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# PAX5 tyrosine phosphorylation by SYK co-operatively functions with its serine phosphorylation to cancel the PAX5-dependent repression of BLIMP1: A mechanism for antigen-triggered plasma cell differentiation

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ABSTRACT

Plasma cell differentiation is initiated by antigen stimulation of the B cell receptor (BCR) and is regulated by BLIMP1. Prior to the stimulation of BCR, BLIMP1 is suppressed by PAX5, which is a key transcriptional repressor that maintains B cell identity. The upregulation of BLIMP1 and subsequent suppression of PAX5 by BLIMP1 are observed after the BCR stimulation. These events are considered to trigger plasma cell differentiation; however, the mechanisms responsible currently remain unclear. We herein demonstrated that the BCR signaling component, SYK, caused PAX5 tyrosine phosphorylation *in vitro* and in cells. Transcriptional repression on the *BLIMP1* promoter by PAX5 was attenuated by this phosphorylation. The BCR stimulation induced the phosphorylation of SYK, tyrosine phosphorylation of PAX5, and up-regulation of BLIMP1 mRNA expression in B cells. The tyrosine phosphorylation of PAX5 co-operatively functioned with PAX5 serine phosphorylation by ERK1/2, which was our previous findings, to cancel the PAX5-dependent repression of BLIMP1. This co-operation may be a trigger for plasma cell differentiation. These results imply that PAX5 phosphorylation by a BCR signal is the initial event in plasma cell differentiation.



## 1. Introductions

PAX5 is a member of the highly conserved paired-box (PAX) domain family of transcription factors. Although B cells express PAX5 from the pro-B to mature B cell stage, its expression is lost during their terminal differentiation into plasma cells [1]. PAX5 plays indispensable roles not only in B-lineage commitment and maintaining the identity of B cells [1,2], but also for checking the initiation of plasma cell differentiation, namely, the terminal differentiation of B cells. PAX5 functions as a transcriptional activator of B-lineage-specific genes and repressor of B-lineage inappropriate genes. It activates CD19, CD79A, and the B-cell linker protein (BLNK), and represses the colony-stimulating factor 1 receptor, Notch1, and FMS-like tyrosine kinase 3 during the pro B to mature B cell stage [2]. PAX5 also suppresses the expression of a key regulator of plasma cell differentiation, B lymphocyte-induced maturation protein 1 (BLIMP1), in germinal center B (GCB) cells [3]. The encounter of the B cell receptor (BCR) with a specific antigen is a trigger for the plasma cell differentiation of GCB cells. It induces the down-regulation of PAX5, up-regulation of BLIMP1, and ultimately, plasma cell differentiation [4]. The molecular mechanisms underlying the initiation of plasma cell differentiation have been partly elucidated. PAX5 suppresses BLIMP1 expression and checks plasma cell differentiation. After the stimulation of BCR by an antigen, the repression of BLIMP1 by PAX5 is abolished, and once BLIMP1 is expressed, it suppresses PAX5. PAX5 is eventually replaced by BLIMP1, which initiates plasma cell differentiation [4]. The abolition of PAX5-mediated repression was previously considered to be the first event in the initiation of plasma cell differentiation [5];

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however, the mechanisms responsible currently remain unclear.

The BCR signal provides survival and differentiation-initiating signals for B lymphocytes in antigen-dependent and -independent ways. The initial event after BCR engagement is the activation of spleen tyrosine kinase (SYK). This tyrosine kinase phosphorylates various kinases, adaptor proteins, and enzymes, and triggers a complex network of signaling pathways downstream of the receptor. For example, SYK phosphorylates BLNK, Bruton's tyrosine kinase (BTK), and phospholipase C γ2, which leads to the activation of the mitogen-activated protein kinase and nuclear factor κ B (NFκB) pathways [6,7]. SYK also activates phosphoinositide-3-kinase (PI3K), leading to the activation of AKT-mammalian target of rapamycin pathway. The resulting signals quickly reach the nucleus and alter gene expression. We previously demonstrated that the BCR signal induced PAX5 phosphorylation at serines 189 and 283 by extracellular signal-regulated kinase (ERK)1/2 [8]. This phosphorylation attenuated the transcriptional repression of BLIMP1 by PAX5 in GCB cells, which was considered to be an initial event in plasma cell differentiation; however, the phosphorylation-defective mutant of PAX5 did not completely abolish BCR signal-induced BLIMP1 expression, implying that another kinase activated by BCR signaling also phosphorylated PAX5 and functioned to cancel the repression of BLIMP1; therefore, we herein searched other kinase components of BCR signaling that phosphorylated PAX5.

## 2. Materials and methods

### 2.1. Cells, antibodies, and reagents

Ramos cells were described previously [8].

Anti-PAX5 C and anti-PAX5 N antibodies that recognize the C-terminal and N-terminal regions of PAX5, respectively, anti-Flag antibody, and anti-human IgM antibody for the BCR stimulation were described previously [8]. An anti-SYK antibody for immunoblotting and anti-phospho-SYK antibody were obtained from Cell Signaling Inc. (Beverly, MA). An anti-SYK antibody for immunoprecipitation was from Santa Cruz Biotechnology (Santa Cruz, CA).

