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B Cell Receptor-ERK1/2 Signal Cancels PAX5-Dependent Repression of BLIMP1 through PAX5 Phosphorylation: A Mechanism of Antigen-Triggering Plasma Cell Differentiation

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Plasma cell differentiation is initiated by Ag stimulation of BCR. Until BCR stimulation, B lymphocyte-induced maturation protein 1 (BLIMP1), a master regulator of plasma cell differentiation, is suppressed by PAX5, which is a key transcriptional repressor for maintaining B cell identity. After BCR stimulation, upregulation of BLIMP1 and subsequent suppression of PAX5 by BLIMP1 are observed and thought to be the trigger of plasma cell differentiation; however, the trigger that derepresses BLIMP1 expression is yet to be revealed. In this study, we demonstrated PAX5 phosphorylation by ERK1/2, the main component of the BCR signal. Transcriptional repression on *BLIMP1* promoter by PAX5 was canceled by PAX5 phosphorylation. BCR stimulation induced ERK1/2 activation, phosphorylation of endogenous PAX5, and upregulation of BLIMP1 mRNA expression in B cells. These phenomena were inhibited by MEK1 inhibitor or the phosphorylation-defective mutation of PAX5. These data imply that PAX5 phosphorylation by the BCR signal is the initial event in plasma cell differentiation. ***The Journal of Immunology*****, 2012, 188: 6127–6134.**

PAX5 is a member of the highly conserved paired-box (PAX) domain family of transcription factors. PAX5 is exclusively expressed from the pro-B to mature B cell stages and is downregulated during terminal differentiation into plasma cells (1). PAX5 is not only indispensable for B-lineage commitment; its continuous expression is essential for maintaining the identity of B cells (2–4). PAX5 functions as both a transcriptional activator of B lineage-specific genes and a repressor of B lineage-inappropriate genes (1) [i.e., it activates *CD19* (5), *CD79A* (6), and B cell linker protein (7) and represses CSF1 receptor (8), *Notch1* (9), and FMS-like tyrosine kinase 3 (10)]. In addition, it checks the initiation of plasma cell differentiation and the terminal differentiation of B cells by repressing B lymphocyte-induced maturation protein 1 (*BLIMP1*) and X box-binding protein 1 (11, 12).

BCR signal plays important roles in the activation, survival, and differentiation of B lymphocytes. The initial event after BCR engagement is the activation of Lyn and Syk. These kinases trigger a complex network of signaling pathways downstream of the receptor, including the Ras-Raf-MEK-ERK1/2 pathway, the Vav-cell division cycle 42-JNK pathway, and the NF-κB pathway (13). The resulting signals quickly reach the nucleus and alter gene expression. The ultimate effects on B cells are profound and vary depending on the maturation state of the cell and on additional signals that the cell receives. For germinal center B (GCB) cells, BCR signal after encountering Ag is known to initiate PAX5 downregulation, BLIMP1 upregulation, and eventually, plasma cell differentiation (14, 15). In these cells, PAX5 suppresses BLIMP1 expression and checks plasma cell differentiation. After BCR stimulation by Ag, BLIMP1 repression by PAX5 is abolished, and once BLIMP1 is expressed, it suppresses PAX5. Eventually, PAX5 is replaced by BLIMP1, which initiates plasma cell differentiation (14). The abolition of PAX5-mediated repression was thought to be the first event to initiate plasma cell differentiation (16); however, the mechanism is still unknown. In this study, we demonstrated that PAX5 was phosphorylated by ERK1/2 in vitro and in vivo at serines 189 and 283. This phosphorylation attenuated the transcriptional repression of BLIMP1 by PAX5. Finally, BCR stimulation induced the phosphorylation of ERK1/2 and PAX5, as well as BLIMP1 mRNA expression in B cells, which were inhibited by MEK1 inhibitor or the phosphorylation-defective mutation of PAX5. These data imply that PAX5 phosphorylation by the BCR signal is an initial event in plasma cell differentiation.

Materials and Methods

Cells, Abs, and reagents

Burkitt lymphoma cell line Ramos cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Anti-HA Ab, anti-ERK2 Ab, and anti-PAX5 Ab (C-20) for immunoblotting, and anti-PAX5 Ab (N-19) for the supershift assay in EMSA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human IgM Ab and anti-mouse IgM Ab for BCR stimulation and anti–phospho-ERK1/2 Ab were from Abcam (Cambridge, U.K.), Jackson ImmunoResearch Laboratories (West Grove, PA), and Cell Signaling (Beverly, MA), respectively. U0126 was obtained from Calbiochem (San Diego, CA).

Plasmids

PAX5/pCDNA, the expression vector for PAX5, was described previously (17). PAX5/pGEX and PAX5(1-279)/pGEX, expression vectors for GST-

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Abbreviations used in this article: BLIMP1, B lymphocyte-induced maturation protein 1; GCB, germinal center B; siRNA, small interfering RNA; UTR, untranslated region.

