Serum withdrawal up-regulates human SIRT1 gene expression in a p53-dependent manner

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Abstract

SIRT1, a nicotinamide adenine dinucleotide (NAD\(^+\))-dependent histone/protein deacetylase, has been extensively studied recently for its critical role in the regulation of physiology, calorie restriction and aging. Studies on laboratory mice showed that expression of SIRT1 can be induced by starvation in a p53-dependent manner and requires the p53-binding sites present in the Sir1 promoter. However, it remains to be determined whether these findings based on rodents apply to human beings. In this paper, we characterized a putative p53-binding element in the human SIRT1 promoter that might be required for the up-regulation of SIRT1 in response to nutritional stress. The p53-binding site in the promoter of human SIRT1 is more deviant from the consensus sequence than the corresponding sequence in the mouse Sir1. There is a C to A change at the second half site in human SIRT1, thus disrupting the core-binding element CWWG in the canonical RRRCWWGGYY. To test whether such sequence change would affect its binding with p53 and the SIRT1 expression under stress, we studied various human cell lines with different p53 status and cells with ectopic expression of functionally distinct p53. We found that serum withdrawal also up-regulates human SIRT1 gene expression in a p53-dependent manner and that the p53-binding element in SIRT1 is required for the up-regulation. Thus, the mechanism responsible for the regulation of SIRT1 expression by p53 is conserved between mice and human beings.

Keywords: SIRT1 • nutritional stress • p53 • gene expression • promoter

Introduction

The silent information regulator 2 (Sir2) family of genes encodes a group of nicotinamide adenine dinucleotide (NAD\(^+\))-dependent histone/protein deacetylases (sirtuins) that are ubiquitously distributed [1, 2]. Sir2, the founding member of the family, was first characterized in yeast, and is a heterostructural component of silent chromatin that is required for transcriptional silencing of the silent mating-type loci, telomeres and rDNA repeats [3–5]. Besides gene silencing, Sir2 proteins were also implicated in the regulation of many biological processes such as cell cycle regulation [6], fatty acid metabolism [7] and lifespan extension [5, 8, 9]. Overexpression of Sir2 extends the lifespan of budding yeast, while its knockout shortens the lifespan by about 50% [8, 10]. A similar paradigm applies to C. elegans in which the dosage of Sir2.1 gene is positively correlated to lifespan [9]. SIRT1, the closest mammalian homologue of the yeast Sir2, has been extensively studied recently [11]. It regulates cell cycle progression, apoptosis, and other metabolic processes by interacting with a number of molecules, including the forkhead transcription factor, the tumour suppressor p53, DNA repair protein Ku70 and PPAR-γ [12–17]. It was found that overexpression of SIRT1 can elicit beneficial phenotypes resembling calorie restriction [18]. Nutrient withdrawal concomitantly augments the expression of the mouse Sir1 and activates the forkhead transcription factor Foxo3a in rodent cells [19]. Moreover, starvation-induced increase in Sir1 expression requires Foxo3a, and stimulation of Sir1 expression by Foxo3a was mediated through physical interaction between Foxo3a and p53.

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which binds to two p53-binding sites present in the mouse Sirt1 promoter [19].

The p53 plays a pivotal role in the maintenance of cellular homeostasis. It binds to DNA in a sequence-specific fashion. The consensus DNA-binding sequence for p53 consists of two decameric motifs or half-sites of the general form RYCCWGGYY (R = A, G; W = A, T; Y = C, T) separated by 0–13 base pairs [20]. Upon binding to DNA targets containing two half-site motifs, p53 functions as tetramers to activate or repress transactivation [21].

A sequence analysis indicated that the p53-binding sites in the promoter of human SIRT1 is more diverent from the consensus sequence than those in the mouse Sirt1. There is a C to A change at the second half site in human SIRT1. With the concern that such a deviation might affect the binding of the DNA sequence to p53, and consequently the transcriptional regulation of SIRT1 by p53 under stress, we determined whether nutritional withdrawal would up-regulate human SIRT1 expression in a p53-dependent manner as in the mouse. Using various human cell lines with different p53 status, we were able to confirm that serum withdrawal also up-regulates human SIRT1 gene expression in a p53-dependent manner.

