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*J Immunol* published online 7 May 2014
http://www.jimmunol.org/content/early/2014/05/07/jimmunol.1302643

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/05/07/jimmunol.1302643.DCSupplemental

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Protein Phosphatase 6 Controls BCR-Induced Apoptosis of WEHI-231 Cells by Regulating Ubiquitination of Bcl-xL

Ryutaro Kajihara,* Hitomi Sakamoto,* Kano Tanabe,* Kazuki Takemoto,* Masayoshi Tasaki,† Yukio Ando,† and Seiji Inui*†

Crosslinking BCR in the immature B cell line WEHI-231 causes apoptosis. We found that Bcl-xL was degraded by polyubiquitination upon BCR crosslinking and in this study explored the mechanism that controls the degradation of Bcl-xL. Ser62 of Bcl-xL was phosphorylated by JNK to trigger polyubiquitination, and this was opposed by serine/threonine protein phosphatase 6 (PP6) that physically associated with Bcl-xL. We show BCR crosslinking decreased PP6 activity to allow Ser62 phosphorylation of Bcl-xL. CD40 crosslinking rescues BCR-induced apoptosis, and we found PP6 associated with CD40 and PP6 activation in response to CD40. Our data suggest that PP6 activity is regulated to control apoptosis by modulating Ser62 phosphorylation of Bcl-xL, which results in its polyubiquitination and degradation. The Journal of Immunology, 2014, 192: 000–000.

B cell receptor signaling induces opposite results depending on the stage of B cell development (1–3). In mature B cells, BCR signaling induces proliferation and differentiation. In contrast, the same BCR engagement leads to cell death by apoptosis in immature B cells. Self-reactive B cells with BCR, which have high affinity for self-antigens, are thus eliminated in the bone marrow (4, 5). The mechanism underlying this difference in BCR signaling remains to be determined. However, Bcl-xL has emerged as one of the molecules that shows different responses to BCR signaling depending on the stage of B cell development. Bcl-xL expression is induced by BCR crosslinking in mature B cells, whereas it decreases in immature B cells following the same stimulation (6, 7). The murine B cell line, WEHI-231, has been widely employed as a model to analyze the mechanism underlying the induction of apoptosis by self-antigens in immature B cells (8, 9). WEHI-231 cells undergo G0/G1 cell-cycle arrest and apoptosis when stimulated with anti-IgM, which mimics Ag stimulation (10, 11).

The B-2 family proteins play an important role in the regulation of apoptosis (12–15). The antiapoptotic members include Bcl-2, Bcl-xL, and other proteins. The proapoptotic members are further divided into two subgroups: Bax subfamily members, which have a similar structure to Bcl-2 and Bcl-xL, with multiple Bcl-2 homology domains, and the BH3-only proteins, which include Bad and Bim (13–15). Bcl-xL has been reported to play critical roles in the apoptosis of lymphocytes (7). Bcl-xL heterodimerizes with proapoptotic molecules and suppresses their activities. Bcl-xL phosphorylation has been reported in response to apoptotic signals (16, 17). Several different kinases have been implicated in the phosphorylation of Bcl-xL including JNK, protein kinase C, and CDK (18, 19). Bcl-xL has been reported to be degraded by the proteasome after the induction of its ubiquitination in some cells (20, 21). However, the relationship between the phosphorylation of Bcl-xL and its ubiquitination has not been clearly elucidated.

The MAPK family consists of three members, ERK, p38 MAPK, and JNK (22). Generally, ERK promotes cell survival, whereas JNK and p38 MAPK are associated with apoptosis (22). Prolonged, rather than transient, activation of JNK has been reported to be involved in apoptosis (23, 24). The induction of apoptosis by JNK may be conveyed by c-Jun, which results in new gene expression. JNK may also control apoptosis by phosphorylating Bcl-2 family members without the need for new protein synthesis (25). Stimuli such as osmotic stress and TNF induce activation of the JNK and induce apoptosis (26). JNK deficiency was reported to result in defects in thymocyte apoptosis (27). In WEHI-231 cells, BCR crosslinking was shown to activate JNK to induce apoptosis. These reports demonstrated the relevance of JNK in the control of apoptosis in immature lymphocytes.