fused full-length and partial PAX5, as well as PAX5/pBGJR, were made by subcloning PAX5 cDNA digested from PAX5/pCDNA with appropriate restriction enzymes into pGEX 5X-1 vector (Pharmacia, Uppsala, Sweden) and pBGJR, a lentivirus expression vector kindly provided by Dr. Stefano Rivella (Memorial Sloan-Kettering Cancer Center). Mutations for serine/threonine-to-alanine substitutions were introduced into PAX5/pCDNA using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All clones were subjected to sequence analysis to confirm the introduction of the correct mutations and to exclude PCR artifacts. The expression vector for HA-tagged CA-MEK1, HA–CA-MEK1/pCMV, was described previously (18). The −1921 to +138 region (relative to the translation start site) of the *BLIMP1* promoter containing putative NF-κB- and PAX5-binding sequences was amplified by PCR, according to a previous report (19), and subcloned into pGL4.20 (Promega, Madison, WI). Two specific primer genes, 5′-TAACAGTGAGTTGATTCACTGGC-3′ (sense) and 5′-CTCGGCGGTCCCTCCTCG-3′ (antisense), were selected on the basis of the sequence of the human *BLIMP1* genomic DNA (accession number AL358952, http://www.ncbi.nlm.nih.gov/nuccore/Al358952). This reporter gene was designated as BLIMP1-luc/pGL4. Expression vectors for NF-κB p50 and p65 were purchased from Addgene (Cambridge, MA).

Transient transfection, lentivirus infection, immunoblotting, immunofluorescence, EMSA, luciferase assay, and in vitro kination assay

Transient transfection, lentivirus infection, immunoblotting, immunofluorescence, EMSA, luciferase assay, and in vitro kination assay were performed as described previously (17, 18, 20).

PAX5 knockdown

The small interfering RNA (siRNA) targeting the 5′-GACTATCCATCCATCATAA-3′ sequence in the 3′ untranslated region (UTR) of *PAX5* was purchased from Sigma-Aldrich (St. Louis, MO) and introduced into Ramos cells with nucleofector (Lonza, Wuppertal, Germany), according to the manufacturer's instructions.

Phosphate-affinity SDS-PAGE

Phosphate-affinity SDS-PAGE was performed similarly to SDS-PAGE, except that Phos-tag acrylamide-containing acrylamide gel was used. Phos-tag acrylamide was obtained from Wako Laboratory Chemicals (Osaka, Japan). In this system, Phos-tag acrylamide binds to phosphorylated amino acids during electrophoresis and slows the migration of phosphorylated proteins, according to the number of phosphorylation sites. Autoradiography or immunoblotting following electrophoresis can detect the phosphorylation of the target protein as band shifts.

Mouse spleen cell isolation

Mouse spleen cells were collected from 10-wk-old BALB/c mice and were used for immunoblotting analysis to detect PAX5 phosphorylation. Mouse spleen B cells were purified from the spleen cells using CD45R (B220) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and subjected to RT-PCR to detect BLIMP1 expression. These cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 U/ml streptomycin.

Real-time PCR

Total RNA was purified using the QIAamp RNA Blood Mini Kit (QIAGEN, Hilden, Germany), and reverse transcription was performed with random hexamers using the Superscript III First Strand kit (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed by standard procedures using TaqMan Universal PCR Master Mix; quantitative PCR primers for human BLIMP1 (Hs00153357), mouse Blimp1 (Mm00476128), GAPDH, or ACTB; and the ABI Prism 7000 Sequence Detection System. All of these reagents, primers, and equipment were from Applied Biosystems (Foster City, CA). Each reaction was performed in duplicate, and results were normalized by GAPDH or ACTB expression.

PAX5 DNA sequencing of clinical samples

Lymph nodes or other tissues containing tumor cells were collected with informed consent from 85 patients diagnosed with diffuse large B cell lymphoma. The sequencing study was approved by the institutional review board of Nagoya University Graduate School of Medicine. Genomic DNA was extracted from those samples with the QIAamp DNA Micro Kit (QIAGEN), according to the manufacturer's instructions. Pyrosequencing was performed for analysis of DNA mutation surrounding codons 189 and 283, according to the manufacturer's instructions. Briefly, a 125-bp sequence of exon 5, including 189 codon, or a 128-bp sequence of exon 7, including 283 codon, was amplified by PCR using biotin-tagged primers. The biotinylated PCR strands were immobilized and purified by streptavidin-Sepharose beads, denatured, and added to annealing buffer containing 250 nM sequencing primer. Sequencing was carried out with the PyroMark Q96 ID system (QIAGEN). DNA sequences corresponding to codons 181–199 and codons 273–292 were analyzed using two sequence primers. Primers used in this analysis are described in Supplemental Table I.

Results

PAX5 is phosphorylated by ERK2 in vitro

It was recently reported that ERK1/2 signal was a key initiation signal for BLIMP1 expression and plasma cell differentiation of GCB cells (21); however, the detailed mechanism of ERK1/2 signal that induces BLIMP1 expression is still unknown. Combined with the open question about the initial event in plasma cell differentiation, these findings gave rise to the speculation that PAX5 phosphorylation by ERK1/2 negatively affected BLIMP1 repression by PAX5 and is the trigger of plasma cell differentiation. To test whether PAX5 can be phosphorylated in response to ERK1/2 signal, we performed an in vitro kinase assay using GST-PAX5 as a substrate. rERK2 efficiently phosphorylated full-length PAX5 and the N-terminal region of PAX5, whereas JNK did not (Supplemental Fig. 1A). Inspection of the PAX5 sequence revealed the presence of eight ERK1/2 consensus sites, S/T-P. Therefore, we introduced a series of alanine substitutions at these sites to map the actual phosphorylation sites by ERK2 and to check the phosphorylation status with the phosphate-affinity SDS-PAGE system. In this system, PAX5 phosphorylation by ERK2 was detected as two shifted bands (Fig. 1A), suggesting that PAX5 had two phosphorylation sites. Substitutions of alanine at codons 189, 283, and 285 (combined mutation), as well as at 283, caused the disappearance of one of the shifted bands, whereas other substitutions, including at codon 285, did not affect the shifted bands (Fig. 1A, Supplemental Fig. 1B, 1C), indicating that the phosphorylation sites were serines 189 and 283. Consistently, all shifted bands disappeared by combined substitution at codons 189 and 283 (Fig. 1A).

