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Original Research Communication

Role of BLNK in Oxidative Stress Signaling in B Cells

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ABSTRACT

BLNK (B cell linker protein) represents a central linker protein that bridges the B cell receptor-associated kinases with a multitude of signaling pathways. In this study, we have investigated the role of BLNK in oxidative stress signaling in B cells. H_2O_2 treatment of B cells induced a rapid tyrosine phosphorylation of BLNK in a H_2O_2 dose-dependent manner, which was inhibited in Syk-deficient DT40 cells. Calcium mobilization in BLNK-deficient as well as Syk-deficient and phospholipase C (PLC)-γ2-deficient cells after H_2O_2 treatment was completely abolished. These were derived from decreased inositol 1,4,5-trisphosphate generation through PLC-γ2 in BLNK-deficient cells. Moreover, viability of BLNK-deficient as well as PLC-γ2-deficient cells after exposure to low doses of H_2O_2 was dramatically enhanced compared with that of the wild-type cells. Furthermore, c-Jun N-terminal kinase activation following high doses of H_2O_2 stimulation, but not low doses of H_2O_2 stimulation, was abrogated in BLNK-deficient as well as Syk-deficient cells. These findings have led to the suggestion that BLNK is required for coupling Syk to PLC-γ2, thereby accelerating cell apoptosis in B cells exposed to low doses of H_2O_2. Antioxid. Redox Signal. 3, 1065–1073.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS), such as the superoxide radical, the hydroxyl radical, and hydrogen peroxide (H_2O_2), are continuously produced in most cells (7). It has been known that excessive generation of ROS is associated with cell injury in a variety of pathological conditions (7). On the other hand, it has been reported that ROS also have normal roles as second messengers in cytokine or platelet-derived growth factor signal transduction (13, 19). H_2O_2 stimulation is known to induce Ca^{2+} mobilization and the activation of protein-tyrosine kinases, mitogen-activated protein kinases and transcription factors (13–17, 20).

Protein-tyrosine kinases play crucial roles in a wide variety of cellular responses, including cellular activation, proliferation, differentiation, and apoptosis (23). Syk, which plays a crucial role in B cell receptor (BCR)-mediated signaling (26), is rapidly tyrosine-phosphorylated by oxidative stress and plays an important role in the transduction of oxidative stress signaling in B cells (15, 16). Genetic studies using Syk-deficient B cells treated with high doses of H_2O_2 (1–5 m*M*) revealed that Syk is essential for the increased tyrosine phosphorylation of cellular proteins, Ca^{2+} release from intracellular stores, and c-Jun N-terminal kinase (JNK) activation (15, 16). B cells are relatively sensitive to H_2O_2 treatment, and high doses of H_2O_2

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induce cell necrosis, whereas cell death induced by low doses of H_2O_2 (10–100 μM) seems to reflect cellular apoptosis in B cells (5, 10, 11). We have recently reported that Syk induces the activation of the phosphatidylinositol 3-kinase (PI 3-kinase)–Akt survival pathway following treatment with low doses of H_2O_2, thereby enhancing cellular resistance to oxidative stress-induced apoptosis (2). However, little is known about the mechanism by which Syk communicates with downstream effectors following oxidative stress.

BLNK (B cell linker protein) is a crucial adapter molecule in signaling (4, 6, 25). BLNK is tyrosine-phosphorylated by Syk following BCR stimulation and associates with phospholipase C (PLC)-γ1, PLC-γ2, Vav, Grb2, and Nck (4). Targeted disruption of BLNK in B cells abolishes PLC-γ2 phosphorylation, Ca^{2+} mobilization, and JNK activation following BCR ligation (8, 9). BLNK requires Syk for coupling to PLC-γ2 and Rac1-JNK in BCR signaling. Moreover, a recent report has suggested that BLNK together with SLP-76 is linked to signaling via Fcγ receptors I and II/III in murine macrophages (1).

In this article, we report the role of BLNK in B cells exposed to oxidative stress. We have found that BLNK is required for coupling Syk to PLC-γ2 activation, thereby accelerating cell apoptosis in B cells exposed to low doses of H_2O_2.