Protein phosphatase 6 (PP6), a serine/threonine phosphatase, belongs to the protein phosphatase 2A (PP2A) subfamily that comprises PP2A, PP4, and PP6 (28). These PP2A subfamily members are sensitive to active site inhibitors, such as okadaic acid (OA) (29, 30). PP6 consists of a catalytic subunit, PP6c, and regulatory molecules including SAPS1, -2 and -3. PP6c is mainly expressed in lymphoid, cardiac, and neuronal cells (31–33). SIT4, a yeast homolog of PP6, was identified as a molecule required for the G1 to S transition of the cell cycle (34). Human PP6 was also reported to be a cell-cycle regulator (32, 35, 36). Kinases and phosphatases have been identified as essential for cell survival and apoptosis (37–39). In particular, it was reported that PP6 was an important regulator of apoptosis (37). In our previous study, PP6 played an important role in the regulation of apoptosis (40). Although PP6 regulates the NF-κB pathway (33), the precise mechanism underlying the regulation of apoptosis by PP6 has not been fully addressed.

The induction of apoptosis in WEHI-231 cells by anti-IgM can be rescued by T cell–derived costimulation signals, such as CD40...
ligation (41). CD40 is a membrane molecule that functions in various ways to activate B cells (42). CD40 is essential for B cell activation, germinal center formation, class switching, and so on (43). CD40 is expressed on the surface of both immature and mature B cells. The mechanism by which CD40 rescues BCR-induced apoptosis remains unclear. The signal transduction of CD40 involves both the NF-κB and MAPK pathways (44, 45).

In the current study, we found that Bcl-xL was degraded by polyubiquitination induced by BCR crosslinking in WEHI-231 cells. Ser62 phosphorylation by JNK triggered this ubiquitination. We investigated whether any phosphatases regulated the apoptosis by interfering with the JNK-induced phosphorylation of Bcl-xL, and found that the PP6 activity was modulated by BCR crosslinking, and PP6 bound to and protected Bcl-xL by dephosphorylating Ser62. CD40 associated with PP6, and CD40 ligation suppressed apoptosis by enhancing the PP6 activity. Together, our results indicate that PP6 plays an important role in the regulation of apoptosis in WEHI-231 cells.

Materials and Methods

Cells and reagents

The WEHI-231 cell line was described previously (46), and the HEK293T (293T) cell line was a kind gift from Dr. Kaisho at Osaka University in Japan. The anti–Bcl-xL-Ab (Abcam, Cambridge, MA), anti–Bcl-xL (pS62) phosphospecific polyclonal Ab (Millipore, Bedford, MA), anti-K48 linkage-specific polyubiquitin Ab (Millipore), anti-JNK Ab (Cell Signaling Technology, Duavers, MA), anti–p-JNK Ab (Cell Signaling Technology), and anti–PP2Ac Ab (Upstate Biotechnology, Lake Placid, NY) were purchased for Western blot and/or immunoprecipitation studies. Anti-PP2Ac was a kind gift from Dr. Brautigan (University of Virginia). The anti-IgM mAb M41 was a kind gift from Dr. Rollink at the University of Basel (Basel, Switzerland). The anti-murine CD40 Ab was purified from LB429 cell culture supernatant (47). MG-132 was purchased from Merck Millipore (Basel, Switzerland). The anti-murine CD40 Ab was purified from LB429 culture supernatant (47). MG-132 was purchased from Merck Millipore (Basel, Switzerland). The anti–Bcl-xL Ab (Abcam, Cambridge, MA), anti–Bcl-xL (pS62) phosphospecific polyclonal Ab (Cell Signaling Technology, Duavers, MA), and anti–PP2Ac Ab (Upstate Biotechnology, Lake Placid, NY) were purchased for Western blot and/or immunoprecipitation studies. Anti-PP2Ac was a kind gift from Dr. Brautigan (University of Virginia). The anti-IgM mAb M41 was a kind gift from Dr. Rollink at the University of Basel (Basel, Switzerland). The anti-murine CD40 Ab was purified from LB429 culture supernatant (47). MG-132 was purchased from Merck Millipore (Basel, Switzerland). The anti–Bcl-xL Ab (Abcam, Cambridge, MA), anti–Bcl-xL (pS62) phosphospecific polyclonal Ab (Cell Signaling Technology, Duavers, MA), and anti–PP2Ac Ab (Upstate Biotechnology, Lake Placid, NY) were purchased for Western blot and/or immunoprecipitation studies. Anti-PP2Ac was a kind gift from Dr. Brautigan (University of Virginia). The anti-IgM mAb M41 was a kind gift from Dr. Rollink at the University of Basel (Basel, Switzerland).

