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Regulation of early T cell development by the PHD finger of histone lysine methyltransferase ASH1

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Abstract

We have previously isolated a mammalian homologue of *Drosophila discs absent, small, or homeotic-1* (*ash1*) from the murine thymus, and recently shown that its SET domain methylates histone H3 lysine 36 (K36). Expression of ASH1 has been reported to be increased in NOD thymocytes in a BDC2.5 clonotype background, but its function in T cell development has remained elusive. Here we report that the *ash1* gene is expressed at high levels in thymocytes of mice deficient for *rag1* or *tcra* genes. ASH1 proteins are present at peri-nuclei and as nuclear speckles in thymocytes. Some of the nuclear ASH1 co-localize with RAG2. Expression of the evolutionarily conserved PHD finger of ASH1 impairs T cell development at the DP stage, and causes increased transcription from the HoxA9 promoter *in vitro*. Moreover, the C-terminal part of ASH1 interacts with HDAC1 repression complexes, suggesting that the PHD finger of ASH1 may be involved in down-regulation of genes for normal development of αβ T cells.

Keywords: Histone modification; Chromatin structure; PHD finger; Bromodomain; HDAC complex; TCR gene rearrangement; Thymus; FTOC

Early stages of αβ T cell development are associated with dynamic changes in gene expression patterns that are regulated primarily by feedback signals from products of successful TCR β and α gene rearrangements [1]. In an attempt to elucidate molecular mechanisms underlying the developmental switch of gene expression patterns in thymocytes, we have carried out a suppression subtractive PCR screening of genes expressed in wild-type C57BL/6 and *rag1*$^{-/-}$ thymocytes and identified a clone that corresponds to the murine homologue of ASH1 (DDBJ Accession Number AF247132; Y.T. and M.T., unpublished). The primary structure of murine ASH1 is highly similar to those of *Drosophila* [2] and human [3] ASH1 and consists of 2958 amino acids (Fig. 1). The N-terminal part of ASH1 is most similar to human SEB (SET oncoprotein/histone chaperone-binding protein), whereas its C-terminal part contains the SET domain, Bromodomain, PHD finger, and BAH (*B*romo-*a*djacent *h*omology) domain. More recently, we have reported that the SET domain of both mammalian and fly ASH1 methylates histone H3 specifically at K36 [4]. While *Drosophila* ASH1 is known to genetically interact with TRITHORAX to regulate Hox gene expression [5], little is known about the biological function of mammalian ASH1. The *ash1* gene is located in the murine type 1 diabetes locus *Idd17* and has been shown to be elevated in NOD mice in a self-reactive BDC2.5 clonotype background, suggesting a potential role of ASH1 for the induction of self-tolerance [6]. Here we show that the PHD finger of ASH1 might be involved in regulation

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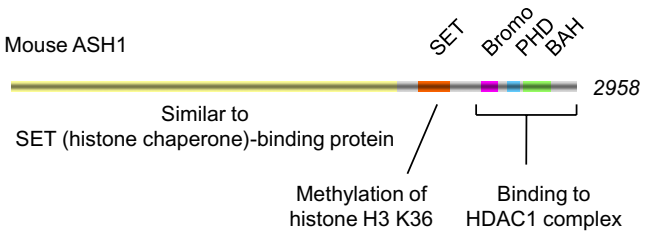

Fig. 1. Schematic representation of murine ASH1. Murine ASH1 consists of 2958 amino acids and has an N-terminal region most similar to a human SEB (SET oncogene/histone chaperone-binding protein), a SET domain, a Bromodomain, a PHD finger, and a BAH (Bromo-adjacent homology) domain.

of early T cell development by interacting with HDAC1 repression complexes.

Materials and methods

Semiquantitative RT-PCR. Equal amounts of reverse transcribed templates were amplified using A25 (*ash1*) and *g3pdh* primer sets by a cycle reaction of 94 °C, 1 min, and 30 cycles of 94 °C, 10 s, 59 °C, 15 s, and 72 °C, 1 min. Products were stained with ethidium bromide after electrophoresis, and staining intensities were quantitated using NIH-Image software. Primers used were as follows:

A25-fwd: AGTGAGGCCGACAGCAGCGA
A25-rev: ACACTGCTCACAAAGGTAGTG
G3PDH-fwd: ACCACAGTCCATGCCATCAC
G3PDH-rev: TCCACCACCCTGTTTGCTGTA

Western blot analysis and immunohistochemistry. Whole cell lysates from *scid* thymocytes and recombinant SET domain and the C-terminal part of ASH1 were analyzed with anti-ASH1 antibodies in the presence or absence of the antigenic peptides derived from the C-terminal end of ASH1. Thymocytes from *scid* mice were stained with anti-RAG2 (rabbit) antibodies and anti-rabbit IgG-FITC followed by staining with anti-ASH1-Alexa568. Thymocytes from wild-type C57BL/6 mice were stained with anti-ASH1-Alexa488. Cells were analyzed by LSM510 Laser Scanning Microscope (Carl-Zeiss). Pull-down assay was carried out using GST-ASH1 C-terminal recombinant protein, thymocyte extract, anti-ASH1 antibody, and anti-HDAC1 antibody (Santa-Cruz sc-8410).

