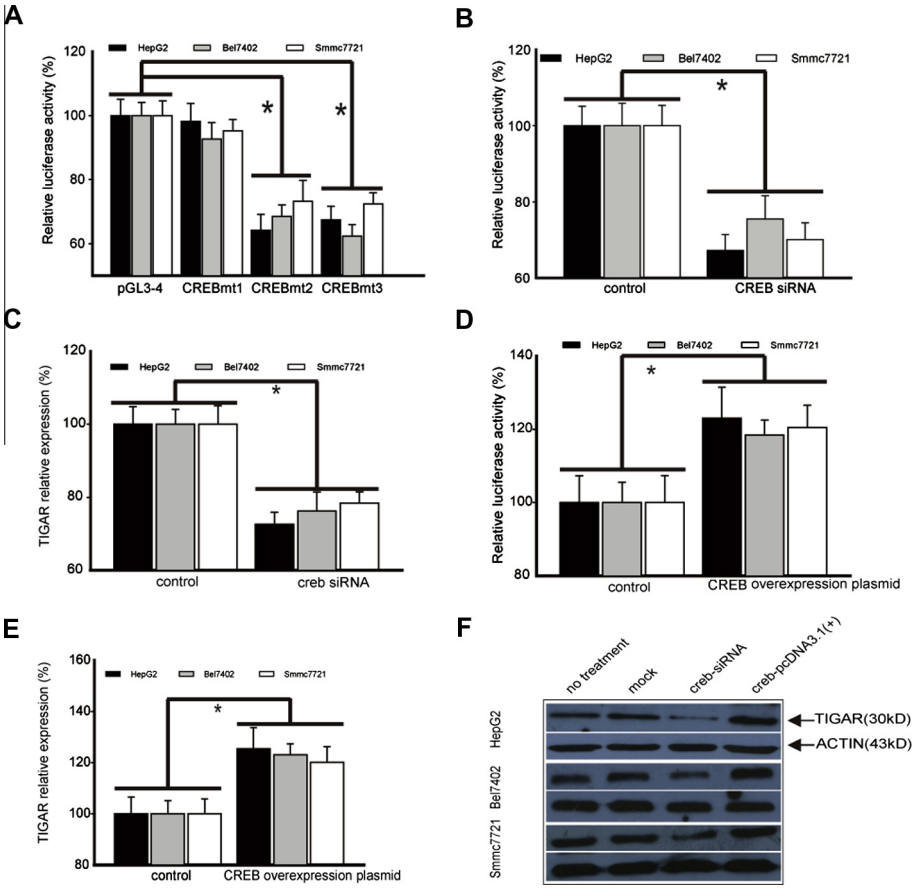

Fig. 2. The effects of treatment and site-directed mutagenesis of CRE on TIGAR promoter activity and expression. (A) Promoter-luciferase plasmid pGL3-4 was subjected to site-directed mutagenesis in order to generate three mutant constructs. The mutated sequences are listed in Table 1. All constructs were transfected into cells, after which promoter activities were measured. The data are represented as the mean firefly/*Renilla* luciferase ratio relative to the activity of the wild construct (designated as 100%). Each point is the mean ± SD of three assays performed in triplicate. Asterisks indicate statistical significance ($P < 0.05$) between groups. (B) Effects of a CREB siRNA on plasmid pGL3-4 activity. Plasmid pGL3-4 and CREB siRNA were co-transfected into cells. Promoter activities were then compared. Luciferase activity in the control pGL3-4 group was designated as 100%. (C) Effects of CREB siRNA on TIGAR gene expression. Control represents mock group. mRNA from the different groups was assessed by RT and real-time PCR. (D) Effects of CREB overexpression on pGL3-4 activity. Cells were subjected to transfected pGL3-4 either alone or with a CREB overexpression vector pcDNA(+3.1). (E) Effects of CREB overexpression on TIGAR gene expression. Treatment with mock transfection cells served as the control. (F) Analysis of the effects of the TIGAR protein expression. After transfection, the levels of TIGAR protein were determined by Western blotting.

(Fig. 4A). Likewise, Fsk strengthened TIGAR mRNA expression significantly, instead of CREB mRNA expression. In addition, it significantly enhanced both TIGAR and p-CREB protein levels, but not t-CREB (Fig. 4B).