Preparation of immature B cells

BALB/c female mice between 6 and 12 wk old were used for the experiments. Animals were housed at the Center for Animal Resources and Development. Experiments were approved by the committee on animal experiments of Kumamoto University. Bone marrow from femurs was harvested, and single-cell suspensions were prepared. B cells were purified using a B cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol. Purified B cells were stained with the mixture of biotinylated anti-IgD (Abcam) and biotinylated anti-CD21 (BioLegend) according to the manufacturer’s protocol and treated with anti-IgM or OA for 24 or 48 h. The relative cell viabilities were analyzed using the Cell Counting kit-8 (Dojindo). In brief, the cells were pulsed with WST-8 for the last 3 h of culture. The absorbance was then measured with an ELISA plate reader at a wavelength of 450 nm.

Cell-viability assay

Cells were cultured in 24-well culture dishes at a density of 1 × 10^5 cells/ml and treated with anti-IgM or OA for 24 h. After treatment, the cells were fixed in 70% ethanol for 3 h and stained with Guava Cell Cycle Reagent (Millipore), assayed in Guava easyCyte 6HT/2L (Millipore), and analyzed using the guavaSoft 2.6 software program (Millipore), according to the manufacturer’s instructions.

Immunoprecipitation and the Western blot analysis

For the immunoprecipitation of polyubiquitinated Bcl-xL, whole-cell lysates (WCL) were prepared in RIPA buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Nonidet P-40) containing 50 µM MG-132 and 10 mM N-ethylmaleimide with freshly added protease inhibitors and phosphatase inhibitors. For coimmunoprecipitation, cells were lysed in buffer containing 25 mM Tris-HCl (pH 7.6), 0.5% Triton X-100, 100 mM NaCl, 1 mM MgCl2, and 2 mM DTT with freshly added protease inhibitors and phosphatase inhibitors. Each cell lysate was precleared by incubation at 4°C with protein G–coupled Sepharose beads for 1 h. These precleared lysates were immunoprecipitated at 4°C by adding specific Abs overnight, followed by 1.5 h of incubation with protein G–coupled Sepharose beads. Immunoprecipitated complexes were washed five times with the buffer used to prepare the cell lysates and then dissolved in SDS sample buffer. The Western blot analysis was performed as described previously (40). Briefly, samples were separated by SDSPAGE and transferred to Immobilon-P membranes (Millipore). The membranes were incubated for 1 h in blocking buffer (TBS containing 0.05% Tween 20 and 5% nonfat dry milk) followed by a 1-h or overnight incubation with the primary Ab in blocking buffer. After extensive washing, the blot was incubated with a secondary Ab for 1 h and processed using the ECL reagents (GE Healthcare, Buckinghamshire, U.K.) according to the manufacturer’s instructions.

In vitro phosphoryase assay

To detect the phosphoryase activity, anti-FLAG immunoprecipitates from FLAG-PP6c–expressing WEHI-231 cells or WCL were prepared as described in the previous paragraph. After washing the precipitates with the reaction buffer (50 mM imidazole and 0.1% 2-ME) the last two times, 90 µl reaction buffer was added to the immunoprecipitates. To assay the WCL, 0.5 µl reaction buffer was added to 5 µl WCL. A total of 0.5 µl ATP (100 µM) plus 0.8-difluoro-4-methylumbelliferyl phosphate (DfMUP, Invitrogen, Carlsbad, CA; dissolved at 100 µM) was added to the precipitates or WCL to start the reaction. After 10 min of incubation at 30°C, 40 µl reaction
mixture was transferred to a 96-well microtiter plate, and the fluorescence (emission 450 nm and excitation 360 nm) was measured in a fluorescence plate reader (MTP-800; Corona Electric, Ibaraki-ken, Japan). The fluorescence of untreated cells was defined as 100%.

Statistical analyses

The Student t test was used to calculate the p values. The differences were considered to be significant at a 5% level.

Results

BCR crosslinking in WEHI-231 cells induces Bcl-xL degradation by ubiquitination

We studied the apoptosis-induction system of WEHI-231 cells that undergo growth arrest and apoptosis following crosslinking of IgM on the cell surface, which mimics the deletion of self-reactive B cells in the bone marrow (8–11). Anti-IgM stimulation induces cell death in 12–24 h. WEHI-231 cells were cultured in the presence of 10 μg/ml of anti-IgM, and expression of the antiapoptotic factor Bcl-xL was assessed by Western blot. BCR crosslinking induced a near-complete loss of Bcl-xL protein levels between 2 and 6 h, which preceded induction of apoptosis in WEHI-231 cells (Fig. 1A).