PAX5 phosphorylation occurred in vivo through ERK1/2 signaling

We next examined whether PAX5 phosphorylation occurred by the activation of ERK1/2 in vivo. Coexpression of the constitutively active mutant of MEK1 (CA-MEK1, an upstream activator of endogenous ERK1/2) in 293T cells caused similar PAX5 phosphorylation as in vitro (i.e., two shifted bands were observed in phosphate-affinity SDS-PAGE and disappeared by the mutation at ERK2 phosphorylation sites determined in vitro) (Fig. 1B). These results indicated that ERK1/2 could also phosphorylate PAX5 in vivo. We demonstrated the schema of the PAX5 structure, pointing out the phosphorylation sites. The amino acid sequences surrounding the phosphorylation sites are conserved evolutionarily, except in zebrafish (Fig. 1C).

Next, we attempted to determine whether endogenous PAX5 was phosphorylated by BCR stimulation. We stimulated BCR by anti-IgM Ab in Ramos cells, a cell line of Burkitt lymphoma, which is thought to be a tumor of GCB cells. Strikingly, BCR stimulation induced strong ERK1/2 phosphorylation and PAX5 phosphorylation in Ramos cells. This phosphorylation was inhibited by the MEK1 inhibitor, U0126, indicating the mediation of PAX5 phosphorylation by ERK1/2 signal (Fig. 2A). Furthermore, to examine whether BCR signal-induced PAX5 phosphorylation

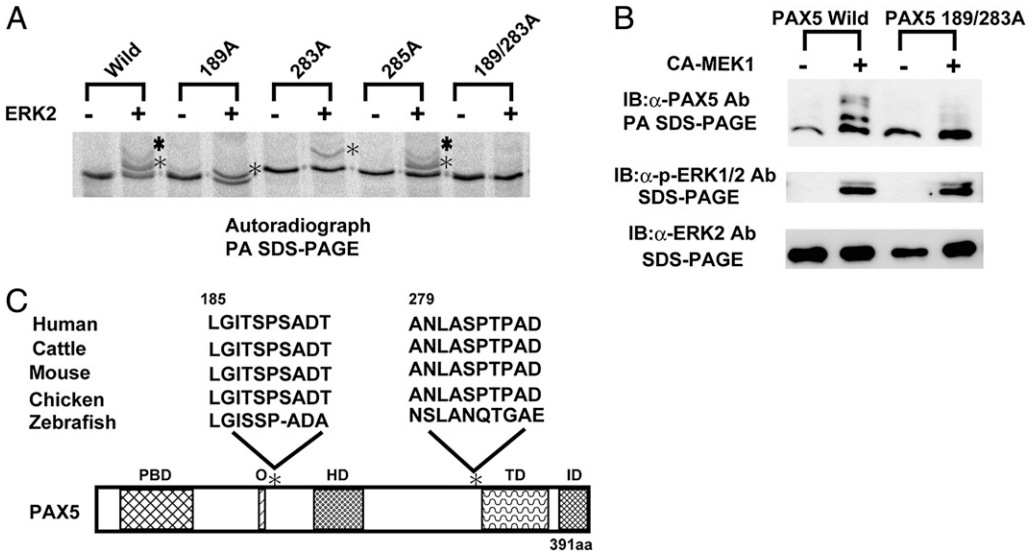

FIGURE 1. PAX5 was phosphorylated by ERK1/2 signal. (**A**) PAX5 was phosphorylated by ERK2 at serines 189 and 283 in vitro. Wild-type PAX5 and PAX5 with the indicated mutations were synthesized in vitro with [^{35}S]-labeling. Mutations are designated by the codon number followed by "A". PAX5 proteins were phosphorylated in vitro with ERK2 and were separated by phosphate-affinity (PA) SDS-PAGE, in which phosphorylated proteins migrate slowly relative to the number of phosphorylation sites. The regular asterisks and bold asterisks indicate PAX5 phosphorylated at one site and two sites, respectively. Mutants of 189A and 283A showed only one shifted band, and all shifted bands disappeared by the mutation of 189/283A, suggesting that serines 189 and 283 were the phosphorylation sites. (**B**) PAX5 was phosphorylated by ERK1/2 in vivo. 293T cells (1×10^5) were transfected with wild-type or mutant PAX5 expression vector (200 ng), with or without cotransfection of constitutively active MEK1 (CA-MEK1) expression vector (200 ng). Cell lysates were separated by PA SDS-PAGE or SDS-PAGE, as indicated, and subjected to immunoblotting (IB). Wild-type PAX5 was phosphorylated by the coexpression of CA-MEK1, but mutant PAX5 was not. (**C**) Conservation of PAX5 phosphorylation sites beyond species. Alignment of amino acid sequences of human, cattle, mouse, chicken, and zebrafish PAX5 corresponding to the phosphorylation sites of human PAX5. Asterisks indicate phosphorylation sites of human PAX5. Amino acid sequences surrounding PAX5 phosphorylation sites are completely conserved, except in zebrafish. HD, Homeobox domain; ID, inhibitory domain; O, conserved octapeptide; PBD, paired box domain; TD, transactivation domain.

occurred at ERK2 phosphorylation sites determined in vitro, we established stable Ramos transfectants of control vector and the expression vectors of wild-type and phosphorylation-defective mutant of PAX5, designated as Control-Ramos, PAX5 Wild-Ramos, and PAX5 189/283A-Ramos, respectively. Because phosphorylation of endogenous PAX5 masked the difference in the phosphorylation status between exogenously expressed wild-type and mutant PAX5 (data not shown), we further introduced the siRNA targeting 3′ UTR of endogenous PAX5 to specifically knockdown endogenous PAX5 of these transfectants. Successful specific knockdown of endogenous PAX5 is demonstrated in Fig. 2B. In this system, BCR signal-induced PAX5 phosphorylation was completely diminished by the mutation at ERK2 phosphorylation sites (Fig. 2C). These results indicated that BCR signal-induced PAX5 phosphorylation was mediated by ERK1/2.

