Identification and preliminary functional analysis of alternative splicing of Siah1 in Xenopus laevis

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Abstract

Siah proteins are vertebrate homologs of the Drosophila ‘seven in absentia’ gene. In this study, we characterized two splicing forms, Siah1a and Siah1b, of the Xenopus seven in absentia homolog 1 gene (Siah1). Overexpression of xSiah1a led to severe suppression of embryo cleavage, while that of xSiah1b was not effective even at a high dose. Competition analysis demonstrated that co-expression of xSiah1a and 1b generated the same phenotype as overexpression of xSiah1a alone, suggesting that xSiah1b does not interfere with the function of xSiah1a. Since xSiah1b has an additional 31 amino acids in the N-terminus compared to xSiah1a, progressive truncation of xSiah1b from the N-terminus showed that inability of xSiah1b to affect embryo cleavage was associated with the length of the N-terminal extension of extra amino acids. The possible implication of this finding is discussed.

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1. Introduction

The seven in absentia homolog (Siah) proteins are homologs of the Drosophila seven in absentia (sina) gene. This gene codes for a protein downstream from the Sevenless tyrosine kinase receptor that degrades the transcriptional repressor Tramtrack and is required for R7 cell fate specification during Drosophila eye development [1–3]. Humans have two unlinked Siah genes, SIAH1 and SIAH2 [4], but mice have two Siah1 (Siah1a and Siah1b) and single Siah2 genes [5]. The Siah1 protein contains an N-terminal RING finger domain, required for interaction with E2 ubiquitin conjugating enzymes [6], as well as a coiled coil domain to form homo- and heterodimers [7]. As an essential component of E3 ubiquitin ligase complexes, Siah1 is responsible for the ubiquitin proteasome degradation that terminates cellular signaling and also for turnover of many key proteins, such as DCC [8], β-catenin [9], Kid [10], c-Myb [11], OBF-1 [12,13], Numb [14], Synaptoophysin [15], TIEG-1 [16], Synphilin-1 [17], Ctip [18], T-STAR [19], Polycystin [20], A4f [21], PHD1 and PHD3 [22], HIPK2 [23], FIH [24,25], PLC epsilon [26] and TRB3 [27].

Siah1a knockout mice exhibit severe growth retardation and male sterility due to a blockade in spermatogenesis [28]. Several splicing variants of SIAH1 appear to play important regulatory roles; for example, SIAH1S, an alternative splicing form of SIAH1, acts as a dominant negative inhibitor of SIAH1 in the regulation of β-catenin activity [29]. SIAH1L, another splicing variant of SIAH1, is induced in response to p53 and plays a key role in the regulation of β-catenin activity [9,30,31].

In Xenopus laevis, overexpression of xSiah2 led to small eyes [32] due to degradation of PHD2 [33], indicating its critical role in retinal development. However, the function of Siah1 in Xenopus embryogenesis still remains largely unknown. In the present study, we report for the first time the identification of two splicing forms of Siah1 in X. laevis and their distinct functional roles.

2. Materials and methods

2.1. Isolation of xSiah1a and xSiah1b in X. laevis

Two Xenopus Siah1 protein sequences were found based on a Blastp search using the human Siah1 coding sequence; one was from X. laevis (NP_001085438), the other was from the Xenopus tropicalis (NP_001015836). The predicted open reading frame (ORF) of the X. laevis gene was longer than that of X. tropicalis. Comparison of the genomic organization of the two ORFs using Blat revealed that the X. laevis Siah1 has an alternative exon in the UTR (Fig. 2A). The full X. laevis Siah1a sequence (GU377277) was cloned by RT-PCR using the forward primer 5'-TCCGTTTTCTTATGGAAGCCGT-3' and the reverse primer 5'-CATGTCACACCAGCTGGCATCTTTGTA-3'. The correct clones were verified by sequencing.
2.2. Plasmid construction and in vitro transcription