Real-time PCR was performed to see whether there was any reduction in mRNA of Bcl-xL after anti-IgM stimulation; as shown in Fig. 1B, there was no change, at least up to 6 h. There were some previous reports that showed that Bcl-xL degraded by the ubiquitin–proteasome pathway in other systems (20, 21). Therefore, we stimulated WEHI-231 cells with anti-IgM in the

**FIGURE 2.** The phosphorylation of Ser<sup>62</sup> of Bcl-xL facilitates its polyubiquitination. WEHI-231 cells were stably transfected with the pCAGGS vector harboring WT FLAG-Bcl-xL (WEHI-Bcl-xL) or phospho-deficient FLAG-Bcl-xL (WEHI-Bcl-xL-S62A). (A) The cell lysates of WEHI-Bcl-xL or WEHI-Bcl-xL-S62A cells were assessed for exogenous expression of FLAG-Bcl-xL. (B) WEHI-Bcl-xL or WEHI-Bcl-xL-S62A cells were stimulated with 10 μg/ml of anti-IgM for the indicated times, and then the cell lysates were subjected to Western blot with anti-FLAG. (C) WEHI-Bcl-xL or WEHI-Bcl-xL-S62A cells were treated with 10 μg/ml of anti-IgM and 10 μM MG-132 for 12 h. The cell lysates were immunoprecipitated (IP) with anti-FLAG. The samples were analyzed by Western blot (WB) with anti-ubiquitin (Ub). (D) WEHI-231, WEHI-Bcl-xL, or WEHI-Bcl-xL-S62A cells were induced to undergo apoptosis by treatment with 10 μg/ml of anti-IgM for 48 h, and the cell-viability levels were measured as described in Materials and Methods. The viability of untreated cells was defined as 100%. The bars represent the means ± SDs from three independent experiments. The results of (A) are representative of two independent experiments, and those in (B)–(D) are representative of three independent experiments. **p < 0.01.
presence or absence of a proteasome inhibitor, MG-132. The degradation of Bcl-xL was almost completely inhibited by the addition of 10 μM of MG-132, and the protein level of Bcl-xL was sustained even after 12 h of anti-IgM stimulation (Fig. 1C). This result suggested that the ubiquitin–proteasome pathway was involved in the regulation of Bcl-xL protein level in BCR-stimulated WEHI-231 cells. To confirm this, endogenous Bcl-xL was immunoprecipitated with anti–Bcl-xL and blotted with anti-ubiquitin before and at various times after anti-IgM stimulation. BCR crosslinking induced the ubiquitination of Bcl-xL in WEHI-231 cells, and this ubiquitination was readily detectable after 6 h of stimulation (Fig. 1D).

**Phosphorylation of Ser^62^ of Bcl-xL is important for its ubiquitination**

In many cases, ubiquitination of a protein is preceded by phosphorylation (49), and the phosphosites are recognized by SCF family E3 ligases (50). We observed phosphorylation of Ser^62^ in Bcl-xL under some apoptotic conditions in the previous studies, although the significance of this phenomenon was not fully addressed (16, 17). We investigated whether Ser^62^ was phosphorylated after BCR stimulation and found that it was indeed phosphorylated within 15 min of stimulation, and the phosphorylation was elevated substantially up to 2 h, after which time, the protein levels dropped (Fig. 1A). The phosphorylation preceded the degradation of Bcl-xL. To more clearly demonstrate that phosphorylation of Ser^62^ is important for the ubiquitination and degradation of Bcl-xL, wild-type (WT) and Ser^62^ to Ala (S62A) mutant cDNAs of Bcl-xL were prepared as described in the Materials and Methods. The proteins were expressed in WEHI-231 cells, and Western blot analysis showed the expression levels of WT and S62A mutant Bcl-xL were comparable (Fig. 2A). However, S62A mutant Bcl-xL was more stable than WT Bcl-xL after BCR stimulation (Fig. 2B). There was a reduction in the levels of both WT and S62A proteins after 6 and 12 h of BCR stimulation, but significantly less degradation of the S62A form of Bcl-xL. Next, WEHI-231 cells transfected with Bcl-xL cDNAs were stimulated with BCR for 12 h. The polyubiquitination of transfected S62A Bcl-xL was greatly diminished compared with that of WT Bcl-xL (Fig. 2C). Apoptosis resistance was compared between WEHI-231 cells expressing either WT or S62A Bcl-xL. The overexpression of WT Bcl-xL in WEHI-231 cells conferred resistance to apoptosis induced by BCR crosslinking (Fig. 2D) as reported previously (9, 51). Although the expression level of S62A mutant protein was comparable with that of WT Bcl-xL (Fig. 2A), the S62A mutant protein conferred more resistance to apoptosis in WEHI-231 cells (Fig. 2D). It was previously reported that BCR crosslinking induced G0/G1 arrest of WEHI-231 cells before the induction of apoptosis (10, 11). Anti-IgM stimulation induced a similar level of G0/G1 arrest in transfectants with WT and S62A mutant Bcl-xL (Supplemental Fig. 1).