We also performed the same experiment in Fig. 2A using mouse spleen cells that were rich in primary B cells. The results were similar to that in Ramos cells. BCR stimulation of mouse spleen cells induced ERK1/2 phosphorylation and PAX5 phosphorylation, which was inhibited by U0126 (Fig. 2D). These results suggested that PAX5 phosphorylation by ERK1/2 in response to BCR stimulation also occurred in primary normal B cells.

ERK1/2 signal canceled PAX5-dependent transcriptional repression of BLIMP1

Next, we set out to identify the effect of phosphorylation on BLIMP1 repression by PAX5. To replicate BLIMP1 repression by PAX5 in the luciferase assay, we constructed a reporter gene containing an ~2-kbp region of *BLIMP1* promoter, including putative binding sites for PAX5, and NF-κB, one of the BLIMP1 expression activators, according to a previous report (19) (Fig. 3A). Before the assay, we focused on enhancement of PAX5 expression by CA-MEK1 coexpression observed in Fig. 1B, because it may affect the luciferase assay comparing PAX5 function with and without CA-MEK1 coexpression. We judged that this enhancement of PAX5 expression is due to a nonspecific effect of ERK1/2 signal on the transcriptional machinery on the T7 or CMV promoter of PAX5/pCDNA, because CA-MEK1 coexpression also enhanced the expression of the phosphorylation-defective mutant of PAX5 (Fig. 1B), indicating that this phenomenon was independent of PAX5 phosphorylation, and BCR stimulation or CA-MEK1 expression in Ramos cells did not affect the expression level of endogenous PAX5 (Fig. 2A, data not shown). Therefore, we investigated the effect of CA-MEK1 coexpression on PAX5 expression in detail (Supplemental Fig. 2A) and adjusted the amount of PAX5 expression vector used for the luciferase assay when it was cotransfected with the CA-MEK1 expression vector to keep the PAX5 expression level constant. A constant expression of PAX5 among luciferase samples was confirmed by immunoblotting (Fig. 3B, *lower panel*).

Luciferase expression was strongly induced by NF-κB expression, which was decreased to 15% of the control level by wild-type PAX5 coexpression (Fig. 3B, *lane 2* versus *lane 4*). Importantly, CA-MEK1 coexpression increased the luciferase expression suppressed by wild-type PAX5 to 80% of the control level (Fig. 3B, *lane 3* versus *lane 5*), indicating that ERK1/2 signal canceled transcriptional repression by PAX5. Furthermore, transcriptional repression by mutant PAX5 was attenuated by CA-MEK1 coexpression to a significantly lesser extent than that by wild-type PAX5 (Fig. 3B, *lane 5* versus *lane 7*, $p < 0.05$), indicating its resistance to ERK1/2 signal-dependent cancellation of the transcriptional repression. These data suggested that PAX5 phosphorylation by ERK1/2 signal played an important role in the abolition of BLIMP1 repression by PAX5.