Materials and methods

Chemicals and reagents

Anti-p53(DO-1)sc-126, anti-SIRT1(B-7) sc-74465 and anti-β-actin(AC-15)sc-68879 were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 and RNA isolation reagent TRIZOL were from Invitrogen (Carlsbad, CA, USA). Dual luciferase reporter assay kit and AMV Reverse Transcriptase were from Promega (Madison, WI, USA). Dulbecco's modified Eagle's medium, McCoy's5A medium, RPMI1640 and foetal bovine serum were obtained from Gibco (Carlsbad, CA, USA). All restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA, USA). Electrophoretic mobility shift assay was performed with the Roche (Basel, Switzerland) DIG Gel shift kit, second generation. Other chemicals were from Sigma (St. Louis, MO, USA) unless otherwise noted.

Cell culture

The human osteosarcoma U2OS and Saos-2 cells were cultured in McCoy's5A medium supplemented with 15% foetal bovine serum and 100 U/ml penicillin-streptomycin. The human hepatoma cell lines HepG2 and Hep3B cells as well as HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum and antibiotics. HEK293 cells were grown in RPMI1640 contained 10% foetal bovine serum. Cells were incubated in the humidified incubator equilibrated with 5% CO₂ at 37°C and passed using standard cell culture techniques.

RNA isolation and real-time PCR analysis

Total RNA was extracted using TRIzol reagents per the protocol provided by the supplier (Invitrogen). The first-strand cDNA was synthesized from 3 μg of total RNA by AMV reverse transcriptase (Promega). SIRT1 mRNAs were measured by TaqMan PCR assay in a 10-μl reaction volume on 96-well plates using the Applied Biosystems 7500 Real Time PCR System. The primers and TaqMan probes were designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). Transcript levels were normalized to GAPDH levels. The sequence of the primers were: human SIRT1 sense: 5’-AGAGGCTTCCTACATGCAAGCTCTTG-3’ and antisense: 5’-GCTGATTTCCCTTTTCTCGCACTCAC-3; probe: 5’-(FAM)ACGGGGCTCACAAGCTC-3’. For human GAPDH sense: 5’-CCAGGTTGGCTCCTCTGACCT-3’ and human p53 antisense: 5’-GGATGTAATGACCAATTTG-3’; probe: 5’-(FAM)AAAGCGGAAACACGTTG-3’. Cycle conditions for hSIRT1 were at 50°C for 2 min., at 95°C for 10 min. and 40 cycles each at 95°C for 15 sec and 60°C for 1 min.

Western blot analysis

Cells were harvested 16 hrs after serum starvation, and lysed with passive lysis buffer (Promega). The protein concentration was determined by BCA protein assay method (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. Samples containing equal amounts of protein (30 μg) were subjected to 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (0.2 μm, GE Healthcare, Little Chalfont, Buckinghamshire, UK). After blocking with 5% skim milk, blots were probed with anti-p53 antibody, anti-SIRT1 antibody or anti-β-actin (Santa Cruz Biotechnology at 1:500 dilution in 1% skimmed milk in TBS-T) at 4°C overnight. After washing with TBS-T, the membranes were treated with horseradish peroxidase-conjugated secondary antibody (1:10,000) for 1 hr and visualized by ECL PLUS (GE Healthcare) as specified by the manufacturer.