**JNK is responsible for the Ser^62^ phosphorylation of Bcl-xL**

A variety of kinases, including JNK, can phosphorylate Ser^62^ of Bcl-xL (18, 19). We tested the effect of a pharmacological JNK inhibitor on the phosphorylation of Bcl-xL in this system. As shown in Fig. 3A, Ser^62^ phosphorylation induced by BCR crosslinking was effectively blocked by a JNK inhibitor SP600125 (used at 10 μM). To investigate the relevance of JNK in the phosphorylation of Bcl-xL during the induction of apoptosis in WEHI-231 cells, the activation of JNK was monitored after BCR crosslinking in WEHI-231 cells. The phosphorylation of JNK was detected 15 min after BCR stimulation (Fig. 3B). Treatment with the JNK inhibitor SP600125 inhibited the degradation of Bcl-xL induced by BCR crosslinking (Fig. 3C). Subsequently, WEHI-231 cells transfected with Bcl-xL cDNA were stimulated by BCR crosslinking for 12 h. The polyubiquitination of transfected Bcl-xL induced by BCR crosslinking was inhibited by the JNK inhibitor SP600125, again indicating the importance of phosphorylation in the regulation of Bcl-xL ubiquitination (Fig. 3D). We further showed JNK was

**FIGURE 3.** The BCR-triggered phosphorylation and ubiquitination of Bcl-xL are dependent on JNK. (A) The phosphorylation status of Bcl-xL was assessed by Western blot after anti-IgM (10 μg/ml) and SP600125 (10 μM) treatment for 1 h in WEHI-231 cells. (B) WEHI-231 cells were stimulated with 10 μg/ml anti-IgM for the indicated times, and the cell lysates were subjected to Western blot with anti-ubiquitin (Ub). The expression level of Bcl-xL was analyzed by Western blot after anti-IgM (10 μg/ml) and SP600125 (10 μM) treatment for 12 h in WEHI-231 cells. (C) WEHI-Bcl-xL cells were treated with 10 μg/ml anti-IgM and 10 μM MG-132, with or without SP600125 (10 μM), for 12 h. The cell lysates were immunoprecipitated (IP) with anti-FLAG. Samples were analyzed by Western blot (WB) with anti-ubiquitin (Ub). (E) WEHI-Bcl-xL cells were treated with 10 μg/ml anti-IgM for 1 h and subjected to IP with anti-Bcl-xL. Samples were analyzed by Western blot with anti-JNK. Results in (A)–(C) and (E) are representative of two independent experiments, and those of (D) are representative of four independent experiments.
Coproductivation with Bcl-xL in WEHI-231 cells, and this binding was enhanced after anti-IgM stimulation (Fig. 3E).

PP6 associates with Bcl-xL

WEHI-231 cells were stimulated with anti-IgM in the absence or presence of OA, a serine threonine PP family phosphatase inhibitor. MG-132 was added to the culture to prevent the degradation of Bcl-xL in this experiment. Ser62 phosphorylation induced by BCR crosslinking was enhanced by the addition of 50 nM of OA (Fig. 4A). MG-132 alone had no effect on the Ser62 phosphorylation level of Bcl-xL even after 12 h of incubation (Supplemental Fig. 2). OA also enhanced the polyubiquitination of Bcl-xL as shown in Fig. 4B. Next, the effect of OA on the induction of apoptosis in WEHI-231 cells was studied. OA itself induced slight apoptosis in WEHI-231 cells and enhanced the apoptosis induced by the BCR crosslinking (Fig. 4C). These results indicate the involvement of phosphatase(s) in the regulation of Bcl-xL ubiquitination and the apoptosis of WEHI-231 cells. Because PP2A subfamily members are similarly affected by OA treatment (29, 30), we tested if any of the type 2A phosphatases were directly associated with Bcl-xL. As shown in Fig. 4D, PP6c specifically associated with Bcl-xL when cotransfected into 293T cells.

