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The characterization of the human Siah-1 promoter[1]

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Abstract Siah-1, the human homologue of *Drosophila* seven in absentia, is related to apoptosis and tumor suppression. Although it was reported that the expression of Siah-1 is induced by p53 and p21/WAF1, little is known about the transcriptional regulation of the Siah-1 gene. To investigate the transcriptional regulation, we isolated and sequenced the genomic fragment of the Siah-1 promoter region. The Siah-1 promoter has no typical TATA box or CCAAT box. Transient transfection assays using reporter plasmids in which the promoter region of the Siah-1 gene was deleted or mutated showed that one Sp1 site was responsible for the basal promoter activity. In Northern blotting analysis, the expression of the Siah-1 gene was upregulated by p53, but activation of the reporter plasmid by the p53 co-transfection assay was not shown, suggesting that a p53 responsive element does not exist in the promoter region we examined in this study but might be present in another region. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Siah-1; Promoter; p53; Sp1

1. Introduction

Siah-1 is a human homologue of the *Drosophila* seven in absentia (SINA), which plays an important role during eye development [1]. Neuronal differentiation of the R7 photoreceptor cell in the *Drosophila* eye requires SINA, the transcription repressor Tramtrack [2] and PHYL, which is induced by the RAS pathway [3]. SINA binds UBCD1 (E2) via the N-terminal RING finger domain and interacts with PHYL, and the complex induces degradation of Tramtrack via the ubiquitin proteasome pathway [4].

Siah family proteins, such as Siah-1 and Siah-2 in human, are widely conserved in *Drosophila* and vertebrates [5]. The Siah-1 gene product also has a RING finger domain that displays 76% amino acid identity with the SINA protein [6]. The N-terminal RING finger domain of the Siah-1 protein also interacts with ubiquitin conjugating enzyme (E2) [7] and promotes degradation of various target proteins via the ubiquitin-proteasome pathway. So far, it has been reported that mammalian Siah proteins promote the proteolysis of DCC, N-CoR and c-Myb via the ubiquitin-proteasome pathway [8–11].

On the other hand, the tumor suppressor gene p53 is most frequently mutated in human malignant tumors [12]. In response to DNA damage, p53 induces apoptosis or cell cycle arrest. p53 stimulates the degradation of c-Myb, which is mediated by induction of Siah-1 in certain types of cells [11]. It was recently revealed that Siah-1 induced by p53 interacts with SIP, SKip and Ebi to promote degradation of β-catenin by the ubiquitin-proteasome pathway [13,14]. Moreover, the expression of Siah-1 was induced in KS cells having lost the malignant phenotype. The KS cells were derived from K562 cells, and the KS cells interestingly reexpressed p53 [15,16], raising the possibility that p53 might induce Siah-1 to suppress the malignant phenotype. Actually, the overexpression of p53 was shown to increase human Siah-1 mRNA in 293 cells [17].

As mentioned above, the Siah-1 gene may be an important molecule for tumor suppression and apoptosis. However, the transcriptional regulation of the Siah-1 gene is unclear. To investigate the expression machinery of the Siah-1 gene, we cloned the Siah-1 promoter, and we clarified the basic structure of the human Siah-1 gene.

2. Materials and methods

2.1. Genome screening of Siah-1 promoter region

A λPS library from human peripheral blood leukocytes (Mo Bi Tec, Göttingen, Germany) was screened by two oligonucleotide probes which contained the Siah-1 cDNA exon 1 sequence. The sequences of the probes are as follows; Siah-b [5′-GACGGAGCGCGT-TGGTGCCAGGACCGGGGT-3′] (sense) and Siah-a [5′-TTCC-CGGCGCCGAGACCGACGGGACACCCT-3′] (antisense). Approximately 3.8×10^6 plaques were lifted on Hybond N nylon filters (Amersham). The filters were hybridized with end-labeled oligonucleotide probes at 50°C overnight and washed at room temperature. Isolated clones were confirmed by Southern blot and sequence analysis using oligonucleotides Siah-a and Siah-b.