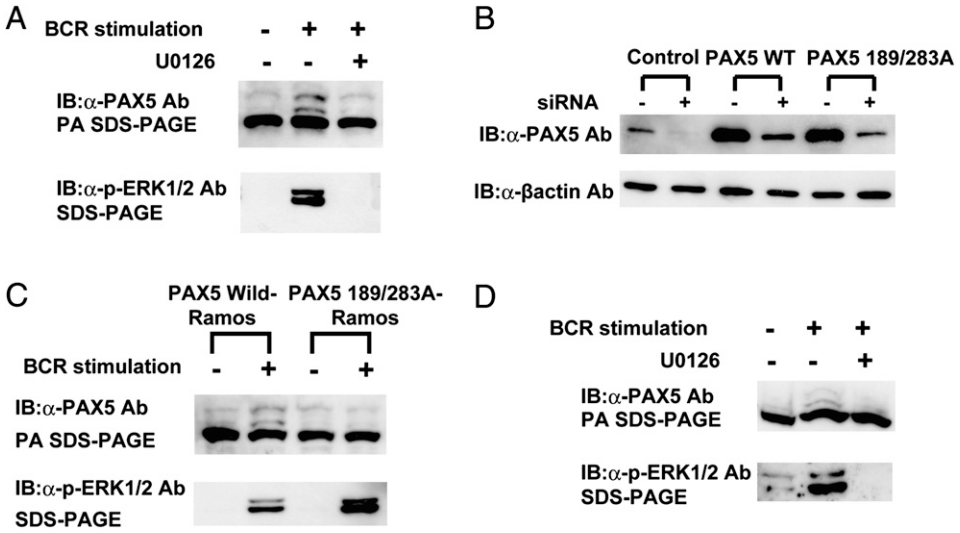

FIGURE 2. PAX5 phosphorylation was induced by BCR stimulation through ERK1/2 signal in B cells. (**A**) MEK1 inhibitor inhibited BCR signal-induced PAX5 phosphorylation. Ramos cells were stimulated with anti-IgM Ab (2 μg/ml) for 10 min. U0126 (10 μM) was added 60 min before stimulation, as indicated. Cell lysates were subjected to immunoblotting analyses, as in Fig. 1B. PAX5 phosphorylation was induced by BCR stimulation, which was inhibited by U0126. (**B**) Specific knockdown of endogenous PAX5 in Ramos cells. The indicated PAX5 stable transfectants of Ramos cells were introduced control siRNA (−) and siRNA targeting the 3′-UTR of endogenous PAX5 (+), as indicated. Twenty-four hours later, cells were lysed and subjected to immunoblotting. Reduction of PAX5 expression by siRNA introduction was much stronger in Control-Ramos cells that expressed only endogenous PAX5 than in PAX5 Wild-Ramos cells and PAX5 189/283A-Ramos cells that expressed endogenous and exogenous PAX5, indicating specific knockdown of endogenous PAX5. (**C**) The mutations at ERK2 phosphorylation sites abolished BCR signal-induced PAX5 phosphorylation. The siRNA targeting the 3′-UTR of endogenous PAX5 mRNA was introduced into PAX5 Wild-Ramos cells and PAX5 189/283A-Ramos cells to knockdown endogenous PAX5 specifically. Then, cells were stimulated with anti-IgM Ab, lysed, and subjected to immunoblotting analyses, as in (A). (**D**) MEK1 inhibitor inhibited BCR signal-induced PAX5 phosphorylation in mouse spleen cells. Mouse spleen cells were treated and analyzed as in (A), except that cells were stimulated with anti-IgM Ab (10 μg/ml) for 10 min.

Of note, mutant PAX5 suppressed the luciferase expression more strongly than did wild-type PAX5 when CA-MEK1 was not coexpressed (Fig. 3B, *lane 4* versus *lane 6*). This is probably due to mild attenuation of wild-type PAX5 ability by weak phosphorylation of PAX5 by constitutively activated ERK1/2 signal in 293T cells, because ERK1/2 was weakly, but constitutively, phosphorylated in 293T cells without CA-MEK1 coexpression (Supplemental Fig. 2B), and administration of U0126 enhanced

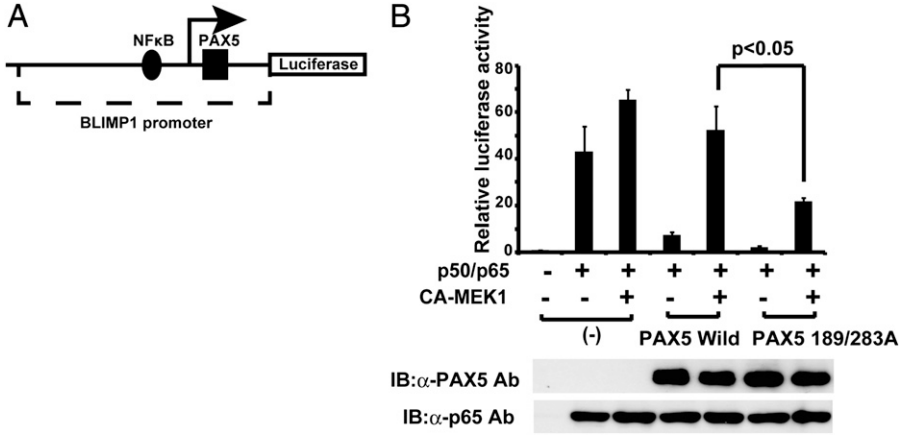

FIGURE 3. PAX5 phosphorylation attenuates transcriptional repression by PAX5. (**A**) Schematic representation of BLIMP1-luc/pGL4, the reporter plasmid for analysis of transcriptional repression by PAX5. The part of the *BLIMP1* promoter (~2 kbp) containing putative NF-κB– and PAX5-binding sequences was subcloned to luciferase reporter plasmid. (**B**) Transcriptional repression by PAX5. Luciferase assay was performed using BLIMP1-luc/pGL4. When CA-MEK1 expression vector was cotransfected, the amount of PAX5 expression vectors was reduced from 100 to 20 ng to avoid elevation of the PAX5 expression level by CA-MEK1 coexpression. Triplicate sets of cells were lysed separately: two sets for the luciferase assay using luciferase assay buffer and the other set for immunoblotting using whole-cell extraction buffer. Luciferase activities in three independent transfection experiments are shown as average values relative to the basal activity observed in control cells (results are the mean ± SD). Cell lysates were also subjected to immunoblotting (IB), as indicated, to confirm the equal expressions of PAX5 (*middle panel*). The expression vectors of NF-κB p50 and p65 were 50 ng each and were not adjusted in cotransfection with CA-MEK1. Equal expressions of NF-κB p65 were also confirmed by immunoblotting (*bottom panel*). Statistical comparisons were performed using the *t* test. Wild-type and mutant PAX5 suppressed NF-κB–induced luciferase expression. Transcriptional repression by wild-type PAX5 was canceled by CA-MEK1 coexpression, whereas that by mutant PAX5 was significantly more resistant to CA-MEK1 coexpression (*lane 5* versus *lane 7*, $p < 0.05$).

the transcriptional repression by wild-type PAX5 but did not affect that by mutant PAX5 and diminished the difference between them (Supplemental Fig. 2C).