4. Discussion

Human TIGAR shares highly conserved sequences with TIGAR of other species [2]; however, it is unclear whether the TIGAR promoter bears this character. We therefore aligned the promoter regions of different vertebrates and found that the TIGAR promoter regions were highly consistent among species (Supplementary Fig. S1). TIGAR function has been well demonstrated [3–8]; however, the mechanisms controlling its regulation are still unclear. Our previous study has demonstrated that SP1 participates in regulating TIGAR promoter activity [21]. Intriguingly, pGL3-4 construct lacking the SP1 domain retained robust promoter activity (Fig. 1), implying that there was an additional cis-element that regulates TIGAR expression. Software predictions, a possible CRE-binding site locating in the promoter region and CREB acting as a TF, were consolidated by results from Fig. 2. By comparing mutant probe sequences, a conclusion can be drawn that these two GT nucleotides contained in the native CRE site play pivotal roles in regulating pGL3-4 activity and are therefore probably indispensable for TIGAR expression. Besides, modulated CREB expression using CREB siRNA or a CREB over-expression plasmid further validate that CREB is responsible for pGL3-4 activity. Furthermore, the hypothesis that the transcription factor CREB is capable of interacting directly with CRE and further forming DNA-protein complex at the TIGAR promoter was testified by EMSA and ChIP assays (Fig. 3). Even though, we did not conduct a super-shift competition assay, the mutant probe could not compete with the native probe or eliminate DNA-protein complex formation, suggesting that the nucleotides in the motif were critical for its interaction with CREB. Forskolin (Fsk), a special agonist of adenylate cyclase that can elevate the cellular content of cAMP [22], provided us further evidence to confirm that CREB was a positive inducer of pGL3-4 (Fig. 4A). Interestingly, it did not change total CREB protein but increased the content of p-CREB instead (Fig. 4B). Fsk upregulated p-CREB, which has functional binding activity, and promoted increased p-CREB binding to the TIGAR promoter region. This subsequently enhanced pGL3-4 activity and TIGAR expression indirectly. This regulatory mechanism was also supported by our studies using CREB siRNA and expression plasmid, which collectively showed that CREB is a positive regulator of TIGAR protein (Fig. 2F).

TIGAR functions as a regulator of glycolysis [4], and therefore has a relationship with glucose as well as energy [7,8]. cAMP, which is a secondary messenger, has been implicated in TIGAR promote activity. Previous data reveal that increased cAMP can lead to double consequences. Firstly, by CRE–CREB interaction, it can induce more TIGAR expression, which contributes to the inhibition of glycolysis. Secondly, it can suppress gene p53 expression and glycolysis through the AMPK signaling pathway [9]. Therefore, increased cAMP can inhibit glycolysis through pathways. However, decreased p53 expression should result in TIGAR down-regulation rather than enhanced expression since p53 has also been shown to induce TIGAR expression [2]. These contradictory results would

