ORIGINAL

Identification of important regulatory region of Th-POK

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Abstract : CD4$^+$ and CD8$^+$ T cells develop from CD4$^+$CD8$^+$ thymocytes. Although it has been reported that expression of the transcription factor Th-POK is important for CD4$^+$ T cell development, the detailed mechanism regulating Th-POK expression is still obscure. By comparing the promoter regions of the Th-POK gene between human and mouse, I found that the region 3600 base pairs (bps) upstream from the transcription initiation site of the Th-POK gene was highly conserved. To identify the important element(s) regulating Th-POK expression in CD4$^+$ T cells, I investigated the promoter activity of this region using a luciferase assay in the human T cell line Jurkat. I identified a positive regulatory element in this region 22 bps in length located 600 bp upstream from the transcription initiation site. This 22 bp element had a consensus binding sequence for SAP-1, which is encoded by the Elk4 gene and is activated by the Erk pathway. These data suggest that the 22 bp element might positively regulate Th-POK expression through Erk-SAP-1 signaling. J. Med. Invest. 57 : 219-223, August, 2010

Keywords **:** *Th-POK, SAP-1, Luciferase activity*

INTRODUCTION

T cells play central roles in the adaptive immune system. Mature T cells develop in the thymus after a series of selection processes. CD4$^+$ T cells develop when CD4$^+$CD8$^+$ double positive (DP) thymocytes confront antigens presented by MHC class II molecules. Antigens presented by MHC class I molecules direct DP cells to retain CD8 expression and silence CD4 expression (1-3). The important transcription factors associated with T cell development include GATA-3, which guides CD4$^+$ T cell maturation (4, 5), and Runx3, which silences CD4 gene expression in CD8$^+$ T cells (6).

The transcription factor Th-POK (also named cKrox), which is encoded by *Zbtb7b*, belongs to a zinc-finger family containing a BTB-POZ domain (7). The crucial role of Th-POK in CD4$^+$ T cell development has been identified by Kappes, *et al.* (8). They reported that two lines of mice with deficient CD4$^+$ T cells not only showed loss of CD4$^+$ T cells but also redirected development of thymocytes that would normally have matured into CD4$^+$ T cells, into CD8$^+$ T cells (9). Further analysis mapped a single point mutation in the second zinc finger domain which lies in the second exon of Th-POK (10). Moderate or prolonged TCR signaling in thymocytes received via MHC class II antigen complexes leads to Th-POK expression and induces the CD4$^+$ T cell fate (11). Taniuchi, *et al.* (12) identified a Runx-binding site in the distal promoter region of Th-POK. They showed that Runx complexes silence Th-POK expression (12) as well as repress CD4 expression in CD8 lineage cells through binding to the CD4 silencer (6, 13). Although Runx complexes silence Th-POK expression (12), the positive regulatory signal(s) for Th-POK expression remains to be clarified.

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In this report, I show that the 22 bp element 600 bp upstream of the transcription initiation site of the Th-POK gene is conserved between human and mouse, and is important for its promoter activity.

MATERIALS AND METHODS

Comparative genomic analysis

The comparative genomic analysis between human and mouse was performed using VISTA tools for comparative genomics (http : //genome.lbl.gov/cgi-bin/GenomeVista).

Construct preparation

Different fragments of the mouse Th-POK proximal promoter were amplified and cloned into pGL3 basic vector (Promega).

Luciferase assay

The 1.2 μg DNA (1 μg pGL3 basic or promoter construct vector plus 0.2 μg pRL-CMV) was transfected into 3.2×10^5 Jurkat cells by using DIMRIE-C lipofection reagent (Invitrogen). Forty-eight hrs after transfection, cells were harvested and analyzed for luciferase activity with Dual Luciferase Assay (Promega) according to the manufacturer's protocol. Firefly luciferase activity was normalized using Renilla luciferase activity.

RESULTS

The important region of the Th-POK promoter lies between 500 bp and 1000 bp upstream of the transcription initiation site

Th-POK is essential for $CD4^+$ T cell development. In order to locate the cis-acting regulatory element critical for Th-POK expression, I performed a comparative genomic analysis of the Th-POK promoter region between human and mouse to identify conserved regions (14). I found that the region from 3600 bp (3.6 kb) upstream of the Th-POK the transcription initiation site to intron-1 (12 kb) region was highly conserved between human and mouse (Figure 1A). When I examined the promoter activity of the entire 3.6 kb region of the mouse Th-POK proximal promoter, I could not detect any significant promoter activity in Jurkat cells, which express endogenous Th-POK. Next, I cloned several shorter regions of the Th-POK promoter into the pGL3 basic vector and tested their promoter activities. I found promoter activity in both the 1000 bp and 1200 bp promoter regions, but not in the 500 bp proximal fragment (Figure 1B), suggesting that the regulatory region of the Th-POK promoter was located between 500 bp and 1000 bp upstream from its transcription initiation site.

