Biochemical and Biophysical Research Communications xxx (2016) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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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ARTICLE INFO

Article history:
Received 18 April 2016
Accepted 12 May 2016
Available online xxx

Keywords:
PAX5
BLIMP1
Phosphorylation
SYK
Plasma cell differentiation

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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http://dx.doi.org/10.1016/j.bbrc.2016.05.067

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

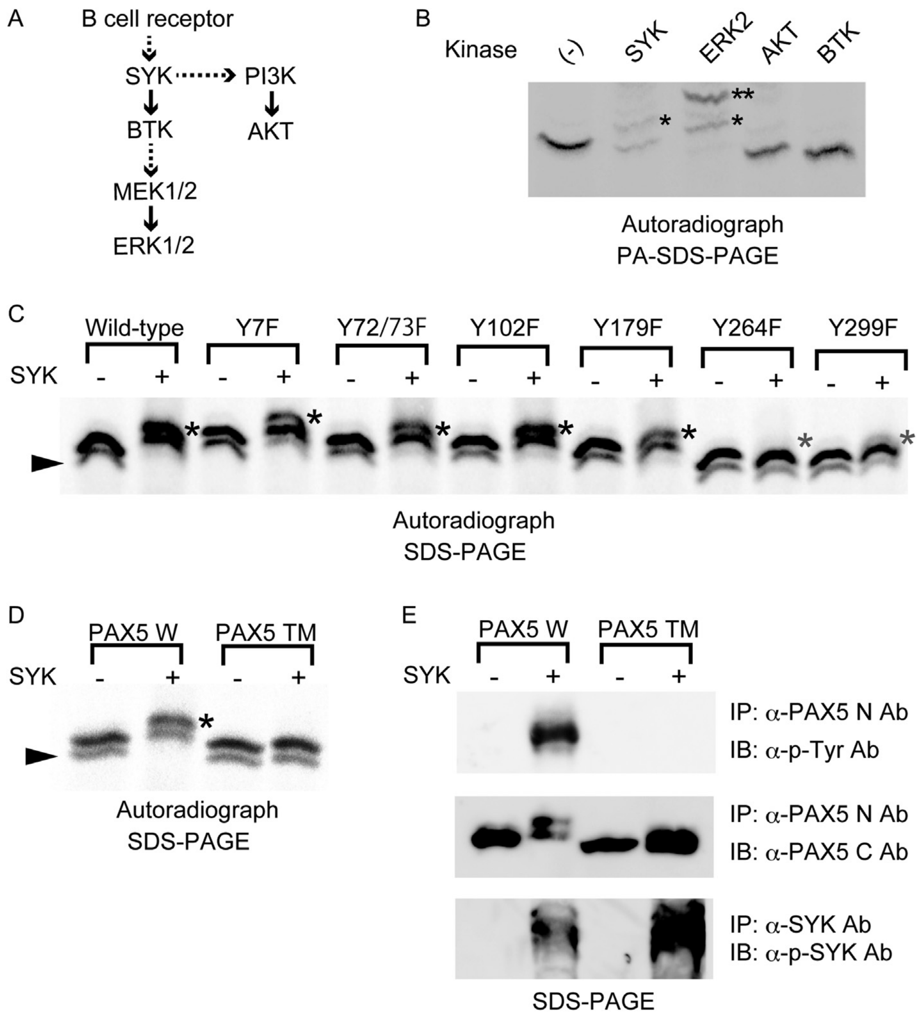

Fig. 1. PAX5 was phosphorylated by SYK. (A) Schematic presentation of BCR signal transduction. Solid and broken arrows indicate direct and indirect phosphorylation, respectively. (B) PAX5 was phosphorylated by SYK and ERK1/2 *in vitro*. Wild-type PAX5 was synthesized *in vitro* with [^{35}S]-labeling, phosphorylated *in vitro* with the indicated recombinant kinases, and separated by PA-SDS-PAGE. The single and double asterisks indicate PAX5 that are phosphorylated at one site and two sites, respectively. (C) Tyrosine 264 and tyrosine 299 were the candidate phosphorylation sites by SYK. Wild-type PAX5 and PAX5 with the indicated mutations were synthesized and phosphorylated by SYK, as in (B), and were separated by usual SDS-PAGE. The asterisks indicate phosphorylated PAX5. Mutants of Y264F and Y299F showed decreases in shifted bands (indicated by gray asterisks). An arrowhead indicates an isoform of PAX5 using a different ATG as the start codon. (D) PAX5 was phosphorylated by SYK at tyrosine 264 and tyrosine 299. Wild-type PAX5 (PAX5 W) and PAX5 with the combined mutations of Y264F and Y299F (PAX5 TM) were subjected to an *in vitro* kination assay, as in (C). (E) PAX5 phosphorylation by SYK in cells. 293T cells were transfected with a wild-type or mutant PAX5 expression vector with or without the co-transfection of the expression vector of SYK. Whole cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies.

co-operatively functioned to cancel the PAX5-induced transcriptional repression through its phosphorylation.