Generation of the SIRT1 reporter constructs

The human SIRT1 fragments with or without the putative p53 response elements were generated by PCR using a common reverse primer R-54 (5’-CCAGGCTTCTTCTCCACTGGCGT-3’) and the following sense primers that are flanked by the restriction sites (restriction sites are underlined): for construct P-158 which contains p53 response elements: 5’-CCCGTCGAAGACGCAAGCTCTCCGCC-3’; for construct P-111 (without p53 response elements): 5’-CCGTCGAAGCGCGCGG-3’; P-158mut which contains two point mutations in the core sequence of p53 response element: 5’-AGCGGAAACGCCTCGCGTCACG-3’. PCR reaction volume on 96-well plates using the Applied Biosystems 7500 Real Time PCR System. The primers and TaqMan probes were designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). Transcript levels were normalized to GAPDH levels. The sequence of the primers were: human SIRT1 sense: 5’-AGAGGCTTCCTACATGCAAGCTCTTG-3’ and antisense: 5’-GCTGATTTCCCTTTTCTCGCACTCAC-3; probe: 5’-(FAM)ACGGGGCTCACAAGCTC-3’. For human GAPDH sense: 5’-CCAGGTTGGCTCCTCTGACCT-3’ and human p53 antisense: 5’-GGATGTAATGACCAATTTG-3’; probe: 5’-(FAM)AAAGCGGAAACACGTTG-3’. Cycle conditions for hSIRT1 were at 50°C for 2 min., at 95°C for 10 min. and 40 cycles each at 95°C for 15 sec and 60°C for 1 min.

Transient transfection and luciferase assay

Transient transfections were performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded in culture medium without antibiotics at a density of 6 × 10⁴ cells/well in a 24-well plate for 24 hrs before transfection. For each well,
0.2 μg of the SIRT1 reporter constructs (deletion and mutated constructs) were cotransfected with 0.02 μg of pRL-TK vector that provides constitutive expression of Renilla luciferase serving as an internal control to normalize transfection efficiencies. Sixteen hours after transfection, cells were harvested, and luciferase activity was measured using Perkin-Elmer 1420 multilabel counter (Waltham, MA, USA). All transfection experiments were carried out in triplicate.

Electrophoretic mobility shift assay (EMSA)

Nuclear extract was prepared from U2OS cells as described previously [22]. The oligonucleotides corresponding to the putative p53-binding site found in the human SIRT1 promoter and its complementary strand were annealed and end-labelled with digoxin using T4 polynucleotide kinase. The sequence of wild-type and mutant p53 response element (sense strand) are 5′-CCACGTGACCCGTTAGTGTTGT-3′ and 5′-CCAGAGACCCGTTAGTGTTGT-3′, respectively. EMSA was performed according to the manufacturer’s instructions. Briefly, 2 μl of 0.4 ng/μl labelled probes were incubated with or without nuclear extract in gel shift binding buffer at room temperature for 20 min. For the competition assay, nuclear extracts were incubated with 100-fold amount of unlabelled competitor oligonucleotides for 10 min. prior to adding labelled wild-type probe. Supershift experiments were performed with a specific p53 antibody. The samples were resolved by 6% non-denaturing polyacrylamide gel, and electrophoretically transferred to a hybond-N nylon membrane (Amersham, Little Chalfont, Buckinghamshire, UK). The nylon membranes were then blocked with the blocking buffer supplied in the kit, incubated with the primary antibody for 1 hr at room temperature, followed by CSPD-conjugated secondary antibody, and visualized by ECL PLUS (GE Healthcare).

Statistical analysis

The statistical significance of differences between experimental groups was calculated using Student’s test. Groups were considered statistically different if P < 0.05.

Results

Up-regulation of human SIRT1 gene expression in response to serum withdrawal was dependent on p53

A previous study showed that expression of the mouse Sirt1 could be induced by acute nutrient withdrawal, and the induction was mediated via two p53-binding sites present in the Sirt1 promoter in a p53-dependent manner [19]. We therefore reasoned that SIRT1 induction by serum deprivation in human cell lines may similarly be regulated in a p53-dependent manner. To determine whether p53 affected the SIRT1 induction in human cells subjected to serum withdrawal, we examined the SIRT1 levels in six cell lines of different p53 status, four cell lines with wild-type p53 (HEK293, HeLa, HepG2, and U2OS) and two p53-null cell lines (Saos-2 and Hep3B), under normal growth condition or under serum starvation. The mRNA and protein levels of SIRT1 were measured by real-time PCR and Western blot, respectively. As shown in Fig. 1A–C, serum withdrawal resulted in a significant induction of SIRT1 at both mRNA and protein levels in the p53-functional, but not p53-null, cells, suggesting that SIRT1 induction by serum withdrawal could be p53 dependent. In consistence with a requirement of p53 function for SIRT1 induction, the p53 level was concomitantly increased 16 hrs after serum withdrawal (Fig. 1C).