MATERIALS AND METHODS

Materials

The RPMI 1640 medium was purchased from ICN Biomedicals. Fetal bovine serum was from GIBCO and Sigma. Protein A-Sepharose CL-4B was from Amersham Pharmacia Biotech AB. Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology Inc. Anti-BLNK antibodies were purchased from Santa Cruz Biotechnology and were provided by Dr. Kurosaki (Kansai Medical University, Moriguchi). Anti-Syk and anti-PLC-γ2 antibodies were purchased from Santa Cruz Biotechnology. Anti-human JNK1 antibody was purchased from Pharmingen. Anti-Akt and anti-phospho-Akt (Ser473) antibodies were purchased from New England Biolabs, Inc.

Cell culture

Syk-deficient (Syk^-), BLNK-deficient ($BLNK^-$), and PLC-γ2-deficient (PLC-$\gamma2^-$) DT40 chicken B cells were provided by Dr. Kurosaki (8, 21, 22). DT40 and Ramos human B cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml kanamycin in a humidified 95% air/5% CO_2 atmosphere. Cells, collected by centrifugation at 400 g for 5 min, were washed with phosphate-buffered saline and resuspended (1×10^7 cells/ml) in Hanks' balanced salt solution. For all of the experiments described here, cells were stimulated with H_2O_2 at 37°C with gentle stirring.

Immunoprecipitation and immunoblotting

Control and stimulated cells (1×10^7 cells/ml) were lysed in ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 100 μM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Lysates were clarified by centrifugation at 12,000 g for 10 min at 4°C. The supernatants were incubated sequentially (1 h for each incubation) with antibodies and protein A-Sepharose CL-4B at 4°C. Whole-cell lysates or immunoprecipitates were analyzed on sodium dodecyl sulfate–polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore). Blots were probed with the indicated antibodies, and immunoreactive proteins were revealed by the enhanced chemiluminescence detection system (DuPont NEN).

Measurement of cytoplasmic free calcium ($[Ca^{2+}]_i$)

Ca^{2+} mobilization studies were performed using Fura 2-AM. Cells (1×10^7 cells/ml) were washed once and loaded with 5 μM Fura 2-AM in Hanks' balanced salt solution. After incubation for 30 min at 37°C, cells were washed once and diluted to 2×10^6 cells/ml. The Fura 2-AM fluorescence was continuously monitored with

a fluorescence spectrophotometer (model F4500, Hitachi) with standard monitor settings at 340 nm and 380 nm excitation and 510 nm emission wavelength.

Measurement of inositol 1,4,5-trisphosphate (IP_3) levels

After H_2O_2 stimulation, IP_3 in DT40 cells was extracted by perchloric acid and measured with a highly specific D-*myo*-[^{3}H]IP_3 assay system (Amersham Life Science) as described by the supplier.

Cell viability

Wild-type and mutant DT40 cells (5×10^5 cells/ml) were stimulated using the indicated concentrations of H_2O_2. After the indicated times, cell viability was determined by the trypan blue dye exclusion method.

DNA fragmentation analysis

Wild-type and mutant DT40 cells (5×10^5 cells/ml) were stimulated with the indicated concentrations of H_2O_2 for 8 h. Then 5×10^6 cells were lysed in 0.5 ml of lysis buffer (10 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA, 200 m*M* NaCl, 0.4% Triton X-100, and 0.1 mg/ml protein K) for 20 min at room temperature followed by incubation with 0.1 mg/ml RNase A for 30 min at 50°C. DNA fragmentation was analyzed using a 2.5% agarose gel in the presence of 0.5 μg/ml ethidium bromide.

Assays for JNK activity

The assay conditions have been described previously (3). In brief, immunoprecipitates with anti-JNK1 antibody and protein A-Sepharose CL-4B were performed in 30 μl of kinase assay buffer containing 1 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and 5 μg of glutathione *S*-transferase-c-Jun (GST-c-Jun) as a substrate. After a 20-min incubation at 30°C, reactions were terminated by the addition of electrophoresis sample buffer and boiling for 5 min. Autoradiography of sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels was carried out using standard procedures, and autoradiographs were quantified using a phosphoimager system (Fuji BAS 2000).