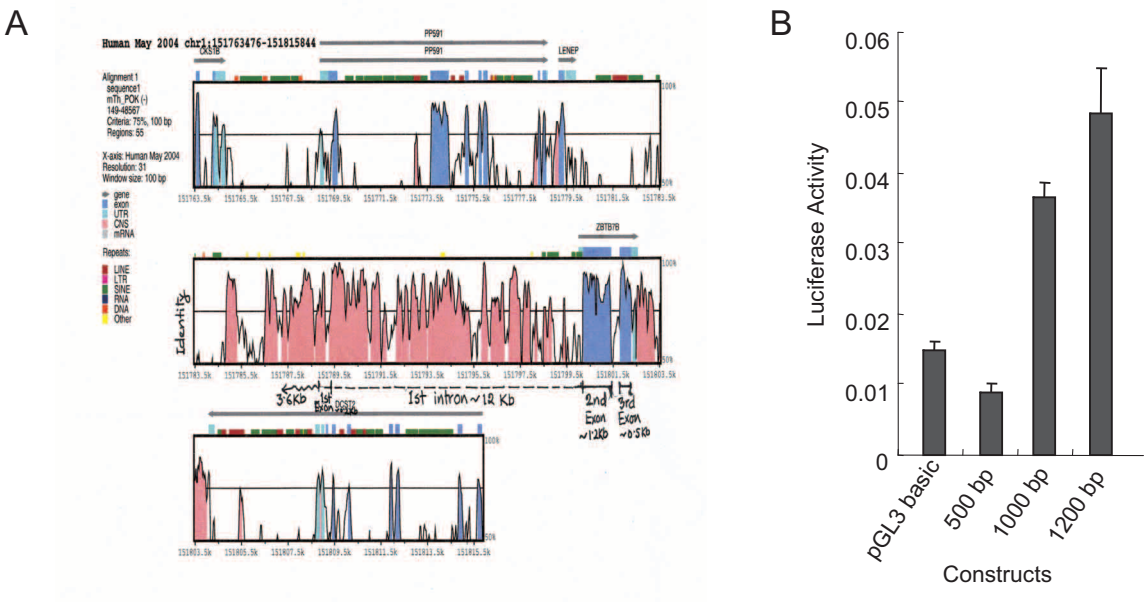

Fig. 1 : One thousand base pairs of noncoding sequence of the Th-POK promoter conserved between human and mouse is important for Th-POK promoter activity. (A) Comparative genomics of the Th-POK gene between human and mouse using the VISTA genomic browser. (B) The 500 bp, 1000 bp and 1200 bp Th-POK promoter regions upstream of the transcription initiation site were cloned into pGL3 basic vector and transfected into Jurkat cells along with control pRL-CMV. Forty-eight hrs after transfection, luciferase activity was measured by a Dual Luciferase Assay. Data shown in (B) are representative of four independent experiments.

Addition of a 22 bp fragment to the 600 bp promoter region increases Th-POK promoter activity

In order to narrow the promoter region containing the regulatory element, I made 100 bp serial deletion mutants from 1000 bp to 500 bp of the promoter region in pGL3. After transfecting these constructs into Jurkat cells, I found increased luciferase activity between the 600 bp and 700 bp promoter constructs (Figure 2A), indicating the regulatory element was located in this 100 bp region. I made mutant promoter constructs with 20 bp serial additions to the 600 bp promoter region of the Th-POK gene and examined each construct's promoter activity. Addition of only 22 bp to the 600 bp construct showed increased luciferase activity comparable to the 700 bp promoter region (Figure 2B). Addition of more base pairs to the promoter region did not increase the activity (Figure 2B), suggesting that the 22 bp fragment was important for Th-POK gene promoter activity.

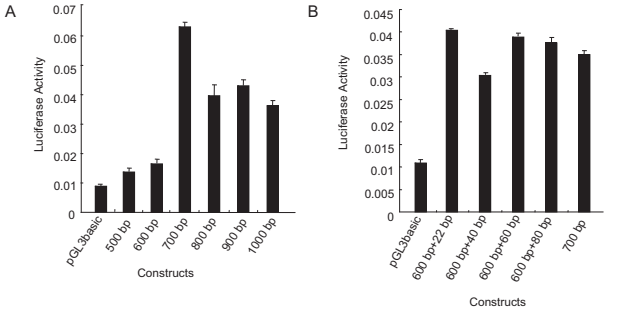

Fig. 2 : Addition of a 22 bp fragment to the 600 bp promoter region increased promoter activity. (A) Deletion of 100 bp serial sections from 1000 bp to 500 bp of the Th-POK promoter region (500 bp, 600 bp, 700 bp, 800 bp, 900 bp) were cloned into the pGL3 basic vector and transfected into Jurkat cells along with control pRL-CMV vector. Forty-eight hrs after transfection, luciferase activity was measured by Dual Luciferase Assay kits. (B) The 20-bp successive additions to the 600 bp Th-POK promoter region were constructed (600 plus 22 bp, 600 plus 40 bp, 600 plus 60 bp and 600 plus 80 bp) using the pGL3 basic vector. The constructs were transfected into Jurkat cells along with control pRL-CMV vector. Forty-eight hrs after transfection, luciferase activity was measured by a Dual Luciferase Assay. Data are representative of four independent experiments.