The p53 response element in the promoter of human SIRT1 gene was required for the induction of the SIRT1 expression by serum withdrawal

The p53 target genes typically contain p53 consensus binding sites in their promoters or other regulatory regions. Mouse Sirt1 gene contains two p53-binding sites in its promoter [19]. However, an analysis using software MatInspector indicated that the p53 response element in human SIRT1 promoter differs from that in the mouse Sirt1 promoter at one position (Fig. 2A). There is a C to A substitution in the second half site. This represents a further deviation from the consensus binding site than the mouse Sirt1 promoter and might impair its binding with p53 and, consequently lead to reduced induction of SIRT1 expression in response to nutritional stress in human cells. To examine this, we first tested if there is a functional difference in the promoters of mouse Sirt1 and human SIRT1. As shown in Fig. 2B, the luciferase reporter containing the human SIRT1 promoter showed about half of the activity of the reporter containing the mouse Sirt1 promoter, under normal growth condition and under nutritional stress. Although human SIRT1 promoter had a lower reporter activity than the mouse counterpart, it was still more than 40 times higher than the promotorless pGL3-basic control. This result indicated that the human SIRT1 promoter may possess a similar function as the one in the mouse, although the p53 responsive element it contains is more deviant from the consensus sequence.

We next focused on the characterization of promoter activity of human SIRT1. Two luciferase reporters driven by the promoter with (P-158) or without (P-111) p53 response element were constructed and transfected into two p53-expressing cell lines, U2OS and HepG2 cells, and luciferase activity was measured after serum deprivation. Consistent with the above findings, the stimulatory effect of serum withdrawal was observed for the construct containing p53 response element, but not in P-111 construct in which the p53 response element was deleted, indicating that the p53 response element is essential for the induction of SIRT1 promoter activity in response to serum deprivation (Fig. 2C). To further confirm this observation, two point mutations were introduced into P-158 construct to generate a p53 mutant construct, P-158mut, that have the core of p53 consensus sequence changed from CGTG to AGTG. The mutation of p53 response element abolished the stimulatory effect of serum withdrawal (Fig. 2C). Notably, although the
Fig. 1 Induction of SIRT1 expression by serum starvation in human cell lines is dependent on p53. The cells were grown under normal nutrients (C) or under serum starvation (S) for 16 hrs. Real-time PCR analysis and Western blot were performed as described in the Material and Methods. Significant induction of SIRT1 expression was observed in p53-expressing cells (HEK293, HeLa, U2OS and HepG2), but not p53-null cells (Saos-2 and Hep3B). GAPDH RNA and β-actin protein were used as normalizing control for RNA and protein quantification assay, respectively. (A) and (B), mRNA levels quantified with real-time PCR. (C) Protein levels determined by Western blot.
A

p53 consensus  
Mus Sirt1 p53 RE  R R C W W G Y Y Y n R R R C W W G Y Y Y
Hum SIRT1 p53 RE  C C A C G T G A C C 0 C G G C  G T G T T G
Hum SIRT1 p53 RE mut  C C A A G A G A C C 0 C G G A G T G T T G

B

C

D
Withdrawal requires p53 protein transfected with luciferase reporter constructs containing SIRT1. Cells and Hep3B cells, which lack endogenous p53 protein, were harvested 16 hrs after treatments and luciferase activities were determined. The activity of the construct containing mouse Sirt1 or human SIRT1 was transfected into HEK293 and U2OS cells and cells were maintained in normal nutrients (C) or under serum starvation (S). Each bar represents the value of mean ± S.E.M. (D) Physical binding of p53 response element with p53. The protein-DNA complexes were resolved by native polyacrylamide gel electrophoresis. The retarded mobility of the oligonucleotide probe was observed in the presence of nuclear extract, lane 2. The formation of DNA-protein complex was competitively inhibited by 100-fold molar excess of unlabelled (cold) wild-type oligonucleotides, lane 3, but not by the same amount of cold mutant oligonucleotides. The retarded mobility of the probe in the presence of p53 protein was supershifted with p53 antibody, lane 5.