2.2. Plasmid construction and mutagenesis

The Siah-1 promoter region with 3528 bp was subcloned into the luciferase reporter plasmid, pGVB2 (Toyo Ink, Tokyo, Japan). Deletion mutants were generated using the Blunt end kit (Takara, Tokyo, Japan) at specific restriction enzyme sites. Plasmids with point mutations at Sp1 sites were generated by the Quick Change Site-Directed Mutagenesis Kit (Stratagene).

2.3. Cell culture

Human embryonic kidney 293 cells, human osteosarcoma Saos-2 cells and breast cancer MCF7 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and incubated at

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[1] The nucleotide sequence of the Siah-1 gene promoter can be found in the DDBJ and GenBank databases with the following accession number: AB072970.

Abbreviations: Siah-1, seven in absentia homologue 1

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37°C in a humidified atmosphere of 5% CO_2. Human erythroleukemia K562 cells were cultured in RPMI with 10% fetal bovine serum.

2.4. Luciferase assay

Breast cancer MCF7 cells were transfected with 2 μg of reporter plasmid by the DEAE-dextran method and the cells were harvested 48 h after transfection. In p53 co-transfection assays, 0.5 μg of reporter plasmid and 0, 25 or 250 ng of p53 expression plasmid were transfected into Saos-2 cells with Lipofectamine Plus reagent (Gibco BRL). After 24 h, the cells were harvested. Luciferase activity was analyzed using the PicaGene luminescence kit (Toyo Ink). Relative luciferase activity was standardized by the protein concentration of each cell lysate. All transfection assays were carried out in triplicate. Each experiment was repeated at least three times. Data are shown as means ± S.D. ($n = 3$). Data were analyzed using the Student's *t*-test and differences were considered significant from controls when $P < 0.05$.

2.5. Northern blotting

Human osteosarcoma Saos-2 cells were plated in 10 cm dishes 24 h before transfection of 10 μg of p53 expression plasmids by Lipofectamine Plus reagent. The 293 cells and K562 cells were treated with the same procedure. Total RNA was extracted at each indicated time. Total RNA (10 μg) was run on a denaturing agarose gel and transferred to a Nylon membrane (PALL, Biodyne). After hybridization with ^{32}P-labeled cDNA probes, signals were detected by autoradiography and with a Bioimaging analyzer (BAS2000; Fuji Film, Tokyo, Japan).

3. Results

3.1. Cloning and characterization of 5′-flanking region of the human Siah-1 gene

We obtained a 16.5 kb Siah-1 genomic fragment containing the 5′-flanking region of exon 1 by screening the human genomic library from peripheral blood leukocytes. We sequenced a 3528 bp fragment upstream of exon 1. As partially shown in Fig. 1, the upstream region of exon 1 from −544 to +183 has an especially high GC content (76.1%). By computer analysis, we could find the elements that are related to differentiation and cell cycle regulation factors, such as GATA-1, CdxA, nuclear factor-κB, v-Myb, E2F and so on (Fig. 1). The 5′-flanking region of the Siah-1 gene has neither typical

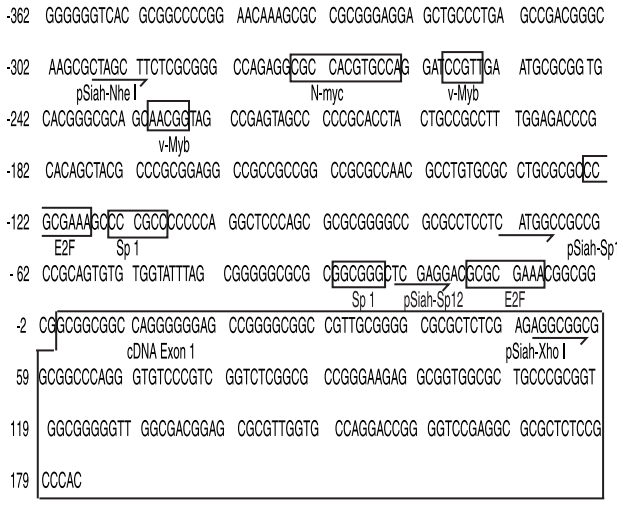

Fig. 1. Sequence of the Siah-1 promoter. The potential binding sites of transcription factors and exon 1 are boxed. Arrows in the DNA sequence indicate the 5′-terminus of deletion mutants. In this report, we assume that the most upstream exon 1 of the Siah-1 cDNA previously reported (GenBank accession number U76247) was at position +1.