RESULTS

BLNK is tyrosine phosphorylated by H_2O_2 stimulation

It has been reported that after BCR stimulation BLNK is rapidly tyrosine-phosphorylated by Syk and is required for coupling Syk to PLC-γ2 and Rac1 in B cells (4, 8). We have previously reported that in DT40 cells treated with high doses of H_2O_2, Syk is required for PLC-γ2 and JNK activation (15, 16). We, therefore, analyzed the tyrosine phosphorylation status of BLNK in B cells exposed to oxidative stress. Immunoprecipitation of BLNK from Ramos cells treated with various concentrations of H_2O_2 demonstrated that BLNK undergoes tyrosine phosphorylation in a H_2O_2 dose-dependent

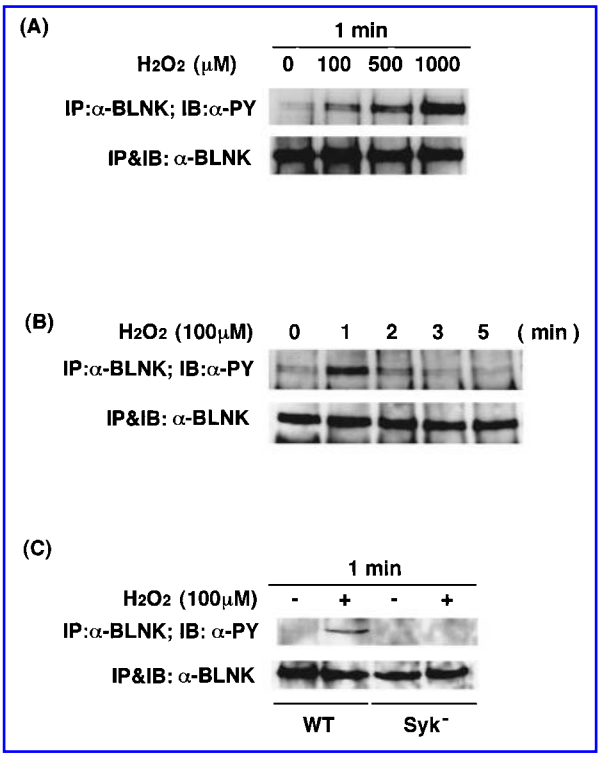

FIG. 1. Tyrosine phosphorylation of BLNK by Syk following oxidative stress. Ramos cells were treated with the indicated doses of H_2O_2 for 1 min (**A**), or with 100 μM H_2O_2 for the indicated times (**B**). (**C**) Wild-type (WT) and Syk-deficient (Syk$^-$) DT40 cells were stimulated with 100 μM H_2O_2 for 1 min. Anti-BLNK immunoprecipitates were subjected to immunoblotting analysis using anti-phosphotyrosine (α-PY) or anti-BLNK antibodies. The results shown are from one representative experiment that was replicated four times. IB, immunoblotting; IP, immunoprecipitation.

manner (Fig. 1A). In B cells exposed to 100 μM H_2O_2, BLNK is tyrosine-phosphorylated. In the following experiments, we examined the role of BLNK in B cells exposed to low doses of H_2O_2 that induce cell apoptosis. In Ramos cells treated with 100 μM H_2O_2, tyrosine phosphorylation of BLNK peaks rapidly at 1 min and returns to near-basal levels by 3 min (Fig. 1B). To determine whether BLNK is phosphorylated by Syk following H_2O_2 stimulation, we examined tyrosine phosphorylation of BLNK in wild-type and Syk-deficient DT40 cells. Anti-BLNK immunoprecipitates obtained from cell lysates were subjected to anti-phosphotyrosine immunoblotting as shown in Fig. 1C. Tyrosine-phosphorylation of BLNK following H_2O_2 stimulation was inhibited in Syk-deficient cells. These findings suggested that Syk induced BLNK phosphorylation in oxidative stress signaling.

Syk in BLNK-deficient cells is normally activated upon H_2O_2 stimulation

To address the function of BLNK in oxidative stress signaling, we utilized BLNK-deficient DT40 cells (8). Comparison of Syk tyrosine phosphorylation in BLNK-deficient cells with that in wild-type cells did not reveal any significant changes (Fig. 2A). Although the overall tyrosine phosphorylation pattern of cellular proteins following low doses of H_2O_2 was weak, the phosphorylation pattern in BLNK-deficient cells was nearly the same as that in wild-type cells (Fig. 2B). These findings suggested that Syk in BLNK-deficient cells was activated normally after oxidative stress.