In vitro T cell differentiation. Retrovirus packaging cell line (Phoenix) was kindly provided by Dr. G. Nolan (Stanford University). Retroviral constructs were transiently transfected into Phoenix cells by a standard calcium phosphate method, and retroviral supernatants were collected between 36 and 72 h. Day 14 fetal thymocytes were cultured in retroviral cocktail containing 100 ng/ml SCF and 20 ng/ml IL-7 (both from Genzyme) for 48 h. Retrovirally transduced thymocytes were then reaggregated with thymic stromal cells and cultured further for 7 days. Differentiation of T cells was assessed by FACS analysis after staining with CD8β-PE and CD4-Cy (both from Pharmingen) using FACScan (Beckton Dickinson) and FlowJo software (Tree Star).

Plasmid construction. The PHD finger of ASH1 was first cloned into pCMV/myc/nuc (Invitrogen) which harbors three nuclear localization signals (nuc) as well as a myc epitope. Subsequently, the PHD/nuc/myc fragment were subcloned into the pMX.IRES.GFP vector which was a kind gift from Dr. T.N. Schumacher (The Netherlands Cancer Institute). The HoxA9-luciferase reporter containing the proximal promoter of human HoxA9 gene will be described elsewhere (Y.T.).

Luciferase assay. The 293T cells were transfected with pCMV-PHD/nuc/myc, pCMV-RL (renilla luciferase), and HoxA9-firefly luciferase. Whole cell lysates were prepared after 48 h and luciferase activities were measured using Dual Luciferase Reporter Assay System (Promega).

Results

Murine ash1 gene is expressed preferentially in immature thymocytes

To elucidate the developmental pattern of *ash1* gene expression in the thymus, we carried out RT-PCR analysis using wild-type C57BL/6 and *rag1*- or *tcra*-deficient thymocytes that exhibit developmental arrest at DN (double negative) and DP (double positive) stages, respectively (Fig. 2A). Expression of the *ash1* gene, as assessed by the amplification of A25 that corresponds to the PHD finger of ASH1, is 10-fold higher in immature thymocytes of $rag1^{-/-}$ and $tcra^{-/-}$ mice than those of wild-type mouse (Fig. 2B). Such a difference in the *ash1* gene expression might simply reflect cell populations in the thymus of these mice. Alternatively, differentiation arrested *rag1*- or *tcra*-null mice may express higher levels of *ash1* because they may not receive feedback signals from pre-TCR or TCR such as in wildtype mice. Northern blot analysis of mouse tissues reveals expression of 10.6 kb *ash1* transcripts preferentially in the brain and thymus (Fig. 2C).

ASH1 proteins are present in peri-nuclei and nuclear speckles

To determine the site where ASH1 is expressed in murine thymocytes, polyclonal antibodies were raised against 18 amino acids of the C-terminal end of ASH1 and were

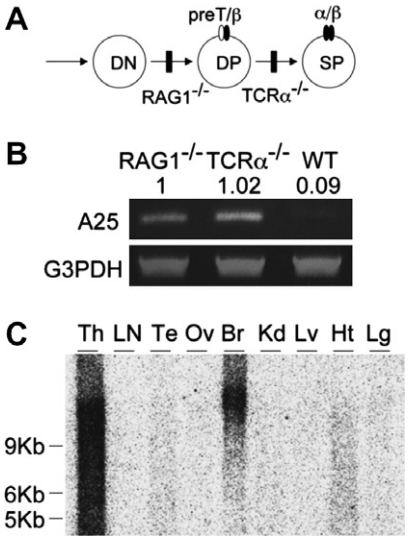

Fig. 2. Expression of ASH1 *in vivo*. (A) Representation of developmental arrest of immature T cells in $rag1^{-/-}$ and $tcra^{-/-}$ mice. (B) RT-PCR analysis of *ash1* (A25) and *g3pdh* genes in wild-type, $rag1^{-/-}$, and $tcra^{-/-}$ thymocytes. The *ash1* gene is expressed at higher levels in differentiation-arrested thymocytes than in wild-type thymocytes. Numbers on each lane indicate density quantitation of each band. (C) Northern blot analysis of the *ash1* gene expression in the thymus (Th), lymph nodes (LN), testis (Te), ovary (Ov), brain (Br), kidney (Kd), liver (Lv), heart (Ht), and lungs (Lg). Ash1 is expressed preferentially in the brain and thymus as a large transcript of approximately 10.6 kb in length.