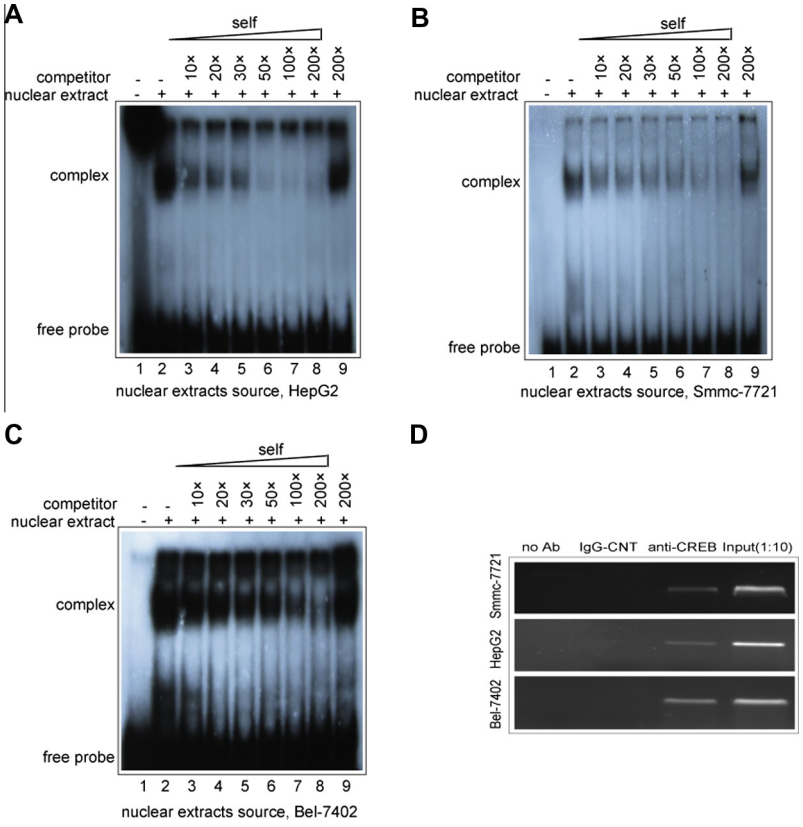

Fig. 3. EMSA and ChIP analysis of the physical interaction between putative CRE motif and the transcription factor, CREB. (A–C) EMSAs were conducted using the nuclear extracts from cell lines. DNA-protein complexes were resolved by native PAGE in TBE buffer. Gels were dried and exposed onto X-ray films overnight. The sample in lane 1 contains only genetic material and serves as an internal control. For competition experiments, indicated fold excess of unlabeled wild-type probe was added from lanes 2 to 8. Lane 9 contains a sample in which a mutant probe was used. Nucleic acid sequences for all probes are listed in Table 1. (D) ChIP assay was used to examine the interaction between the CRE motif and CBP. Experiments were performed with chromatin derived from cell lines. Immunoprecipitated chromatin was used for the PCR assay using specific primers and the amplicon was resolved on a 3% agarose gel. The expected size of the bands was 181 bp. Input DNA was used as a positive control for amplification.

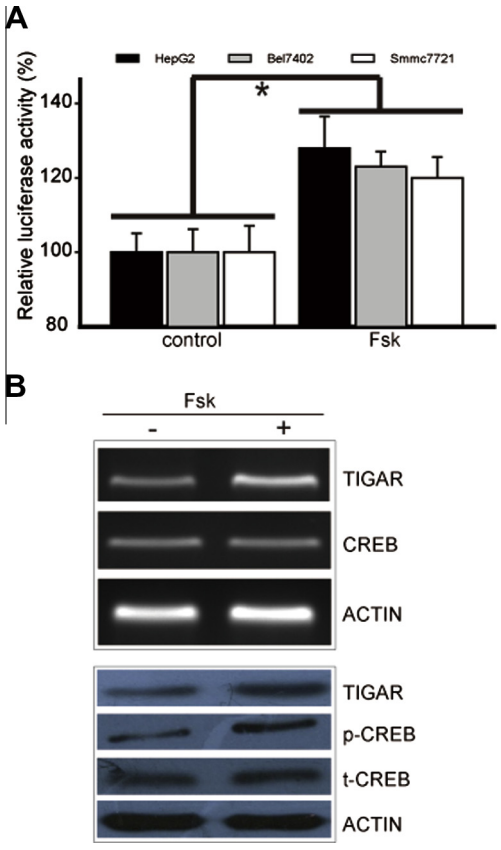

Fig. 4. Effects of forskolin on TIGAR promoter activity and protein expression. (A) After transfection with pGL3-4, cells were treated with or without forskolin (Fsk) for another 6 h. Promoter activities were determined and control groups were designated as 100%. The asterisk represents statistical significance between groups. (B) Analysis of Fsk effects on multiple proteins. Cells were grown in a medium containing Fsk for 72 h. After that cells were collected and lysed and then proteins were extracted. Expression of total CREB protein (t-CREB) and phosphorylated CREB (p-CREB) was then analysed.

suggest that there are other potential regulatory pathways that regulate p53-induced TIGAR expression. Under certain circumstances, one pathway may take priority over another. In our previous study, SP1 was identified as an activator of the TIGAR promoter [21]. This led us to extrapolate that SP1 and CREB could exert synergistic or additive effects on TIGAR promoter activity. However, results from experiments in which SPI and CREB siRNA or expression vectors were co-transfected suggest that they play equal and parallel roles in TIGAR promoter activity (Supplementary Fig. S2). But we prefer to believe that CREB acts as a more important role than SP1 because TSS locates within CRE. This hypothesis was mostly based on the fact that CREB may create a core promoter architecture that is suitable for the assembly of a pre-initiation complex [23]. Further research on the transcription factors that control TIGAR gene expression will be required in order to fully determine how it is regulated.

Acknowledgments

We would like to thank Professor Longhua Hu for discussions and assistance in data analysis.

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.bbrc.2013.08.098.

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