We investigated the effects of PAX5 tyrosine phosphorylation on its DNA-binding ability to clarify the mechanism of the cancelation of PAX5-dependent transcriptional repression; however, neither phosphorylation by SYK nor tyrosine phosphorylation site mutations affected the DNA-binding ability of PAX5 (Supplemental Figure S2).

3.3. The BCR signal induced BLIMP1 expression in B cells through the tyrosine and serine phosphorylation of PAX5

We determined whether tyrosine phosphorylation of PAX5 is induced by BCR signaling and contributes to BCR signaling-induced BLIMP1 expression. We initially established stable transfectants of Ramos cells, a cell line of a GCB cell tumor, expressing PAX5 W, PAX5 TM, and PAX5 TM/SM and designated as PAX5 W-Ramos, PAX5 TM-Ramos, and PAX5 TM/SM-Ramos, respectively. Since the phosphorylation of endogenous PAX5 masked differences in the phosphorylation status between exogenously expressed wild-type and mutant PAX5, we further introduced siRNA targeting 3′ UTR of endogenous PAX5 to specifically knockdown the endogenous PAX5 of these transfectants. The successful specific knockdown of endogenous PAX5 was demonstrated in Fig. 3A. BCR of these transfectants was stimulated by the anti-IgM antibody. In this system, the BCR signal induced PAX5 tyrosine phosphorylation, which was completely diminished by the mutation at the SYK phosphorylation sites (Fig. 3B). These results indicated that BCR signal-induced PAX5 tyrosine phosphorylation was mediated by SYK. We then examined BLIMP1 mRNA expression after the BCR stimulation in parental Ramos cells (Parental-Ramos) and these transfectants. BLIMP1 mRNA induction by the BCR stimulation was observed in PAX5 W-Ramos at a similar level to that in Parental-Ramos, while it was partly impaired in PAX5 TM-Ramos and completely abolished in PAX5 TM/SM-Ramos (Fig. 3C). These results indicated that PAX5 phosphorylation by SYK and ERK1/2 co-

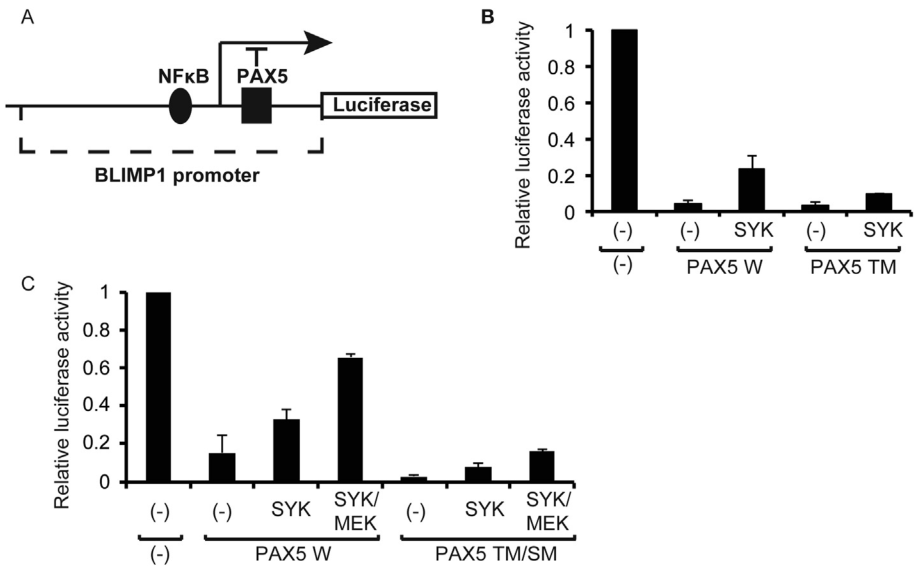

Fig. 2. Tyrosine and serine phosphorylation of PAX5 co-operatively functions to attenuate transcriptional repression by PAX5. (A) Schematic representation of the reporter plasmid for the analysis of transcriptional repression by PAX5. The luciferase reporter plasmid contains putative NF-κB- and PAX5-binding sequences. NF-κB induces luciferase expression while PAX5 represses it. (B) PAX5 phosphorylation by SYK attenuated transcriptional repression by PAX5. A luciferase assay was performed using the reporter plasmid described in (A). The expression vectors of NF-κB p50 and p65 were transfected in all samples. The expression vectors of PAX5 and SYK were co-transfected as indicated. The luciferase activities of the duplicated samples in 2 independent transfection experiments are shown as average values relative to the basal activity observed in control cells (results are the mean ± SD). (C) Co-operation of tyrosine and serine phosphorylation for the cancelation of PAX5-dependent repression. A luciferase assay was performed, as in (B).

operatively functioned for the BCR signal-induced expression of BLIMP1 in B cells.