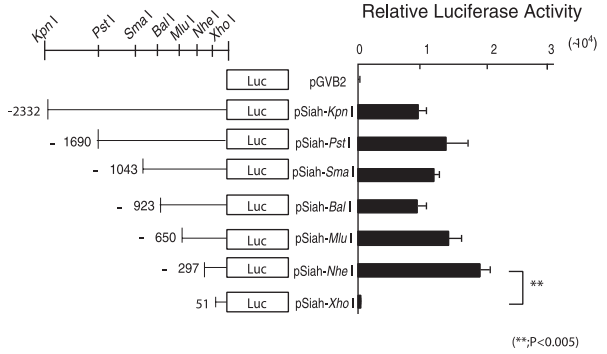

Fig. 2. Deletion analysis of the Siah-1 promoter in MCF7. Each plasmid (2 μg) was transiently transfected into MCF7 cells by the DEAE-dextran method. Luciferase activity was analyzed 48 h after transfection. Relative luciferase activity was standardized by protein concentration. Data are shown as means ± S.D. ($n = 3$). $^{**}P < 0.005$.

TATA box nor CCAAT box, but there are three copies of Sp1 sites. We also found two incomplete p53 consensus sequences which have one point mutation in two copies of a 10 bp motif 5′-PuPuPuC(A/T)(A/T)GpyPyPy-3′ [18] at positions from −3236 to −3207 and −618 to −596. We could not identify the transcriptional start site of the Siah-1 gene using a primer extension assay, Cap Site cDNA dT (Nippon Gene) or RNase protection assay presumably due to the GC-rich sequence of exon 1.

3.2. Promoter activity of the Siah-1 gene

The 2515 bp DNA fragment of the 5′-flanking region of exon 1 was subcloned into the pGVB2 luciferase reporter plasmid and a transient transfection assay was performed (Fig. 2). The pSiah-*Kpn*I/−2332 construct demonstrated enough luciferase activity compared with that using negative vector pGVB2. This result indicates that the 2515 bp DNA fragment (pSiah-*Kpn*I) has authentic promoter activity. Next, we constructed several deletion mutants and measured each of their promoter activities. We observed the maximum promoter activity when the deletion mutant pSiah-*Nhe*I/−297 was transfected into MCF7 cells. The luciferase activity of pSiah-*Nhe*I was higher than those of the longer constructs, pSiah-*Mlu*I/−650 or pSiah-*Bal*I/−923, which suggests the existence of a repression site between −923 and −298. The luciferase activity significantly decreased between pSiah-*Nhe*I

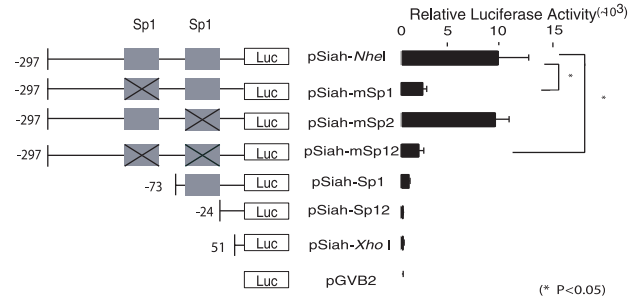

Fig. 3. Mutation analysis to identify the basal promoter activity of the Siah-1 gene in MCF7. Each construct is shown schematically on the left. pSiah-mSp1, pSiah-mSp2 and mSp12 mutants contained the following sequence: pSiah-mSp1 (−114 to −109: cccgcc caagtt), pSiah-mSp2 (−31 to −26: ggcggg aacttg) and pSiah-mSp12 plasmid has two mutations of the Sp1 sites. Data are shown as means ± S.D. ($n = 3$). $^{*}P < 0.05$.