PP6 controls the phosphorylation and ubiquitination of Bcl-xL

To determine whether PP6 regulates the phosphorylation and degradation of Bcl-xL, PP6c cDNA was transfected into WEHI-231 cells. The overexpression of PP6c in WEHI-231 cells diminished the apoptosis induced by BCR crosslinking (Fig. 5A). This effect was due to the activity of PP6 because this difference was not observed in the presence of OA, a potent inhibitor of PP6 (30). The phosphorylation of Ser62 of Bcl-xL after BCR crosslinking was also decreased in these cells (Fig. 5B). To confirm this result, an inactive form of PP6c, PP6c-D84N (52), was prepared and transfected into WEHI-231 cells. Overexpression of inactive form PP6c had no effect on the resistance to apoptosis or the phosphorylation of serine 62 of Bcl-xL induced by BCR crosslinking (Fig. 5A, 5B). Of note, the degradation of Bcl-xL was inhibited in WEHI-231 cells overexpressing PP6c (Fig. 5C). The polyubiquitination of Bcl-xL induced by BCR crosslinking was also diminished by PP6c overexpression (Fig. 5D). To elucidate the mechanism by which Bcl-xL is regulated by PP6, we studied whether the JNK activation was compromised in PP6c-overexpressing WEHI-231 cells. The JNK activation, as monitored by the phosphorylation of JNK, was not significantly different between the parental cells and the transfectant (Fig. 5E). Therefore, we next tested whether PP6c associated with JNK before or after anti-IgM stimulation. No association between JNK and PP6c was observed under either condition (Fig. 5F). In contrast, PP6c associated with Bcl-xL in WEHI-231 cells, and this association was decreased by BCR crosslinking (Fig. 5G). These results suggest that PP6 controls the phosphorylation of Bcl-xL.

CD40 associates with PP6c and controls its activity

We then studied whether the PP6 activity was altered after BCR crosslinking in WEHI-231 cells. An in vitro phosphatase assay was performed using DiFMUP as a substrate, as described in the Materials and Methods. The phosphatase activity in the anti-FLAG precipitate decreased after BCR crosslinking (Fig. 6A). It is well established that CD40 crosslinking rescues BCL-1-induced apoptosis (41). CD40 ligation was reported to activate the NF-kB pathway and increase the transcription of the Bcl-xL gene, which has an NF-kB binding site (53). We therefore investigated whether PP6 associated with CD40, because PP2Ac was reported to bind to CD28, a T cell activation molecule (54). First, CD40 cDNA was transfected into 293T cells together with PP6c cDNA, and PP6c was coprecipitated with CD40 (Fig. 6B). The association was specific to PP6c, because PP2Ac did not bind to CD40 nor did control Ab coprecipitate PP6c (Fig. 6B). The association between endogenous PP6c and Bcl-xL was then tested in greater detail. As shown in

FIGURE 4. An OA-sensitive phosphatase is involved in regulating the phosphorylation status of Bcl-xL. (A) WEHI-Bcl-xL cells were stimulated with 10 μg/ml anti-IgM in the presence or absence of OA (50 nM) for the indicated time periods. MG-132 was also added to the media to prevent Bcl-xL from being degraded. WCL were prepared and tested for the presence of p-Bcl-xL and total Bcl-xL. (B) WEHI-Bcl-xL cells were incubated with 10 μg/ml anti-IgM and 10 μM MG-132, with or without OA (50 nM) for 12 h. The cell lysates were immunoprecipitated (IP) with anti-FLAG. Samples were analyzed by Western blot (WB) with anti-ubiquitin (Ub). (C) WEHI-231 cells were induced to undergo apoptosis by treating them with 10 μg/ml anti-IgM in the presence or absence of OA (50 nM) for 24 h, and the cell viability was measured as described in Materials and Methods. The viability of untreated cells was defined as 100%. The bars represent the means ± SDs from three independent experiments. **p < 0.01. The 293T cells were transfected with FLAG-PP1c, PP2Ac, PP4c, or PP6c in combination with Bcl-xL cDNA. After a 48-h incubation, the cells were harvested and subjected to IP with anti-Bcl-xL. Then, immunoblotting was performed with anti-FLAG. The results shown in (A) and (D) are representative of two independent experiments. The results shown in (B) and (C) are representative of three independent experiments. **p < 0.01.
Fig. 6C, we found that endogenous CD40 also associated with PPP6c. Next, we tested if CD40 ligation increased the PP6 activity in WEHI-231 cells. Cells expressing FLAG-PP6c were stimulated with anti-CD40 for 1 h, and the lysate was precipitated with anti-FLAG. The PP6 activity increased after CD40 ligation, as shown in Fig. 6A, and CD40 ligation reversed the decrease in PP6 activity in BCR-stimulated WEHI-231 cells. CD40 stimulation also diminished the phosphorylation of Bcl-xL induced by BCR crosslinking (Fig. 6D). Based on these results, we propose a model in which the stability of Bcl-xL is controlled by the phosphorylation of Ser 62 (Fig. 7). This phosphorylation status is regulated by JNK and PP6, both of which are under the control of BCR crosslinking.