BCR signal-induced PAX5 phosphorylation increased BLIMP1 expression in B cells

To further confirm the BLIMP1 derepression by BCR stimulation, we first examined BLIMP1 mRNA expression after BCR stimulation in Ramos cells. BLIMP1 mRNA expression was induced by BCR stimulation following ERK1/2 and PAX5 phosphorylation, and U0126 inhibited the induction of BLIMP1 expression (Fig. 4A). Next, we used Ramos transfectants with specific knockdown of endogenous PAX5. Exogenous expression of wild-type and mutant PAX5 reduced BLIMP1 expression. BCR stimulation relieved BLIMP1 repression by wild-type PAX5, whereas repression by mutant PAX5 was resistant to BCR stimulation (Fig. 4B). These data indicated that BCR signal-induced BLIMP1 expression was mediated by PAX5 phosphorylation by ERK1/2. Notably, in contrast to the luciferase assay in Fig. 3B, no difference in the repression of BLIMP1 was observed between wild-type and mutant PAX5 transfectants of Ramos cells when there was no BCR stimulation (Fig. 4B, *lane 3* versus *lane 5*). This occurs because Ramos cells have no constitutive ERK1/2 activation, which is different from 293T cells (Supplemental Fig. 2B).

We also examined BCR signal-induced BLIMP1 expression in mouse spleen B cells. BCR stimulation of mouse spleen B cells induced BLIMP1 mRNA expression, which was inhibited by U0126 (Fig. 4C). Taken together with BCR signal-induced PAX5 phosphorylation in these cells (Fig. 2D), these data implied that BCR signal-induced BLIMP1 derepression through PAX5 phosphorylation by ERK1/2 might also occur in primary B cells.

PAX5 phosphorylation did not affect DNA-binding ability or cellular localization

To clarify how PAX5 phosphorylation attenuated its function, we used EMSA to investigate the effect of PAX5 phosphorylation on DNA-binding ability. PAX5, synthesized in vitro, was incubated with radiolabeled oligomers containing the PAX5-binding sequence in the *CD19* promoter. The obtained single band was competed by the presence of a 200-fold molar excess of non-radiolabeled oligomers and was supershifted by anti-PAX5 Ab but not by control rabbit IgG (Fig. 5A). Wild-type and mutant PAX5 were subjected to in vitro kination, with or without ERK2, and then to EMSA. No difference in DNA-binding activity was observed, regardless of phosphorylation by ERK2 and phosphorylation-defective mutation (Fig. 5A). These results indicated that PAX5 phosphorylation did not affect its DNA-binding ability.

Next, we examined the alteration of PAX5 cellular localization by coexpression of CA-MEK1 or phosphorylation-defective mutation. Overexpressed wild-type and mutant PAX5 localized diffusely in the nucleus, and this was not affected by coexpression of CA-MEK1, suggesting that cellular localization of PAX5 was not altered by its phosphorylation (Fig. 5B). The molecular mecha-