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer’s instructions, and cDNAs were synthesized using random primers and M-MLV (Promega). PrimerSTAR (Takara) was used to amplify xSiah1a, xSiah1a-n9, xSiah1a-n16, xSiah1a-22 and xSiah1b, which were cloned into the pCS2plus. Primers used are as following: xSiah1a forward primer (5’-CAT-GAGCCGACAGCTCGTACGCAATC-3’); xSiah1a-n9 forward primer (5’-TATGAGTGACCCGAACGGAAGAAAGAAATG-3’); xSiah1a-n16 forward primer (5’-TATGTTGCTCAGCTCGTTGCGGTAGTG-3’); xSiah1a-22 forward primer (5’-TATGTTGCTACGATGAAAGGAGGTCTGC-3’); xSiah1b forward primer (5’-ACGGCGATGAACTCTACCTCAGACG-3’) and a shared common reverse primer (5’-CTGCCAATTCAGCACATTGAGATCG-3’). For the xSiah1a-P2A-GFP construct, the reverse primer was (5’-GCACATTGAGATCGTAGTTTATTC-3’), and PCR products were inserted into pCS2-P2A-GFP. xKid was amplified with the forward primer (5’-GAATGGTTCTTACTGGGCCTCCCCAAAGAG-3’) and the reverse primer (5’-GCTGGAGATGCTGCTCAGGATATTTGCCT-3’). The resulting fragment was cloned into pCR3.1-flag vector. All constructs were verified by sequencing.

To generate mRNA, constructs were linearized by NotI. Capped mRNA in vitro transcription was carried out using an mMESSAGE Machine Sp6 kit (Ambion). Synthesized mRNA was purified using an RNAeasy kit (Qiagen).

RT-PCR was carried out using following primers: xSiah1a forward primer (5’-CAAGGCGAACAGGAAGAGGCA-3’), xSiah1b forward primer (5’-TGACCCGAACGGAAGGAAAGA-3’), and reverse primer (5’-GCAGGTTGGGCAGCAAGTGAG-3’), ODC (ornithine decarboxylase) forward primer (5’-TGAATTGGATGAAAGTGGCAAGG-3’) and reverse primer (5’-CAGGGCTGGGTTTAACACACAGAT-3’).

2.3. Embryo manipulation

Xenopus laevis embryos were obtained from HCG-induced eggs and in vitro fertilization, and were dejellied in 2% cysteine, then
cultured in 0.1 MBS. In vitro transcribed mRNA and MG132 (Beyotime) were injected into the embryos at the 1 or 2 cell stage. Embryos were fixed in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EDTA and 3.7% formaldehyde) for 1 h, washed in ethanol three times and stored at −20 °C in ethanol. For β-galactosidase staining, embryos were fixed in MEMFA for 20 min at room temperature, and then washed in PBS twice before staining in a buffer containing 1 mg/ml X-gal, 20 mM K₃Fe(CN)₆, 20 mM K₄Fe(CN)₆, and 2 mM MgCl₂. For DAPI whole mount staining, MEMFA fixed embryos were washed with PBS five times, stained with DAPI for 30 s, and then washed in PBS five times.

2.4. Cell transfection and Western blotting analysis

293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were homogenized with RIPA lysis buffer.
Beyotime), and protease inhibitor cocktail (Sigma–Aldrich) and 100 mM PMSF were added according to the manufacturer’s recommendation. Proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membrane (Millipore). Anti-flag (Sigma–Aldrich) and anti-GAPDH (Abcam) antibody were diluted as recommended by the suppliers. Detection was by HRP-labeled secondary antibodies and ECL.

3. Results and discussion

3.1. Isolation of xSiah1a and xSiah1b

Searching the Xenopus protein database using the human SIAH1 protein sequence found one X. tropicalis Siah1 (xtSiah1) and one X. laevis Siah1 (xSiah1). Searching the nucleotide sequences in the X. tropicalis genome using Blat tools revealed large differences in the 5′ untranslated region. The xSiah1 has an upstream initiation codon and encoded an additional 31 amino acids compared to human Siah1s (Fig. 1). We then obtained the short xSiah1 through prediction based on bioinformatics using xSiah1 sequences, was and designated this as xSiah1a (GU377277), while the long form was designated xSiah1b.

Searching the X. tropicalis genome for xSiah1a and xSiah1b using the Blat program revealed that xSiah1a and xSiah1b shared most sequences except the N-terminus. XSiah1b consists of two exons and encodes a 313 amino acid protein, while xSiah1a consists of three exons and encodes a 282 amino acid protein (Fig. 2A). The sequences are conserved between humans and Xenopus (Fig. 1). These splicing variants have also been found in humans, but their functional significance is not known.

Expression analysis during different stages of embryogenesis revealed that mRNAs for xSiah1a and xSiah1b are both maternal and can be detected at all developmental stages analyzed (Fig. 2B). XSiah1a and xSiah1b are abundantly present before the gastrulation stages (Fig. 2B), indicating their potential roles in Xenopus embryogenesis.