4. Discussion

The prominent exchange of PAX5 with BLIMP1 after the BCR stimulation by an antigen was previously considered to be a key molecular event in plasma cell differentiation. Once BLIMP1 is expressed by overcoming the repression caused by PAX5, it suppresses PAX5, which allows for the stronger expression of BLIMP1. PAX5 may be quickly replaced by BLIMP1 through this positive feedback [4]. A previous study reported that abolition of the PAX5-mediated repression of BLIMP1 occurred before the down-regulation of PAX5 expression levels without a decrease in the DNA-binding ability of PAX5 [5]. The mechanism underlying the BCR signaling-induced attenuation of PAX5 functions has yet to be clarified. In order to provide an insight into this issue, we proposed PAX5 serine phosphorylation by ERK1/2 in our previous study and its tyrosine phosphorylation by SYK in the present study. The BCR stimulation induced increases in BLIMP1 expression that were 3.0-, 1.6-, and 1.1-fold that in PAX5 W-Ramos, PAX5 TM-Ramos, and PAX5 SM/TM-Ramos, respectively (Fig. 3C, comparing lane 3 vs. lane 4, lane 5 vs. lane 6, and lane 7 vs. lane 8, respectively). Furthermore, the expression of BLIMP1 was 1.5-fold stronger in PAX5 SM-Ramos in the same experiment in our previous study [8]. The result that TM and SM both severely impaired the responsiveness of PAX5 to the BCR stimulation indicated that the tyrosine and serine phosphorylation was required for PAX5 to respond sufficiently to BCR signaling. This may enhance the specificity of BCR signaling as a differentiation trigger. The schema of this putative model is shown in the Graphical abstract.

The exact mechanisms by which tyrosine and serine phosphorylation attenuates the PAX5-dependent repression of BLIMP1 have not yet been elucidated. The tyrosine and serine phosphorylation of PAX5 did not affect the DNA-binding ability of PAX5 in the present study (Supplemental Figure S2) or our previous study [8]. Grg4, a member of the Groucho co-repressor family, have been shown to play a role in transcriptional repression by PAX5 [10]. PAX5 phosphorylation may affect associations with these co-repressors.

A fusion gene between the potent enhancer of the IGH gene and PAX5 promoter have been detected in non-Hodgkin's lymphoma patients with the chromosomal translocation, t(9; 14)(p13; q32) [11], and the oncogenicity of this fusion gene was confirmed in its transgenic mouse [12]. These findings suggest that the sustained repression of BLIMP1 by over-expressed PAX5 impairs plasma cell differentiation and leads to lymphoma. The recent finding that BLIMP1 inactivation by multiple mechanisms such as homozygous deletions, mutations, and repression by constitutively active BCL6 is frequently observed in activated B cell-like diffuse large B cell lymphoma patients further supports this possibility [13]. These findings also raise the possibility that mutations impairing the tyrosine and/or serine phosphorylation of PAX5 cause lymphoma through the sustained repression of BLIMP1. Previous studies reported that mutations in the *PAX5* gene by aberrant somatic hypermutation were frequently observed in lymphoma patients [14,15]. Mutation(s) at the tyrosine and serine phosphorylation sites have not been reported, yet; however, the sequencing analyses were mainly performed in the 5′ region of the gene in those studies although the phosphorylation sites of PAX5 are rather in the C-terminal half. Furthermore, mutations outside the phosphorylation sites have the possibility to abolish PAX5 phosphorylation by affecting its association with kinases. Some of the reported PAX5 mutations in lymphoma patients might impair PAX5 phosphorylation and its function.

Disclosure of conflicts of interest

T.N. received research funding from FUJIFILM Corporation. H.K. received research funding from Bristol-Myers Squibb, Chugai Pharmaceutical Co. LTD., Kyowa Hakko Kirin Co. LTD., Dainippon Sumitomo Pharma, Zenyaku Kogyo, FUJIFILM Corporation, and Nippon Boehringer Ingelheim Co. LTD. The other authors have no potential conflicts of interest.