Deletion of the 22 bp element reduces Th-POK promoter activity

In order to confirm the importance of the identified 22 bp element in the Th-POK promoter, I deleted this 22 bp element from the 700 bp construct (Figure 3A) and performed luciferase assays in Jurkat cells. I found that deletion of the 22 bp fragment

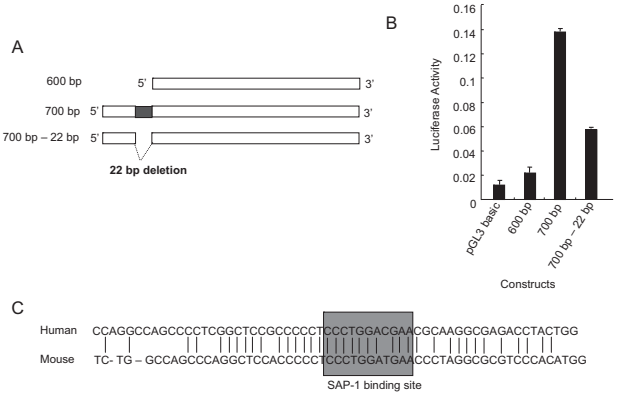

Fig. 3 : Binding of SAP-1 to the 22 bp region controls Th-POK promoter activity. (A) Schematic diagram of deletion mutants from the 700 bp promoter region. (B) Constructs shown in Figure 3A were transfected into Jurkat cells along with control pRL-CMV vector. Forty-eight hrs after transfection, luciferase activity was measured using Dual Luciferase Assay kits. (C) The putative SAP-1 binding site in the Th-POK promoter region is conserved between human and mouse. Data shown in (B) are representative of four independent experiments.

caused a significant decrease in luciferase activity (Figure 3B), indicating that this 22 bp element was critical for Th-POK gene promoter activity. Next, I searched for transcription factor binding sequences in the 22 bp element. Interestingly, I found the binding sequence for SAP-1 (encoded by the Elk4 gene) in this element, which is highly conserved between human and mouse (Figure 3C), suggesting the importance of SAP-1 in Th-POK expression.

DISCUSSION

Mounting evidence has shown that Th-POK is the master regulator for $CD4^+$ T cell development (10). Runx transcription factors favour $CD8^+$ T cell development by inhibiting both the expression of CD4 (by binding to the CD4 silencer) (6, 13) and Th-POK (by binding to the Th-POK silencer) (12). Suppression of such Th-POK silencing induces robust $CD4^+$ T cell fate decisions (3). However, it remains unclear how Th-POK expression is activated. In this report, I show that the Th-POK promoter has a 22 bp element critical for activating gene expression.

The 22 bp element that I identified as a positive regulator for Th-POK gene expression contained an SAP-1 binding sequence. It has been shown that SAP-1 activity is regulated by the Erk pathway and that TCR stimulation activates Erk signaling (15,

16). Indeed, SAP-1 deficient mice show defective positive selection, which is mediated through the TCR, indicating that SAP-1 activity is regulated by TCR signaling (16). Furthermore, moderate or prolonged TCR signaling leads to Th-POK expression in thymocytes (11) and TCR signaling in peripheral $CD8^+$ T cells derepresses Th-POK expression (17). Therefore, this study suggests that TCR-mediated SAP-1 activation might promote Th-POK gene expression via SAP-1 binding to the 22 bp element in the Th-POK promoter.

Setoguchi, *et al.* (12) showed that the binding of Runx to the enhancer of the Th-POK gene is important for $CD8^+$ T cell development and that the deletion of such Runx-binding sequences leads to a robust $CD4^+$ T cell pool (18). However, the 3.6-kb region did not show significant Th-POK promoter activity in this study, although Jurkat cells express endogenous Th-POK. Collectively, these findings suggest that expression of the Th-POK gene is regulated by several factors including Runx.

In conclusion, these data describe a regulatory mechanism of Th-POK crucial for $CD4^+$ T cell development. The identification of molecules that control Th-POK expression will help clarify the molecular mechanisms required to exert $CD4^+$ T cell-specific functions.

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