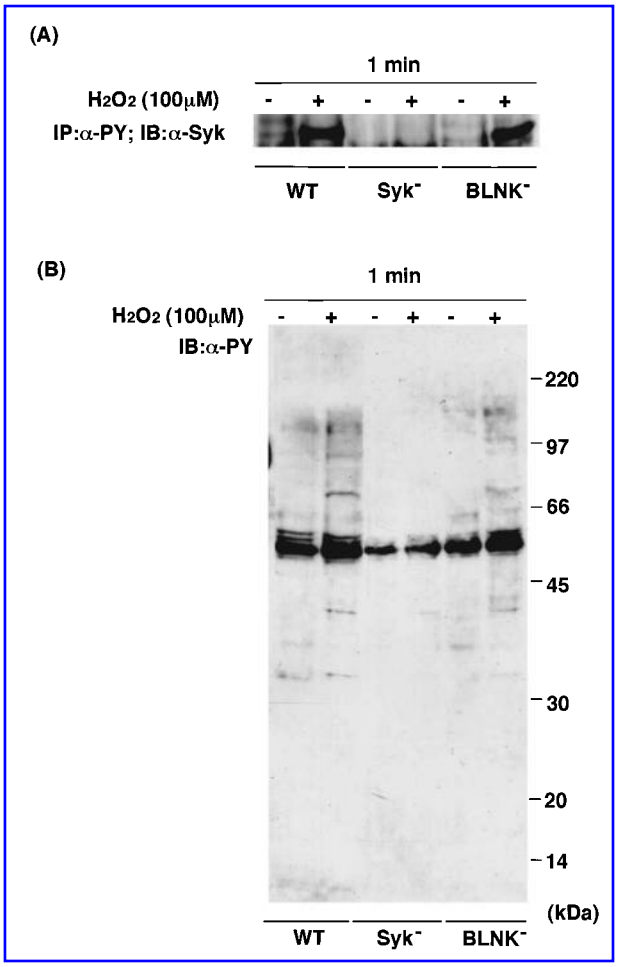

FIG. 2. Tyrosine phosphorylation of Syk and cellular proteins in BLNK-deficient cells following oxidative stress. Wild-type (WT), Syk-deficient (Syk^-), and BLNK-deficient ($BLNK^-$) DT40 cells were stimulated with 100 μM H_2O_2 for 1 min. Anti-phosphotyrosine (α-PY) immunoprecipitates from cell lysates were immunoblotted with anti-Syk antibody (**A**). Whole cell lysates were subjected to immunoblotting analysis with anti-phosphotyrosine antibody (**B**). The results from one representative experiment that was repeated four times are shown.

BLNK is required for PLC-γ2 activation following H_2O_2 stimulation

We have previously showed that an increase in $[Ca^{2+}]_i$ after oxidative stress is mediated by both an extracellular Ca^{2+} influx and a Ca^{2+} release from intracellular pools, and only the latter is dependent on Syk (15, 16). We, therefore, examined whether BLNK is involved in coupling Syk to Ca^{2+} mobilization after oxidative stress. H_2O_2 treatment (100 μM) of wild-type cells induced a rapid increase in $[Ca^{2+}]_i$, whereas BLNK-deficient as well as Syk-deficient cells showed a relatively slow and small increase in $[Ca^{2+}]_i$ after H_2O_2 stimulation (Fig. 3A). These findings indicated that BLNK was required for coupling Syk to Ca^{2+} mobilization after oxidative stress.

Previous studies have suggested that the Ca^{2+} release from intracellular pools after oxidative stress is dependent on IP_3 generation following the activation of PLC-γ2 (3, 15). As shown in Fig. 3A, Ca^{2+} mobilization in BLNK-deficient cells was almost the same as that in PLC-γ2-deficient cells. Furthermore, IP_3 generation upon H_2O_2 stimulation was abolished in BLNK-deficient as well as PLC-γ2-deficient