**Up-regulation of SIRT1 in response to serum withdrawal requires p53 protein**

If up-regulation of the human SIRT1 expression is p53 dependent, one would predict that this stimulatory effect by serum withdrawal would be abolished in p53-deficient cells. Towards this end, Saos-2 cells and Hep3B cells, which lack endogenous p53 protein, were transfected with luciferase reporter constructs containing SIRT1 promoter. As shown in Fig. 3A, luciferase activities of the reporters driven by the SIRT1 promoter were not significantly increased by serum deprivation in the absence of p53 protein, although the p53 response element was present.

To further address the impact of p53 expression on up-regulation of human SIRT1 promoter, Saos-2 and Hep3B cells were cotransfected with the P-158 construct and the expression vectors encoding either wild-type p53 or a truncation mutant. The introduction of the wild-type p53 led to a significant induction of SIRT1 upon serum withdrawal. In contrast, cotransfection of a vector expressing p53 truncation mutation was unable to restore the stimulatory effect of serum withdrawal in p53-deficient cells (Fig. 3B).

**Discussion**

Collectively, our results demonstrated that up-regulation of human SIRT1 expression in response to serum withdrawal is p53 dependent. This conclusion was supported by several lines of evidence. First, serum withdrawal resulted in a significant induction of SIRT1 at the mRNA and at the protein level in p53-expressing cells, but not p53-null cells. Second, deletion or mutation of the p53 response element in the SIRT1 promoter abolished the stimulatory effect of serum deprivation. Third, the SIRT1 promoter was unable to drive the luciferase reporter in response to serum starvation in p53-null cells. Fourth, cotransfection assay indicated that re-introduction of wild-type p53, but not truncated (mutant) p53, could restore the stimulatory effect of serum withdrawal in p53-deficient cells. These findings indicated that the up-regulation of human SIRT1 expression under the nutrient stress is mediated in a p53-dependent manner.

Sirtuins are up-regulated by various biological stresses including caloric restriction. Caloric restriction has been shown to extend lifespan and prevent numerous diseases associated with aging in mammals [10, 17, 23–26]. The induction of SIRT1 expression under various stress conditions probably acts as a protective adaptation response. Nemoto et al. reported that acute nutrient withdrawal could simultaneously augment the expression of the Sirt1 and activate the Foxo3a, a forkhead transcription factor, in rodent cells. Knockdown of Foxo3a expression inhibited the
starvation-induced increase in Sirt1 expression. Furthermore, they found that stimulation of Sirt1 transcription by Foxo3a was mediated via a p53-binding site (two half sites) in the mouse Sirt1 promoter, and there was a nutrient-sensitive physical interaction between Foxo3a and p53. Sirt1 expression was not induced in starved p53-deficient mice [19]. These results suggested that in mammalian cells, p53, Foxo3a, and SIRT1, which are all implicated in aging, constitute a nutrient-sensing pathway. Consistent with these observations, we found that serum withdrawal also up-regulates human SIRT1 gene expression in a p53-dependent manner and the p53-binding element in SIRT1 is required for the up-regulation.