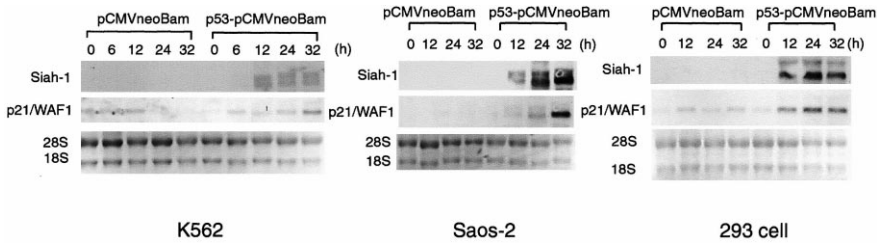

Fig. 4. Induction of Siah-1 mRNA by wild-type p53. Northern blot analysis was performed using total RNA from the indicated cell lines transfected with a p53 expression plasmid or a negative control plasmid at the indicated time. The filter was probed with Siah-1, and rehybridized with p21/WAF1 cDNA.

and pSiah-*Xho*I/+51. We considered that there might be important elements for the basal promoter activity between −297 and +50.

3.3. Two Sp1 sites are involved in transcriptional activation of the Siah-1 promoter

We found two Sp1 sites between −297 and +50 (Fig. 1). Therefore, we hypothesized that these two Sp1 sites might contribute to the basal promoter activity of Siah-1. To determine whether these Sp1 sites are responsible for basal promoter activity, we constructed reporter plasmids containing a deletion or point mutation in two Sp1 sites (Fig. 3). A significant reduction was observed using pSiah-mSp1, pSiah-Sp1 and pSiah-mSp12. These constructs have deletions or point mutations in the 5′-Sp1 site (−114 to −109). These results indicated that the 5′-Sp1 site was important for basal promoter activity, and that the 3′-Sp1 site (−31 to −26) does not contribute to the basal promoter activity.

3.4. Transcriptional activation of Siah-1 by the tumor-suppressor gene p53

Since the Siah-1 promoter region has two incomplete p53 consensus sequences from −3236 to −3207 and from −618 to −596, we examined whether its expression is induced by wild-type p53 via the promoter region. First, we carried out Northern blotting to examine the endogenous Siah-1 induction by p53 using three cell lines, the osteosarcoma cell line Saos-2 (p53 null), the human kidney epithelial cell line 293 (p53 is inactivated by infection of adenovirus) and the human erythroleukemic cell line K562 (p53 mutation) (Fig. 4). The pCMVneoBam vector was used as a negative control for the p53 expression plasmid. We detected two transcripts that might be alternative splicing products, which have been previously reported [15]. The induction of Siah-1 mRNA was increased in a time-dependent manner by wild-type p53 transfection but not by a control vector in each cell line. The expression of p21/WAF1 increased simultaneously with Siah-1 expression (Fig. 4).

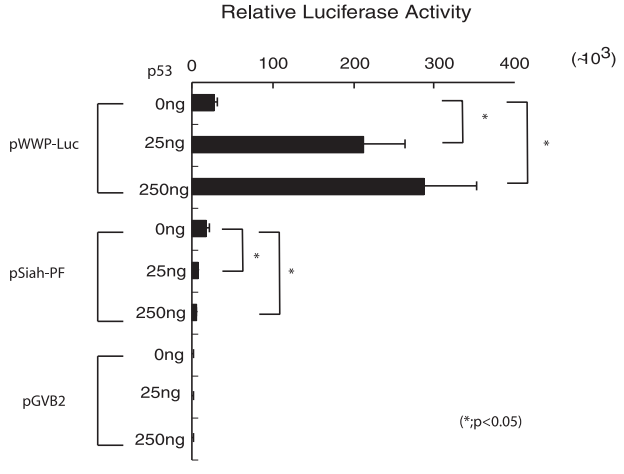

Fig. 5. p53 does not transactivate the Siah-1 promoter in Saos-2 cells. The full-size Siah-1 reporter plasmid, pSiah-PF, containing the promoter region spanning from −3345 to +183 and p21/WAF1 reporter plasmid, pWWP-Luc, as a positive control, were transiently transfected with p53 expression plasmid, or a negative control plasmid. Luciferase activity was analyzed 24 h after transfection. Data are shown as means ± S.D. ($n = 3$). *$P < 0.05$.