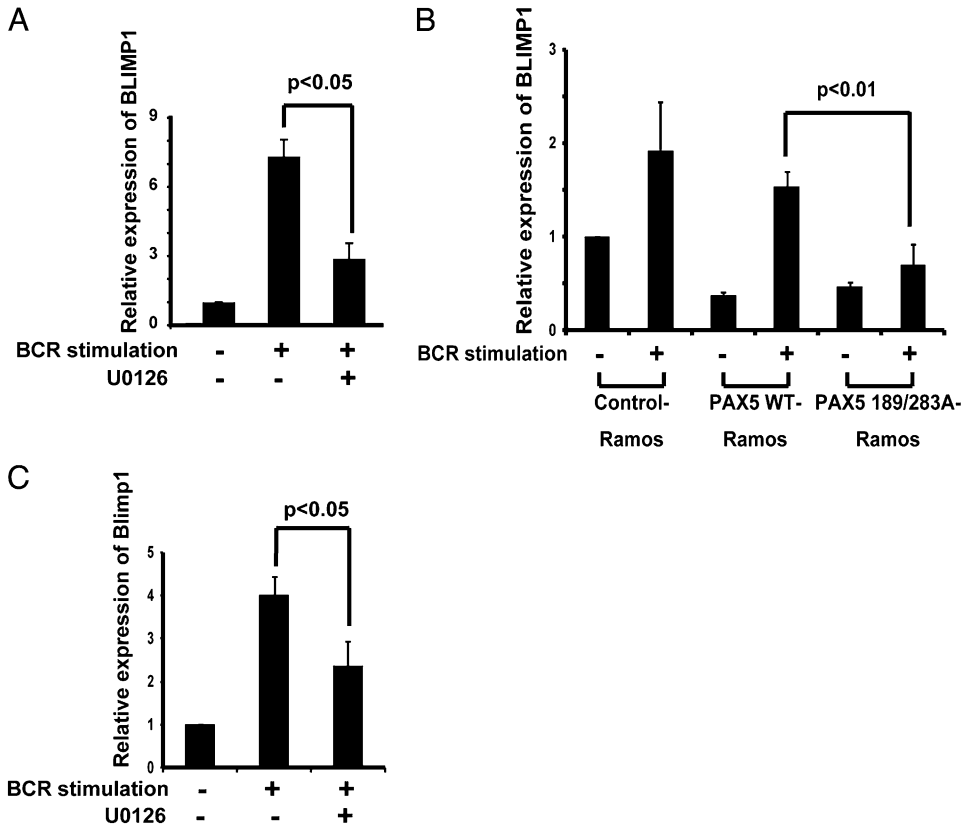

FIGURE 4. PAX5 phosphorylation plays an important role in BCR signal-induced BLIMP1 expression. (**A**) MEK1 inhibitor inhibited BCR signal-induced BLIMP1 expression. Ramos cells were treated as in Fig. 2A. Eight hours after stimulation, RNA was harvested, and mRNA expression of BLIMP1 was quantified by real-time RT-PCR. The relative mRNA expression levels reflect duplicate values from two independent experiments. Statistical comparisons were performed by using the *t* test. (**B**) Phosphorylation-defective mutant of PAX5 abolished BCR signal-induced BLIMP1 expression. Control-Ramos, PAX5 Wild-Ramos, and PAX5 189/283A-Ramos cells were introduced siRNA for specific knockdown of endogenous PAX5 and stimulated with anti-IgM Ab for BCR stimulation as in Fig. 2C. RNA harvest and quantification of BLIMP1 mRNA were performed as in (A). (**C**) MEK1 inhibitor inhibited BCR signal-induced BLIMP1 expression in mouse spleen B cells. Mouse spleen B cells, purified as described in *Materials and Methods*, were treated and analyzed as in (A), except that cells were stimulated with anti-IgM Ab (10 μg/ml), and RNA extraction was performed 1 h after stimulation.

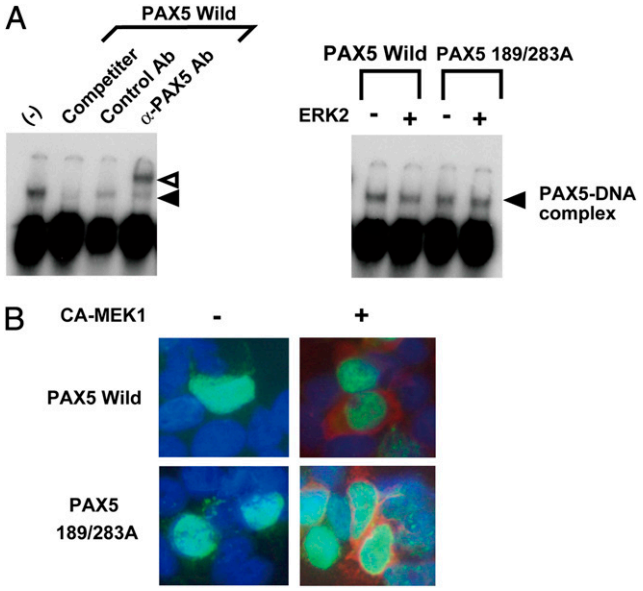

FIGURE 5. PAX5 phosphorylation did not affect DNA-binding ability or cellular localization. (**A**) Phosphorylation of PAX5 did not affect DNA-binding ability. Equal amounts of PAX5 synthesized in vitro were incubated with ^{32}P-labeled oligonucleotide probe containing the PAX5-binding sequence of the *CD19* promoter in the presence or absence of a 200-fold molar excess of unlabeled oligonucleotide (competitor), normal rabbit IgG (control Ab), or anti-PAX5 Ab (*left panel*). PAX5–DNA complex (black arrowhead) was visualized with an imaging analyzer. Supershifted band is indicated by the white arrowhead. Equal amounts of wild-type and mutant PAX5 were first subjected to in vitro phosphorylation, with or without ERK2, and then applied to EMSA (*right panel*). DNA-binding ability of PAX5 was not affected by phosphorylation. (**B**) Phosphorylation of PAX5 did not affect its localization. 293T cells were transfected with the expression vectors for wild-type and mutant PAX5, with or without HA-tagged CA-MEK1. Localization of PAX5 was observed with immunofluorescence staining using anti-PAX5 Ab with Alexa Fluor 488-conjugated secondary Ab (green), anti-HA Ab with Alexa Fluor 568-conjugated secondary Ab (red), and DAPI (blue). Original magnification ×400. Diffuse nuclear localization of PAX5 was not affected by the coexpression of HA-tagged CA-MEK1 or phosphorylation site mutations.

nisms through which PAX5 phosphorylation abolishes transcriptional repression of BLIMP1 are unknown.

Discussion

The data presented in this article support our speculation that PAX5 phosphorylation by ERK1/2 negatively affected BLIMP1 repression by PAX5. It is reported that BLIMP1 repression by PAX5 is abolished after BCR stimulation by Ag. Once BLIMP1 is expressed, it suppresses PAX5, which allows greater expression of BLIMP1. This positive feedback loop enables quick replacement of PAX5 with BLIMP1, which initiates plasma cell differentiation (14). Kallies et al. (16) investigated the first event of plasma cell differentiation in detail; they reported that the abolition of PAX5-mediated repression of BLIMP1 was the first event to initiate plasma cell differentiation and that the mechanism was neither a decrease in the DNA-binding ability of PAX5 nor downregulation of the PAX5 expression level and was yet to be revealed. PAX5 phosphorylation by ERK1/2 could be a clue to this uncertainty. The schema of this putative model is shown in Fig. 6.

