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Possible involvement of Helios in controlling the immature B cell functions via transcriptional regulation of protein kinase Cs

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ABSTRACT

The transcription factor Ikaros family consists of five zinc-finger proteins: Ikaros, Aiolos, Helios, Eos and Pegasus; these proteins except Pegasus are essential for development and differentiation of lymphocytes. However, in B lymphocytes, the physiological role of Helios remains to be elucidated yet, because its expression level is very low. Here, we generated the Helios-deficient DT40 cells, *Helios*$^{-/-}$, and showed that the Helios-deficiency caused significant increases in transcriptions of four protein kinase Cs (PKCs); PKC-δ, PKC-ε, PKC-η and PKC-ζ, whereas their expressions were drastically down-regulated in the Aiolos-deficient DT40 cells, *Aiolos*$^{-/-}$. In addition, *Helios*$^{-/-}$ was remarkably resistant against phorbol 12-myristate 13-acetate (PMA)/ionomycin treatment, which mimics the B cell receptor (BCR)-mediated stimulation. In the presence of PMA/ionomycin, their viability was remarkably higher than that of DT40, and their DNA fragmentation was less severe than that of DT40 in the opposite manner for the Aiolos-deficiency. The resistance against the PMA/ionomycin-induced apoptosis of *Helios*$^{-/-}$ was sensitive to Rottlerin but not to Go6976. In addition, the Helios-deficiency caused remarkable up-regulation of the Rottlerin-sensitive superoxide (O_2^-)-generating activity. These data suggest that Helios may contribute to the regulation of the BCR-mediated apoptosis and O_2^--generating activity, via transcriptional regulation of these four PKCs (especially PKC-δ) in immature B lymphocytes. Together with previous data, our findings may significantly help in the understanding of the B lymphocyte-specific expressions of PKC genes and molecular mechanisms of both the BCR-mediated apoptosis involved in negative selection and the O_2^--generating system in immature B lymphocytes.

1. Introduction

The normal development of B lymphocytes requires various transcription factors, including E2A [1], EBF1 [2], Pax5 [3], PU.1 [4], Ikaros family proteins [5,6] and so on [7,8]. The Ikaros family consists of five zinc-finger proteins: Ikaros, Aiolos, Helios, Eos and Pegasus; these proteins except Pegasus are essential for development and differentiation of lymphocytes [9,10]. They play important roles as tumor suppressors; their down-regulation causes leukemias and lymphomas in mice [6,11–13]. Ikaros family transcription factors participate in the control of intracellular signaling pathway mediated by B cell receptor (BCR) [14–19]. For instance, Ikaros critically regulates the pre-BCR-mediated cell cycle arrest, and also promotes tumor suppression through its cooperation with downstream molecules of the pre-BCR signaling pathway in acute lymphoblastic leukemia cells [15]. The disruption of Aiolos in mice showed that most splenic B lymphocytes were differentiated to follicular mature B lymphocytes, suggesting that BCR-delivered maturation signals are enhanced [17]. On the other hand, although Helios is constitutively expressed in hematopoietic tissues [9], it is mainly detected in T lymphocytes after differentiation and involved in T lymphocyte development and function [9,20–23]. Helios is also expressed in B lymphocytes [9,24,25], and silencing of Helios is critical for normal function of B lymphocytes [24]. However, the physiological role including BCR-signaling of Helios in B lymphocytes remains to be elucidated.

Gene targeting techniques using chicken immature B cell line DT40 [26] are excellent methods to study physiological functions of various genes in immature B lymphocytes [27–29]. Concerning study on the BCR-signaling, Ikaros-deficiency in DT40 induced the BCR-signaling defect with reduced phospholipase C-γ2 phosphorylation and impaired intracellular calcium mobilization [16]. In addition, disruption of Aiolos in DT40 caused drastic acceleration of the BCR-mediated apoptosis [18,19]. We also revealed that lack

Abbreviations: BCR, B cell receptor; O_2^-, superoxide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate

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of Aiolos accelerated apoptosis mediated by the BCR stimulation through transcriptional regulation of protein kinase Cs (PKCs) and elevation in cytochrome *c* release [19]. Recently, interestingly, it was reported that Helios is expressed even in DT40 [29]. These results suggest that Helios may also participate in controlling the BCR-signaling pathway of DT40, as well as Ikaros and Aiolos.