3.2. Xenopus cleavage was exclusively suppressed by xSiah1a

Siah1 is involved in the cell cycle, apoptosis and proliferation [10,34,35]. Stable overexpression of SIAH1 in a human lung cancer MCF7 cell line showed cell growth inhibition due to perturbed mitosis through degradation of Kid [10,35], a protein needed for chromosome alignment in metaphase and that should be degraded in anaphase to allow for chromosome separation [36]. Gene ablation of Siah1a in mice demonstrated that Siah1a is also required for traversing metaphase during meiosis I of spermatogenesis [28].

In our experiments, overexpression of xSiah1a through mRNA injection was found to suppress cleavage of Xenopus embryos in a dose-dependent manner (Fig. 3A) and eventually led to severe
apoptosis (data not shown). However, xSiah1b was unable to suppress Xenopus embryo cleavage, even at a high dose (Fig. 2B). Overexpression of human SIAH1 in cell lines showed cell growth inhibition and more than 50% cells formed multinucleated giant cells [35]. DAPI staining showed that the enlarged cells suppressed by xSiah1a overexpression had giant nuclei (Fig. 3E). Overexpression of xSiah1a has been suggested to disturb mitosis during Xenopus early cleavage. To better trace the mRNA distribution in Xenopus embryos, we used 2A peptide [37] to link xSiah1a with GFP (Fig. 3D) so that GFP positive cells would express xSiah1a. Co-injection of MG132, a well-known inhibitor of the proteosome with xSiah1a-P2A-GFP diminished the phenotype of cell cycle suppression (Fig. 3C), indicating that suppression of embryo cleavage with xSiah1a-P2A-GFP diminished the phenotype of cell cycle suppression (Fig. 3C), indicating that suppression of embryo cleavage by xSiah1a is proteosome dependent. In contrast, injection of 2 ng xSiah1b mRNA into embryos had no significant effect, even at a later tadpole stage (data not shown). This result suggests that xSiah1b is inactivated in Xenopus.

Siah1 can form homo- or heterodimers with other Siah1 proteins through its C-terminal domain [7]. To determine whether xSiah1b can affect xSiah1a activity by formation of a heterodimer with xSiah1a, a low dose of xSiah1a was co-injected with a high dose of xSiah1b. The effect was the same as injection of xSiah1a alone (Fig. 4A), thus eliminating the possibility that xSiah1b serves as an additional 15 amino acids in the N-terminal than does SIAH1 transcriptional level. Transient overexpression of SIAH1L, which has overexpression by mRNA injection demonstrated that xSiah1b activity was correlated with the length of the N-terminus (Fig. 4B). Overexpression by mRNA injection demonstrated that xSiah1b activity was correlated with the length of the N-terminus (Fig. 4B). On the other hand, xSiah1a and xSiah1b had the same effect with respect to degradation of substrate xKid when transfected into 293T cells (Fig. 4D), indicating that other protein(s) in the Xenopus embryo is (are) likely to bind to xSiah1b through the 31 N-terminal amino acids, resulting in inhibition of xSiah1b. Clearly additional efforts are required to determine the detailed mechanism.

In summary, this study reports, for the first time, the presence of alternative splicing variants of Siah1—xSiah1a and 1b—in early embryogenesis of X. laevis. xSiah1b seems to be inactivated in Xenopus. xSiah1 activity also is apparently regulated at the posttranscriptional level. Transient overexpression of SIAH1L, which has an additional 15 amino acids in the N-terminal than does SIAH1 (Fig. 1), in a 293 cell line resulted in cell growth arrest [31]. Injection of SIAH1 mRNA at 1.5 ng per egg caused hypodorsalization of Xenopus embryos due to downregulation of β-catenin signaling but had no effect on early cleavage during Xenopus embryogenesis [9]. Overexpression of xSiah2, which is conserved with xSiah1a in its C-terminal, also did not show suppression of embryo cleavage at 2 ng per embryo (data not shown) but resulted in the development of small eyes in the tadpole stage [32,33]. A recent report suggests that suppression of Siah1 activity can reduce tumor growth and prolong survival of the mice with neuroangiogenesis [38]. Our present results may prove useful in providing more insight into the regulation of xSiah1 activity in a Xenopus model system.

Acknowledgments

This work was supported in part by Funds from the Key Project of Knowledge Innovation Program of the Chinese Academy of Sci-

References