3.5. The Siah-1 promoter activity is downregulated by p53

We constructed a Siah-1 reporter plasmid containing two incomplete p53 consensus sequences, pSiah-PF (Fig. 5), and performed a transient transfection assay with pSiah-PF and an increasing amount of p53 expression plasmid using Saos-2 cells (Fig. 5). Unexpectedly, the luciferase activity of pSiah-PF was decreased by p53 in a dose-dependent manner. On the other hand, the luciferase activity of the p21/WAF1 reporter plasmid, pWWP-Luc, as a positive control was increased by p53 in a dose-dependent manner.

4. Discussion

4.1. The characterization of the Siah-1 promoter

It has been reported that the Siah-1 gene is related to apoptosis and cell growth inhibition and is induced by the p53 tumor suppressor gene and p21/WAF1 [19,20]. However, little was known about the machinery of Siah-1 expression. Therefore, we clarified the basic structure and activity of the Siah-1 promoter. At first, we cloned and sequenced the Siah-1 genomic fragment. We searched for putative transcription factor binding sites in the 5′-flanking region of the Siah-1 gene. We found elements related to differentiation and cell cycle regulation factors. Siah-1 may mediate signals of these factors and induce differentiation and cell cycle regulation. Further investigation will be required to clarify the mechanisms of expression and physiological function of the Siah-1 gene.

4.2. Sp1 site is important for promoter activity of Siah-1

We found two Sp1 sites spanning from −114 to −109 and from −31 to −26 (Fig. 1). The 5′-Sp1 site (−114 to −109) was shown to be essential for the basal promoter activity (Fig. 3). There was neither a TATA box nor a CCAAT box in the proximal promoter region of the Siah-1 gene. TATA-less promoters such as housekeeping gene promoter are GC-rich and

contain a CCAAT box and a GC box, like the Sp1 site, as core promoter elements instead of a TATA box [21,22]. The Sp1 site (−114 to −109) may act as a core promoter element in the Siah-1 gene promoter.

4.3. Regulation of Siah-1 by p53

The overexpression of p53 in 293 cells induced Siah-1 mRNA undergoing cell growth inhibition [17]. We also confirmed that Siah-1 mRNA was increased by p53 overexpression in three different cell lines (Fig. 4). Next, we carried out a p53 co-transfection reporter assay to investigate whether Siah-1 expression might be regulated by p53 via the Siah-1 promoter region, which we isolated in this study. However, our reporter plasmid had no response to p53. These results indicated that p53 might activate the Siah-1 gene through another region. For example, other p53 target genes such as GADD45, DR5, p53R2, IGF-BP3 and PUMA [23–28] have p53 responsive elements in the intron region. The Siah-1 gene may also have intronic p53 responsive elements. Otherwise, Siah-1 induction by p53 may not be caused by transcription but by mRNA stabilization.

4.4. Further possibilities for clinical application using the Siah-1 promoter

In this study we confirmed that p53 induces Siah-1 gene expression. p53 is inactivated in half of malignant tumor cells, which cause disordered cell proliferation. Therefore, activation of Siah-1 should compensate for the loss of function of p53. We proposed that methods for upregulating p53 target genes would be useful for cancer therapy, and termed this method 'gene-regulating chemotherapy' [29,30,31]. As a model of this, histone deacetylase inhibitors, such as butyrate or trichostatin A, stimulate the p21/WAF1 gene, a p53 target gene, through the Sp1 sites of the promoter independent of p53, resulting in cell cycle arrest [32,33]. Therefore, our findings and methods in this study may be suitable for screening the regulators of Siah-1 expression. The screening of these regulators may be important in the development of novel cancer therapy as well as understanding the physiological function of Siah-1.

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