Therefore, to clarify the function of Helios in the BCR-signaling pathway, we generated and analyzed the Helios-deficient DT40 mutant, *Helios*$^{-/-}$. Our results showed that Helios may regulate BCR-mediated apoptosis via controlling gene expression of several PKCs. In immature B lymphocytes, cross-linking of BCR induces their apoptosis, but antigen binding to BCR triggers their activation and proliferation [30,31]. Therefore, cross-linking of the BCR in immature B lymphocytes is thought to function as a mechanism to exclude self-reactive B cell clones (negative selection), although the regulation mechanisms of BCR-mediated apoptosis still remain unclear. In addition, we also report that the O_2^--generating activity is regulated by Helios in DT40. These novel findings should profit to clarify the participations of Helios in molecular mechanisms of negative selection and B cell-specific regulation of the O_2^--generating system in immature B lymphocytes.

2. Materials and methods

2.1. Materials

PMA, Go6976 and Rottlerin (Calbiochem, Darmstadt, Germany), ionomycin (Sigma, St Louis, MO) were obtained.

2.2. Generation of Helios-deficient DT40 cells

We constructed Helios-disruption vectors as follows (Fig. 1A). Partial genomic Helios DNA fragments were obtained from DT40 genomic DNA by means of PCR based on nucleotide sequences from the Web Bursal database and confirmed by the PCR sequencing protocol. The upstream fragment, an *Eco*RI/*Bam*HI digested 2.8-kb PCR fragment (obtained using sense primer 5′-GATTGTAAGGAACAAGAGCCTGTGATGGAC-3′ from exon 7 and antisense primer 5′-TCTGGATCCTGGATAGCCAAGTCTCATGAC-3′ from exon 8 (*Bam*HI site was underlined)), and the downstream fragment, an *Asc*I/*Xho*I digested 1.9-kb PCR fragment (obtained using sense primer 5′-TTAGGCGCGCCAGCTGATACAGTCTCAAAT-3′ from exon 8 and antisense primer 5′-ACTCTCGAGTGAAGTTGGGGTAGTTCC-3′ from intron 8 (*Asc*I and *Xho*I sites were underlined)) were transferred into the pBluescript II vector. Two cassettes, carrying hygromycin and blasticidin S resistance genes, transcribed by the chicken β-actin promoter, were inserted between the upstream and downstream arms. Transfection was carried out essentially as described [32,33].

2.3. Southern blotting

Genomic DNAs were digested with *Hin*dIII, separated in a 0.8% agarose gel, transferred to a Hybond N^+ membrane, and then hybridized with ^{32}P-labeled probe Helios (see Fig. 1A) as described [32,33].