The degradation of Bcl-xL is regulated by JNK and PP6 in normal immature B cells

We next investigated whether the phenomena observed in WEHI-231 cells were applicable to normal immature B cells. We prepared immature B cells from the bone marrow of BALB/c mice. BCR crosslinking induced a decrease of Bcl-xL in immature B cells, as indicated in Fig. 8A. MG-132 treatment inhibited this decrease, suggesting a role of ubiquitination in this process. BCR crosslinking also induced the phosphorylation of Ser 62 of Bcl-xL in immature B cells (Fig. 8B). The addition of a JNK inhibitor, SP600125, reduced the Ser 62 phosphorylation of Bcl-xL, just as it did in WEHI-231 cells (Fig. 8B). Anti-IgM treatment induced the phosphorylation of JNK in normal immature B cells (Fig. 8C). We also found that the Ser 62 phosphorylation of Bcl-xL was enhanced by the addition of OA (Fig. 8D). We next examined whether BCR crosslinking induced a decrease in PP6 activity in normal immature B cells. Because a sufficient amount of endogenous PP6 was not available for immunoprecipitation from normal immature B cells, we used WCL to monitor the phosphatase activity. As shown in Fig. 8E, the phosphatase activity was reduced after BCR crosslinking and enhanced after CD40 ligation (Fig. 8E). The reduced phosphatase activity induced by BCR crosslinking was reversed by CD40 ligation (Fig. 8E), Fig. 6C, we found that endogenous CD40 also associated with PPP6c. Next, we tested if CD40 ligation increased the PP6 activity in WEHI-231 cells. Cells expressing FLAG-PP6c were stimulated with anti-CD40 for 1 h, and the lysate was precipitated with anti-FLAG. The PP6 activity increased after CD40 ligation, as shown in Fig. 6A, and CD40 ligation reversed the decrease in PP6 activity in BCR-stimulated WEHI-231 cells. CD40 stimulation also diminished the phosphorylation of Bcl-xL induced by BCR crosslinking (Fig. 6D). Based on these results, we propose a model in which the stability of Bcl-xL is controlled by the phosphorylation of Ser 62 (Fig. 7). This phosphorylation status is regulated by JNK and PP6, both of which are under the control of BCR crosslinking.

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which was in agreement with the observation in WEHI-231 cells (Fig. 6A).

**Discussion**

The WEHI-231 cell line has served as an excellent model to study apoptosis in immature B cells (8, 9). It was reported that Bcl-xL played an important role in the life and death decision-making process in mature versus immature B cells (6, 7). Cellular apoptosis is controlled by Bcl-2 family proteins (12–15). Bcl-xL and Bcl-2 seem to function in the same apoptotic pathway, and they are especially important in the control of apoptosis in lymphocytes, in which both proteins are abundantly expressed (7, 9). The overexpression of either molecule conferred resistance to BCR-induced apoptosis in WEHI-231 cells (9, 51). Although there was a report that demonstrated the existence of heterogeneity in the WEHI-231 cell line (55), our study confirmed the expression and importance of Bcl-xL in the resistance of WEHI-231 cells to apoptosis. It was recently reported that Ser62 phosphorylation was involved in cell-cycle blockade (56), and anti-IgM stimulation of WEHI-231 was also shown to cause cell-cycle blockade (10, 11). Therefore, we assessed the effects of overexpressing WT and S62A mutant Bcl-xL on the cell-cycle blockade induced by BCR crosslinking. Our results showed that there was no apparent effect on the cell cycle associated with the expression of either molecule (Supplemental Fig. 1). The differences in the results may be explained by the differences in the cell types examined and the employed stimulus between the previous paper (56) and our present study.