### 2.2. Plasmids

PAX5/pCDNA for *in vitro* translation/transcription and mammalian expression, PAX5/pGEX for GST-fused PAX5, and PAX5/pBGJR for lentivirus infection were described previously [8]. Flag-SYK/pFlag for mammalian expression was made as described previously [9]. Mutagenesis for tyrosine-to-phenylalanine substitutions were performed as previously described [8].

### 2.3. Transient transfection, lentivirus infection, immunoblotting, immunofluorescence, EMSA, luciferase assay, in vitro kination assay, knockdown of PAX5, and real-time PCR

These were performed as described previously [8]. Recombinant SYK, BTK, and AKT were obtained from Carna Biosciences (Kobe, Japan).

## 3. Results

### 3.1. PAX5 is phosphorylated by SYK in vitro and in cells

We first examined whether PAX5 was phosphorylated by the major kinase components of BCR signaling such as SYK, BTK, and AKT (see the Introduction section). The positions of these kinases in the BCR signaling cascade are shown in Fig. 1A. Recombinant ERK2 and SYK efficiently phosphorylated radio-labeled PAX5, which was synthesized by an *in vitro* translation/transcription method, and caused a band shift in electrophoresis using phosphate-affinity (PA) SDS-PAGE (Fig. 1B). In this system, Phos-tag™ binds to phosphorylated amino acids during electrophoresis and slows the migration of phosphorylated proteins. Autoradiography or immunoblotting are subsequently used after electrophoresis to detect the phosphorylation of the target protein as band shifts.

The synthesized partial PAX5 including amino acids 1 to 313 was also phosphorylated by SYK *in vitro* (data not shown); therefore, we introduced a series of phenylalanine substitutions for tyrosines to this region in order to map the actual phosphorylation sites by SYK. Since the phosphorylation of PAX5 by SYK was found to be detected also by usual SDS-PAGE as a band shift (Fig. 1C), we used usual SDS-PAGE to detect PAX5 phosphorylation by SYK. Substitutions of tyrosine at codons 264 and 299 caused a reduction in the shifted band, whereas other substitutions did not affect the shifted band (Fig. 1C), indicating that these tyrosines were the phosphorylation sites. Consistently, the shifted band completely disappeared with the combined substitutions at codons 264 and 299 (Fig. 1D). Although we did not examine the effects of substitutions of tyrosines in the C-terminal region of PAX5, these results strongly indicated that the phosphorylation sites of PAX5 by SYK were tyrosines 264 and 299. We designated PAX5 with these combined substitutions as PAX5 TM (a tyrosine mutant). Both phosphorylation sites were nearby the transactivation domain of PAX5 and the amino acid sequences surrounding the phosphorylation sites were evolutionarily conserved (Supplemental Figure S1).

We next determined whether PAX5 tyrosine phosphorylation occurs by SYK in cells. The co-expression of SYK with PAX5 in 293T cells caused similar PAX5 phosphorylation to that observed *in vitro*; namely, tyrosine phosphorylation of wild-type PAX5 (PAX5 W) was detected by an anti-phospho-tyrosine antibody, whereas PAX5 TM showed no tyrosine phosphorylation (Fig. 1E).

### 3.2. PAX5 tyrosine phosphorylation by SYK co-operatively functions with its serine phosphorylation to cancel the PAX5-dependent repression of BLIMP1

We investigated the effect of PAX5 phosphorylation by SYK on the PAX5-dependent repression of BLIMP1 in a luciferase assay. We used the reporter gene containing an approximately 2-kbp region of the *BLIMP1* promoter, including putative binding sites for PAX5 and NFκB (Fig. 2A). Using this reporter gene, we observed the transcriptional repression of NFκB-induced luciferase by PAX5, as described in our previous study [8]. As shown in Fig. 2B, NFκB-induced luciferase expression was decreased to less than 5% of the control level by the co-expression of wild-type PAX5. This PAX5-dependent transcriptional repression was attenuated by the co-expression of SYK (Fig. 2B lane 2 vs. lane 3), while the transcriptional repression by PAX5 TM was more resistant to the co-expression of SYK (Fig. 2B lane 3 vs. lane 5), suggesting that PAX5 tyrosine phosphorylation by the SYK signal plays an important role in the abolition of BLIMP1 repression by PAX5.

We examined co-operation of the tyrosine phosphorylation of PAX5 with its serine phosphorylation by ERK1/2, which we previously reported, in the cancelation of the PAX5-dependent repression of BLIMP1. We used CA-MEK1, a constitutive active mutant kinase of ERK1/2, to activate ERK1/2. The co-expression of CA-MEK1 and SYK attenuated transcriptional repression by PAX5 more than the co-expression of SYK alone in the luciferase assay (Fig. 2C lane 3 vs. lane 4). Furthermore, the combined PAX5 mutant of the tyrosine and serine phosphorylation sites (PAX5 TM/SM) showed strong resistance to the SYK and CA-MEK1-induced cancelation of transcriptional repression by PAX5 (Fig. 2C lane 4 vs. lane 7). These results indicated that SYK and ERK1/2 signaling