The p53 protein is estimated to regulate several hundred target genes that are involved in pathways like apoptosis, DNA damage repair and cell growth arrest [27]. According to in vitro experiments, the p53 protein binds specifically to a palindromic consensus sequence, RRRWWGGYYY (R = A, G; W = A, T; Y = C, T) separated by 0–13 base pairs [20], with nearly all response elements containing at least one mismatch; in vivo results have suggested that the spacer region may be much smaller [27]. It was suggested that the ‘rules of engagement’ for p53 response elements may differ

Fig. 3  p53 protein is required for the induction of human SIRT1 promoter activity by serum withdrawal. (A) Constructs with (P-158) or without p53 response element (P-111) were transfected into either p53-positive cells (U2OS and HepG2) or p53-null cells (Saos-2 and Hep3B), and the relative luciferase activity was determined. The activity under normal nutrient (C) was arbitrarily set to 100%, and the relative luciferase activity under starved condition (S) was calculated accordingly. Each bar represent the value of mean ± S.E.M. (B) Saos-2 and Hep3B cells were cotransfected with the P-158 construct along with the expression plasmids encoding either wild-type p53 (p53 wt) or a truncation mutant (p53mut), and the luciferase activity was determined. The activity under normal nutrient (C) was arbitrarily set to 100%, and the relative luciferase activity under starved condition (S) was calculated accordingly. Each bar represents the value of mean ± S.E.M.
depending on the activated pathway, particularly in the regulation of apoptosis and cell-cycle checkpoints [28]. Although some p53-binding sites match the consensus sequence quite well, others can be consensus poor and yet they are all essential, and efficient, in the transactivating process [29]. Although p53-binding sites in human SIRT1 promoter differ from those in mouse Sirt1 promoter, our experiments indicated that this response element is essential and sufficient to mediate starvation-induced increase in SIRT1 expression. Interestingly, a comparison of mouse Sirt1 and human SIRT1 promoters in their ability to drive luciferase reporter revealed that the fragment in human SIRT1 promoter was about half as efficient as the one in mouse Sirt1 promoter. Probably, the sequence variation in p53-binding element in human SIRT1 promoter, which is more deviant from the consensus p53-binding sequence, rendered a lower p53-binding capacity and led to a lower transcriptional activity. Nevertheless, the p53-binding element in human SIRT1 promoter is functional in response to nutrient stress. This finding suggests that the mechanism responsible for p53-mediated regulation of SIRT1 expression is conserved between mice and human beings.

It should be noted that although the up-regulation of SIRT1 expression stimulated by serum starvation requires p53 and Foxo3a, SIRT1 also down-regulates p53 and Foxo3a proteins [16, 30, 31]. SIRT1 has been demonstrated to bind and deacetylate p53 in vitro and in vivo and to attenuate its ability to transactivate its downstream target genes, such as p21 for cell-cycle arrest and Bax for apoptosis [16, 30]. Overexpression of sirtuins has been shown to inhibit p53-dependent apoptosis in response to DNA damage and oxidative stress [16]. SIRT1 interacts with Foxo3a in a similar manner as it does with p53. In response to serum withdrawal, Foxo proteins move from cytoplasm to the nucleus, where they function as transcription factors to activate or repress a suite of genes, including SIRT1. On the other hand, SIRT1 deacetylates and represses the activity of Foxo3a [31], paralleling its effect on p53. Thus, under nutritional stress, p53 and Foxo proteins promote SIRT1 expression, which serves as a protective mechanism against excessive apoptosis. Thanks to deacetylation activity of SIRT1, p53 and Foxo proteins are only kept at appropriate levels. Therefore, SIRT1 interacts with p53 and Foxo in a feedback loop. Such feedback loops are critical in the maintenance of homeostasis.

It remains to be determined how serum withdrawal activates p53. A recent study showed that glucose deprivation can activate AMPK, which in turn activates p53, via phosphorylation on serine 15 and induces cell cycle arrest [32]. However, it is unknown if changes of SIRT1 expression are involved in this process.

In summary, our results demonstrate that the up-regulation of human SIRT1 expression in response to serum withdrawal is p53 dependent. The mechanism responsible for the regulation of SIRT1 expression is conserved between mice and human beings. These findings should be helpful in understanding other factors involved in the regulation of SIRT1 gene expression in human beings.

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