It should be noted that Ramos cells or primary B cells from mouse spleen did not undergo plasma cell differentiation as the result of BCR stimulation with anti-IgM Ab (data not shown), despite the stimulation-induced phosphorylation of ERK1/2 and PAX5 and BLIMP1 expression. We could not keep the primary B cells alive for longer than a few days and could not estimate the differentiation. With regard to Ramos cells, one possible reason is that ERK1/2 phosphorylation induced by anti-IgM Ab stimulation was transient, peaking 10 min after stimulation and returning to basal levels ~2 h later. The kinetics of PAX5 phosphorylation were similar to those of ERK1/2 phosphorylation and, consistent with these kinetics, BLIMP1 mRNA expression was also transient and returned to the basal level within 24 h (data not shown). After anti-IgM Ab stimulation, BLIMP1 expression in Ramos cells is not sufficient to suppress PAX5 and initiate the above-described positive-feedback loop to replace PAX5 with BLIMP1. BCR stimulation with Ag is not the only stimulation required for plasma cell differentiation. Stimulation with cytokines, such as IL-2, IL-4, IL-10, and IL-21, and contact-dependent engagement of CD40 on B cells by CD40L (CD154), expressed by activated CD4$^+$ T cells, are required for plasma differentiation (22–26). Costimulation with these cytokines and T cells might prolong and enhance ERK1/2 phosphorylation and PAX5 phosphorylation and enable enough BLIMP1 expression to initiate plasma cell differentiation. The other possible reason is that Ramos cells, a lymphoma cell line, have impaired differentiation, as do tumor cells. BLIMP1 expression is induced by the cooperation of transcription factors, such as STAT3, IRF-4, and NF-κB (27, 28). Normal GCB cells express these factors properly and are ready to respond to BCR stimulation, which might enable a rapid and substantial increase in BLIMP1 expression in response to even transient PAX5 phosphorylation.

Other researchers reported the phosphorylation of PAX family proteins by the MAPK superfamily. PAX2 is phosphorylated by JNK at the transactivation domain (29), and both ERK1/2 and

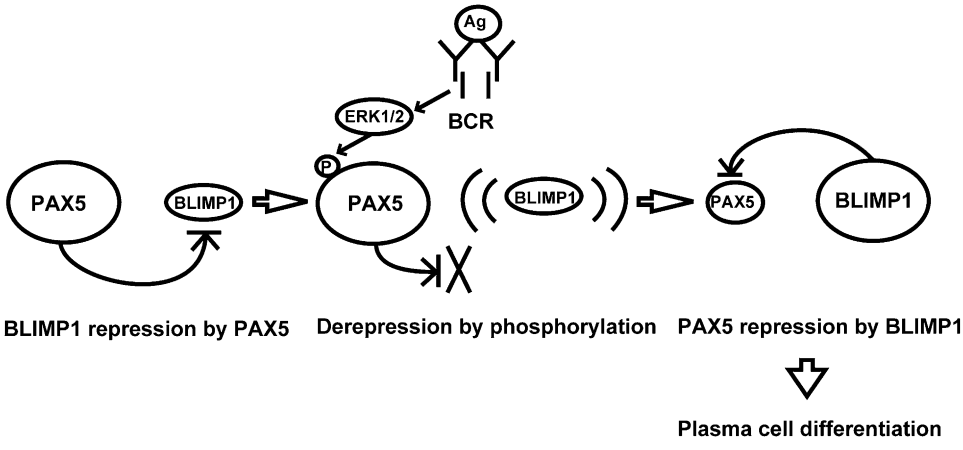

FIGURE 6. Putative schematic model of plasma cell differentiation triggered by PAX5 phosphorylation. PAX5 represses BLIMP1 expression during B cell development (*left*). When BCR signal is induced by Ag, ERK1/2 signal activation and PAX5 phosphorylation occur simultaneously, and repression of BLIMP1 is attenuated by PAX5 phosphorylation (*middle*). Once BLIMP1 is expressed, it suppresses PAX5 (*right*). Finally, PAX5 is replaced with BLIMP1, and plasma cell differentiation is initiated.

p38 phosphorylate PAX6 at the same sites: serines 376 and 413 and threonine 323 (30). This phosphorylation enhances the transcriptional activities of PAX family proteins. In addition, PAX6 phosphorylation by homeodomain-interacting protein kinase 2 (31), sumoylation of PAX6 (32), and acetylation of PAX5 by p300 (33) are reported to be posttranslational modifications of PAX family proteins, and all enhance the transactivation of PAX family proteins; therefore, negative regulation of PAX5 function by phosphorylation seems to be unique. Serines of PAX5 phosphorylation sites are not conserved in any other PAX family proteins. PAX5 is the only PAX family protein that regulates the differentiation of hematopoietic cells and might obtain a unique method to respond to extracellular signals. Furthermore, the cancellation of PAX family-dependent transcriptional repression by phosphorylation may also be unique to PAX5, although the effect of phosphorylation on transcriptional repression by other members of the PAX family has not been investigated.

The aberrant expression of normal PAX5 protein by the fusion gene between the potent enhancer of the *IGH* gene and the *PAX5* promoter was found in non-Hodgkin's lymphoma patients with the chromosomal translocation, t(9;14)(p13;q32) (34, 35). The oncogenicity of this fusion gene might be explained by impaired initiation of plasma cell differentiation due to sustained repression of BLIMP1 by overexpressed PAX5. Similarly, phosphorylation-defective mutation of PAX5 might impair plasma cell differentiation and cause lymphoma; therefore, we examined the genome DNA sequence surrounding PAX5 phosphorylation sites in 85 cases of diffuse large B cell lymphoma, but no mutation was found (data not shown; information on samples and sequencing methods is described in *Materials and Methods*). PAX5 mutations in lymphoma cells, if they exist, might be at the ERK1/2 binding site of PAX5, which is currently unknown.

In summary, our study provides evidence for an ERK1/2 pathway that phosphorylates PAX5 in response to BCR stimulation; this increase in PAX5 phosphorylation may associate with attenuated transcriptional repression by PAX5, derepression of BLIMP1, and initiation of plasma cell differentiation. This work provides new insight into the regulation of PAX5 function and establishes a novel relationship among the BCR signal, ERK1/2, and PAX5.

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