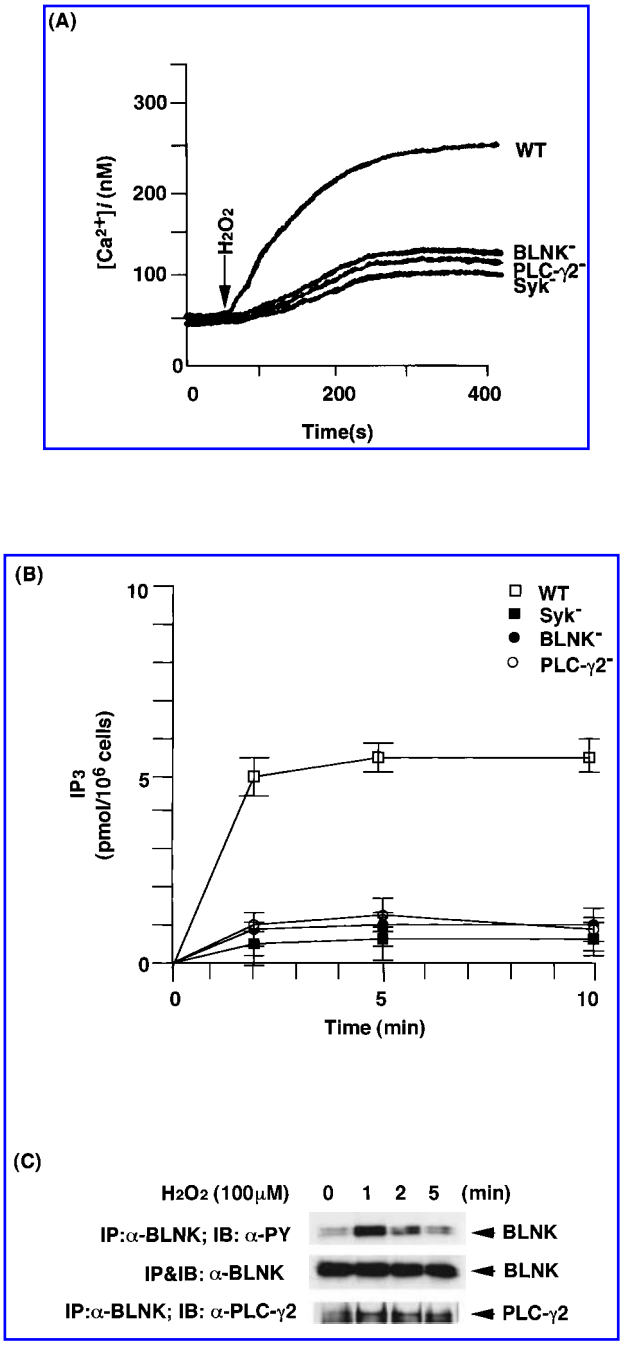

FIG. 3. Calcium mobilization in BLNK-deficient cells following oxidative stress. (**A**) Calcium mobilization analysis. $[Ca^{2+}]_i$ levels in Fura-2-loaded DT40 cells were monitored by a spectrophotometer after stimulation with 100 μM H_2O_2. (**B**) IP_3 generation. DT40 cells were stimulated with 100 μM H_2O_2 for the indicated times, and IP_3 production was measured as described in Materials and Methods. The data represent the means ± SD from four independent experiments. (**C**) Association of phosphorylated BLNK with PLC-γ2. Ramos cells were stimulated with 100 μM H_2O_2 for the indicated times. Anti-BLNK immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine (α-PY), anti-BLNK, and anti-PLC-γ2 antibodies. The results from one representative experiment that was repeated four times are shown.

cells (Fig. 3B). After H_2O_2 stimulation, PLC-γ2 associated with BLNK in a BLNK phosphorylation-dependent fashion (Fig. 3C). These observations indicated that during oxidative stress, BLNK was essential for PLC-γ2 activation followed by Ca^{2+} release from intracellular storage.

BLNK-mediated PLC-γ2 activation accelerates cell apoptosis following H_2O_2 stimulation

In BCR signaling, activation of PLC-γ2 through Syk is required for B cell apoptosis (22). We have previously reported that Syk plays a role in the protection of B cells from oxidative stress-induced apoptosis (2). We examined the role of BLNK in oxidative stress-induced cell death. Wild-type and various mutant cells were treated with low doses of H_2O_2; and cell viability was determined by the trypan blue dye exclusion method. Viability of Syk-deficient cells after exposure to H_2O_2 was dramatically decreased compared with that of the wild-type cells (Fig. 4A and B). On the other hand, BLNK-deficient as well as PLC-γ2-deficient cells treated with low doses of H_2O_2 showed a higher percentage of cell viability than wild-type cells. Furthermore, DNA ladder formation was detected in B cells treated with low doses of H_2O_2. As shown in Fig. 4C, BLNK-deficient cells treated with H_2O_2 exhibited a reduction in apoptotic response based on DNA fragmentation. These findings indicate that BLNK has the role of acceleration for oxidative stress-induced apoptosis through PLC-γ2 activation.