BCR crosslinking induced the polyubiquitination and degradation of Bcl-xL in WEHI-231 cells. This ubiquitination was preceded by the phosphorylation of Ser62 of Bcl-xL. Previous findings showed that Bcl-xL was degraded following its ubiquitination in response to apoptosis-inducing signals (20, 21). UV irradiation, oxidative stress, and antimitotic drugs were all demonstrated to induce the degradation of Bcl-xL by the proteasome. Phosphorylation was also implicated in the regulation of functions of Bcl-2 family members, although the impact of phosphorylation on resistance to apoptosis was inconsistent (16, 25). The phosphorylation of Ser62 of Bcl-xL abolished its antiapoptotic activity (16). Several kinases, including JNK, protein kinase C, and CDK, have been reported to phosphorylate this site (18, 19). Although JNK may mediate a transcription-dependent apoptotic pathway, it was also shown that the effects of UV did not require new gene expression to induce apoptosis (25). However, the relevant kinase involved in the BCR-induced apoptosis had been unclear, and the relationship between the phosphorylation of Bcl-xL at Ser62 and its ubiquitination has not been clearly demonstrated. In this study, we showed that JNK was responsible for the Ser62 phosphorylation and demonstrated that this phosphorylation was essential for Bcl-xL ubiquitination.

Phosphatases and kinases are like two sides of a coin, and when phosphorylation regulates a specific signal transduction pathway,
dephosphorylation must also play a role in the same pathway to counteract the effect (57). PP2A, which is a ubiquitously expressed serine/threonine phosphatase, plays an important role in the regulation of cellular functions, including cell-cycle control and survival (28). Previous studies showed that PP2A dephosphorylated Bcl-2 family members, including Bcl-2 and Bcl-xL, and controlled its antiapoptotic activity (58, 59). In this study, we showed that PP6, which belongs to the PP2A subfamily, played an important role in the regulation of Bcl-xL. This finding was not surprising, because it was also previously reported that PP6 was important in the regulation of apoptosis (37, 40), and PP6 is highly expressed in lymphoid cells (32, 33). In some cases, phosphatases directly associate with the kinases that they regulate (60). However, an association between PP6 and JNK was not observed in either the nonphosphorylated or phosphorylated forms of JNK (54). In contrast, PP6 associated with Bcl-xL, and this association decreased after BCR crosslinking. Therefore, we propose that PP6 controls the phosphorylation of Bcl-xL by working on Bcl-xL, rather than JNK (Fig. 7).

It is well established that activation signals, such as IL-4, LPS, and CD40, can rescue WEHI-231 cells from BCR-induced apoptosis (41). CD40 induces NF-κB activation and upregulates the transcription of Bcl-xL, which has an NF-κB binding site in its promoter region (53). PP2A was shown to associate with CD28, a T cell activation marker, but not with CD40 (54). Our results confirmed that PP2A did not bind to CD40. However, CD40 associated with PP6. We then tested if the PP6 activity was involved in the regulation of Bcl-xL phosphorylation following BCR activation. BCR crosslinking decreased the phosphatase activity, and CD40 ligation increased the PP6 activity. Furthermore, the BCR-induced decrease of PP6 activity was diminished by CD40 ligation. Therefore, as an additional mechanism to rescue WEHI-231 cells from BCR-induced apoptosis, CD40 may stabilize Bcl-xL by controlling its phosphorylation status.

BCR crosslinking induces apoptosis in immature B cells (1–5) and transitional B cells in the neonatal spleen (61). Transitional B cells are subdivided into T1 and T2 cell subpopulations, and the adult spleen also contains these transitional T1 cells (62). We prepared immature B cells from bone marrow and showed that BCR crosslinking induced the degradation of Bcl-xL by the proteasome. It was reported in a previous study that BCR crosslinking induced a much lower amount of Bcl-xL in T1 cells compared with T2 cells (62). Our present study demonstrated that Bcl-xL degradation was controlled by the induction of polyubiquitination resulting from the Ser62 phosphorylation by JNK. We further showed that a protein phosphatase was involved in the control of Bcl-xL degradation. These results confirmed that the mechanism observed in WEHI-231 cells was applicable to normal immature B cells.