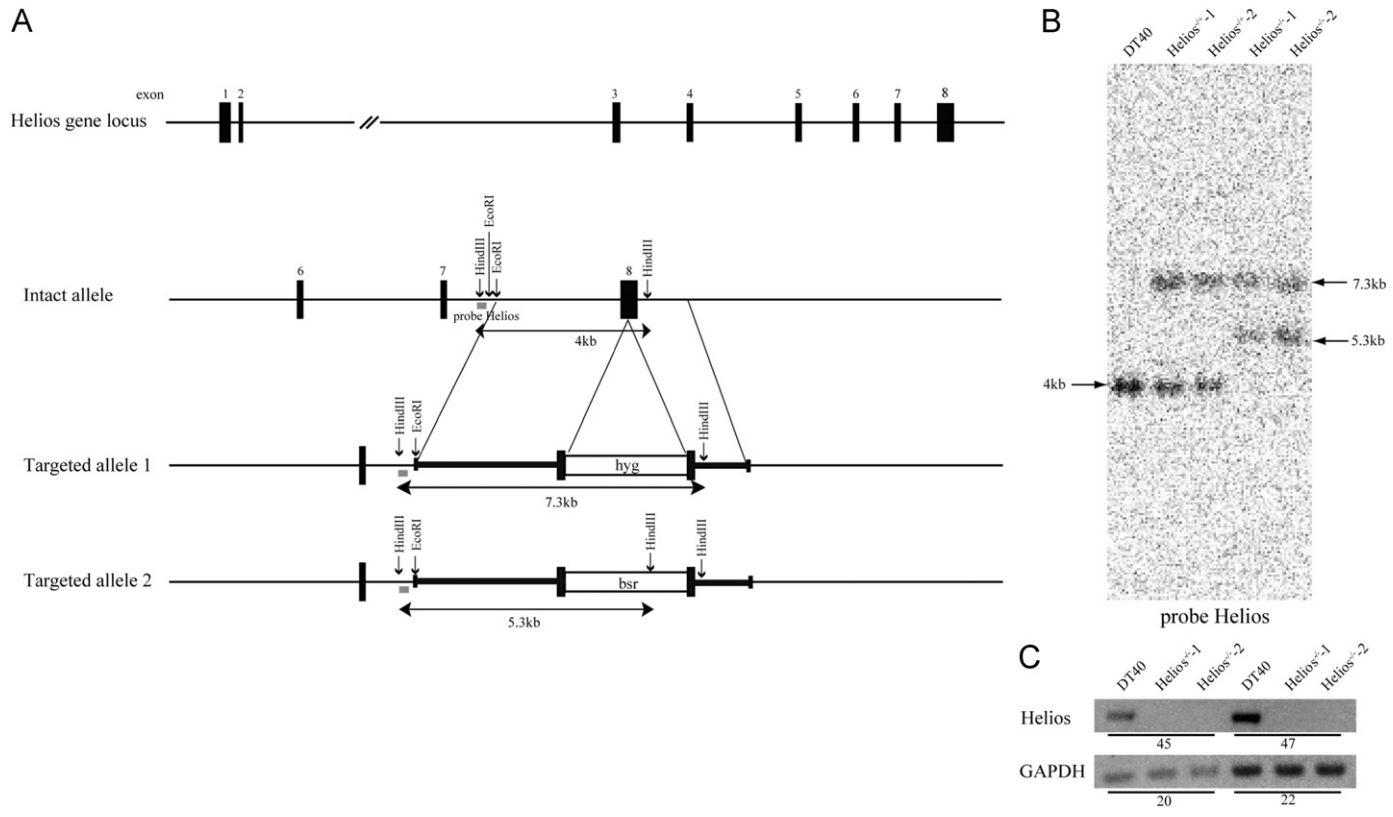

Fig. 1. Generation of the Helios-deficient mutants *Helios*$^{-/-}$. (A) Genomic organization and schematic diagram of the homologous recombination resulting in disruption of the first (Targeted allele 1) and second alleles (Targeted allele 2) of the chicken Helios gene. Locations of exons are indicated by solid boxes with appropriate designations. White boxes indicate drug resistance cassettes: hygromycin resisitance (hyg) and blasticidin S resistance (bsr). Location of probe Helios is indicated by a gray bar. Only relevant restriction sites are indicated. Possible relevant fragments obtained from *Hin*dIII digestion are shown with their lengths in kb. (B) Southern blotting of homologous recombination events in the Helios gene. Genomic DNAs were prepared from DT40, two heterozygous mutants *Helios*$^{-/+}$ and two homozygous mutants *Helios*$^{-/-}$. The *Hin*dIII fragments were analyzed with probe Helios. (C) Semiquantitative RT-PCR. Total RNAs were extracted from DT40 and two independent *Helios*$^{-/-}$, and the Helios mRNA level was determined by semiquantitative RT-PCR as described in Section 2. Numbers under the panels indicate cycle numbers of PCR.