We have previously reported that Syk induces the activation of the PI 3-kinase–Akt survival pathway following oxidative stress, thereby enhancing cell survival against oxidative stress (2). We examined whether BLNK is involved in phosphorylation of Akt upon H_2O_2 stimulation. Whereas in Syk-deficient cells Akt phosphorylation was almost completely abolished, in BLNK-deficient cells 60% of Akt phosphorylation remained following H_2O_2 stimulation (Fig. 4D). PLC-γ2-deficient cells showed the same level of Akt phosphorylation as BLNK-deficient cells following H_2O_2 stimulation (data not shown). These results indicated that BLNK was partly involved in Akt activa-

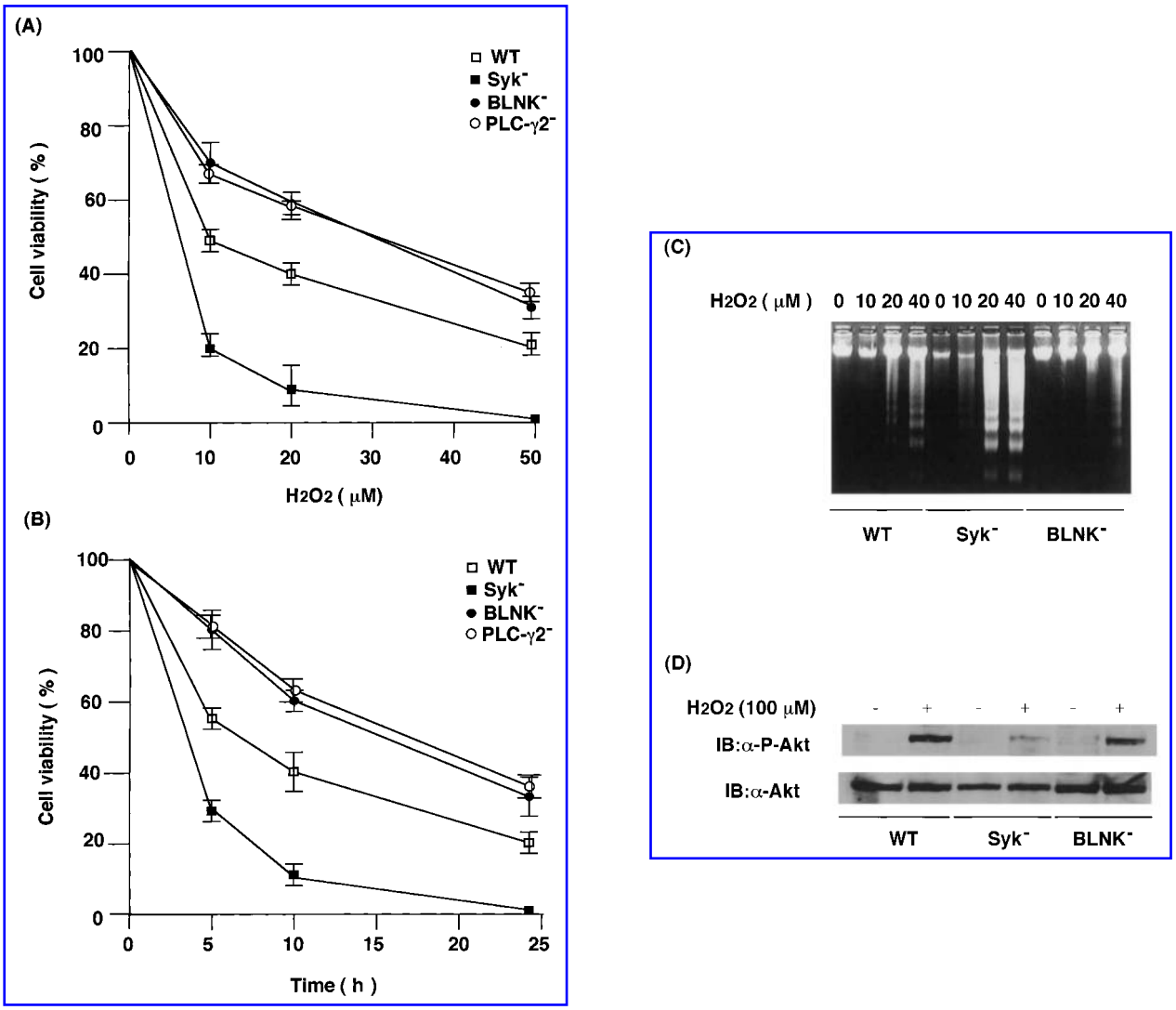

FIG. 4. Cell apotosis and Akt phosphorylation after treatment with low doses of H_2O_2. Wild-type (□), Syk-deficient (■), BLNK-deficient (●), and PLC-γ2-deficient (○) DT40 cells were treated with low doses of H_2O_2 for 10 h (**A**), or with 20 μM H_2O_2 for the indicated times (**B**), and cell viability was determined by the trypan blue dye exclusion method. Results shown are means ± SD from three independent experiments. (**C**) Wild-type, Syk-deficient, and BLNK-deficient DT40 cells were treated with low doses of H_2O_2 for 8 h, and cell lysates were subjected to DNA fragmentation analysis as described in Materials and Methods. (**D**) Wild-type, Syk-deficient, and BLNK-deficient DT40 cells were treated with 100 μM H_2O_2 for 5 min, and whole cell lysates were subjected to immunoblotting analysis using anti-phospho-Akt or anti-Akt antibodies. The results from one representative experiment that was repeated four times are shown.