Please cite this article in press as: Y. Inagaki, et al., 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, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.05.067

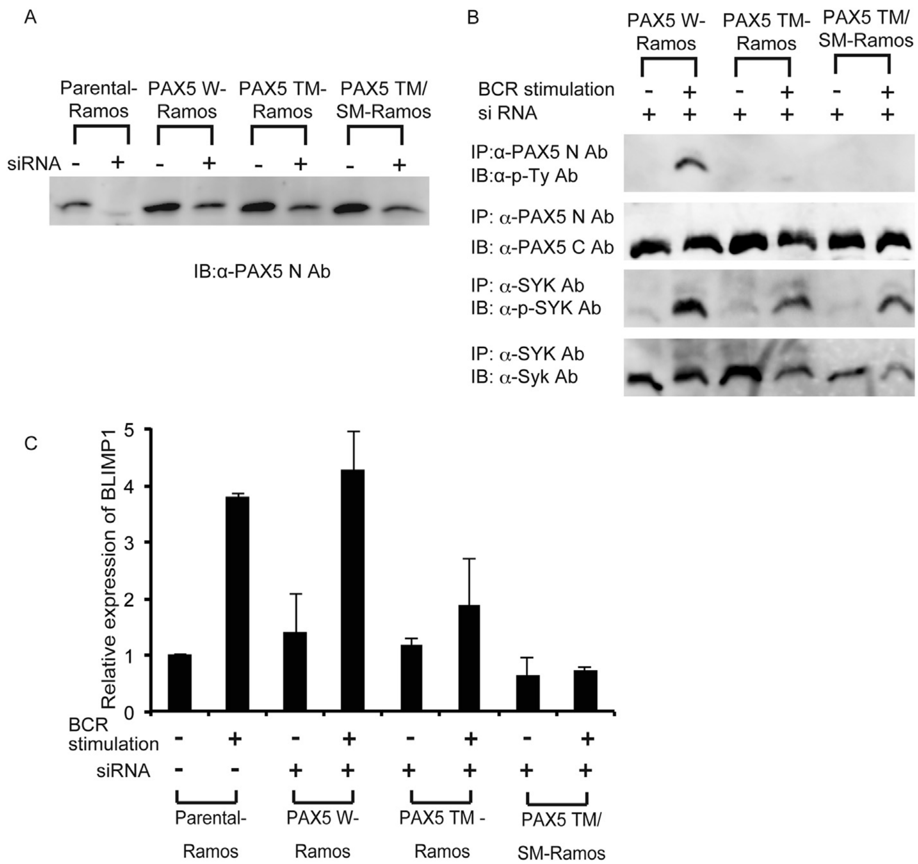

Fig. 3. PAX5 tyrosine phosphorylation co-operatively functioned with its serine phosphorylation for BCR signal-induced BLIMP1 expression. (A) Specific knockdown of endogenous PAX5 in Ramos cells. The indicated PAX5 stable transfectants of Ramos cells were introduced into control siRNA [siRNA (−)] and the siRNA targeting the 3′-UTR of endogenous PAX5 [siRNA (+)]. Cells were lysed 24 h later and subjected to immunoblotting. A similar level of PAX5 reduction occurred with the introduction of PAX5-targetting siRNA in all Ramos transfectants, and PAX5 expression was diminished in Parental-Ramos expressing only endogenous PAX5, indicating the successful specific knockdown of endogenous PAX5. (B) Mutations at the SYK phosphorylation sites abolished BCR signal-induced PAX5 tyrosine phosphorylation. PAX5-targeting siRNA was introduced into indicated Ramos transfectants to specifically knockdown endogenous PAX5. Cells were then stimulated with an anti-IgM antibody, lysed, and subjected to immunoprecipitation and immunoblotting as in Fig. 2A. (C) Resistance of the phosphorylation-defective mutant of PAX5 to BCR signal-induced BLIMP1 derepression. Control siRNA and PAX5-targeting siRNA were introduced into parental Ramos cells and Ramos transfectants, as in (A). Eight hours after the BCR stimulation, RNA was harvested and the mRNA expression of BLIMP1 was quantified by real-time RT-PCR. The relative mRNA expression levels presented reflect duplicate values from two independent experiments.

Acknowledgments

We are very grateful to Yoko Matsuyama and Chika Wakamatsu for their technical assistance. This work was supported by MHLW KAKENHI Grant Number H26-Kakushintekigan-Ippan-133, and JSPS KAKENHI Grant Numbers 23591381, 25118711, 25293218, and 25670449. This study was also supported by the Program to Disseminate a Tenure Tracking System, MEXT, Japan.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.05.067.

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