tion upon H_2O_2 stimulation, but this fact could not explain the high viability in BLNK-deficient cells exposed to low doses of H_2O_2.

BLNK couples Syk to JNK activation following high doses of H_2O_2 stimulation, but not low doses of H_2O_2 stimulation

We have previously demonstrated that JNK activation is dependent on Syk in B cells treated with high doses of H_2O_2 (16). As shown in Fig. 5, after treatment with 2 mM H_2O_2, JNK was activated in wild-type cells, whereas activation of JNK was completely abrogated in BLNK-deficient and PLC-γ2-deficient as well as Syk-deficient cells. It has been reported that BCR-induced JNK activation is dependent on both PLC-γ2 and Rac1 and BLNK is required for coupling Syk to both of them (8). Our data suggested that unlike BCR signaling, BLNK mediated chiefly PLC-γ2-dependent JNK activation following oxidative stress. On the other hand,

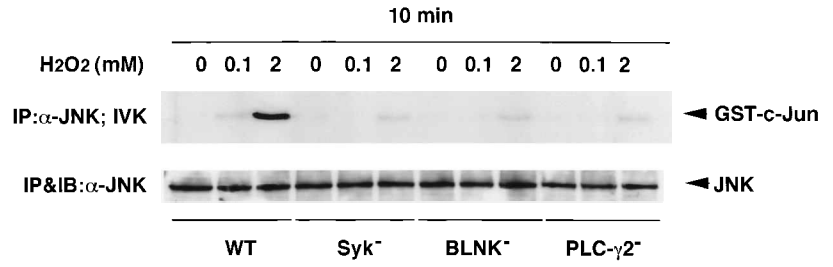

FIG. 5. Oxidative stress-induced JNK activation in BLNK-deficient cells. After DT40 cells were stimulated with 100 μM or 2 mM H_2O_2 for 10 min, cell lysates were immunoprecipitated with anti-JNK antibody, and JNK activity in the immunoprecipitate was determined using a specific *in vitro* kinase assay (IVK) as described in Materials and Methods. Results shown are from one representative experiment that was replicated four times.

in B cells stimulated with 100 μM H_2O_2, JNK is hardly activated. Although it has also been shown that a high level of JNK activity is correlated with the induction of apoptosis (24), we suspected that JNK had little, if any, role in B cell apoptosis induced by low doses of H_2O_2.

DISCUSSION

BLNK represents a central linker protein that bridges Syk with a variety of downstream effector proteins, including PLC-γ2, Vav, Grb2, and Nck in BCR-stimulated cells (4, 6, 8, 9, 25). Syk plays a key role not only in BCR signaling, but also in the oxidative stress signaling in B cells. Oxidative stress induces rapid enhancement of Syk tyrosine-phosphorylation, which triggers Ca^{2+} mobilization and JNK activation (15, 16). BLNK is phosphorylated by Syk in a H_2O_2 dose-dependent manner. BLNK-deficient cells lack Syk-dependent Ca^{2+} mobilization and JNK activation following treatment with low or high doses of H_2O_2. These findings suggest that BLNK plays a crucial role in coupling Syk to downstream effectors in oxidative stress signaling as well as BCR signaling.

Although the degree and period of BLNK phosphorylation in B cells treated with low doses of H_2O_2 are very low and short (Fig. 1), Syk-dependent Ca^{2+} mobilization through PLC-γ2 occurs, whereas JNK activation is not exhibited. On the other hand, high doses of H_2O_2 induce strong tyrosine phosphorylation of BLNK followed by Syk-dependent increase of $[Ca^{2+}]_i$ (data not shown) and JNK activation. We think that BLNK in a BLNK phosphorylation-dependent manner has a selective role in coupling Syk to a multitude of signaling pathways following oxidative stress. Further studies are required to clarify the selective role of BLNK in oxidative stress.

We have attempted to show the subcellular localization of Syk, BLNK, and PLC-γ2 in B cells after oxidative stress. It was observed that in B cells treated with high doses of H_2O_2, but not with low doses of H_2O_2, BLNK and PLC-γ2 translocated to the membrane fraction, and defect of BLNK abolished the translocation to the membrane fraction and the tyrosine phosphorylation of PLC-γ2 (data not shown). Although we failed to show the translocation of PLC-γ2 to the membrane fraction in B cells exposed to low doses of H_2O_2, IP_3 generation by PLC-γ2 was dependent on BLNK. These findings suggest that BLNK plays an important role in the codistribution of PLC-γ2 with membrane-localized phosphatidylinositol 4,5-bisphosphate, which is a substrate for PLC-γ2.

It is interesting that BLNK-deficient as well as PLC-γ2-deficient cells exhibit a reduction of oxidative stress-induced apoptosis. It has been reported that both activation of protein kinase C and Ca^{2+} mobilization through PLC-γ2 are required for BCR-induced apoptosis (18). Our data indicate that PLC-γ2 is required also for oxidative stress-induced apoptosis. Moreover, one prominent feature of H_2O_2 in apoptosis is to release the mitochondrial protein cytochrome *c* into the cytosol, thereby leading to the activation of the caspase cascade and DNA fragmentation (12). Release of cytochrome *c* in wild-type, Syk-deficient, and BLNK-deficient cells following H_2O_2 stimulation is not differ-

ent (data not shown). Therefore, the difference of cell viability in wild-type and BLNK-deficient cells following oxidative stress seems to be dependent on PLC-γ2-mediated pathways. On the other hand, oxidative stress triggers the activation of the P I3-kinase–Akt survival pathway. We have previously suggested that Syk is essential for the activation of the Akt survival pathway in B cells and enhances cellular resistance to oxidative stress-induced apoptosis (2). In Syk-deficient cells exposed to H_2O_2 stimulation, both an increase of $[Ca^{2+}]_i$ and Akt activation are abrogated, resulting in reduced resistance to oxidative stress-induced apoptosis. On the other hand, in BLNK-deficient cells, an increase of $[Ca^{2+}]_i$ is inhibited and 60% of Akt activation remains, thereby enhancing cell viability following oxidative stress. These findings have led to the suggestion that the balance between an increase of $[Ca^{2+}]_i$, release of cytochrome *c*, and activation of the Akt survival pathway is a key factor in regulating apoptosis following oxidative stress in B cells.

In summary, our findings suggest a crucial role of BLNK in oxidative stress signaling. BLNK is required for coupling Syk to Ca^{2+} mobilization following oxidative stress and accelerates apoptosis through PLC-γ2.

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ABBREVIATIONS

BCR, B cell receptor; BLNK, B cell linker protein; $[Ca^{2+}]_i$, cytoplasmic free calcium; GST-c-Jun, glutathione *S*-transferase-c-Jun; H_2O_2, hydrogen peroxide; IB, immunoblotting: IP, immunoprecipitation; IP_3, inositol 1,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PLC, phospholipase C; ROS, reactive oxygen species.

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