Syk has been demonstrated to play a crucial role in oxidative stress signaling in B cells. Here we report that Syk is required for the activation of the phosphatidylinositol (PI) 3-kinase-Akt survival pathway in B cells exposed to oxidative stress. Phosphorylation and activation of the serine-threonine kinase Akt were markedly increased in B cells treated with H₂O₂. In Syk-deficient DT40 cells treated with low doses of H₂O₂ (10–100 μM), Akt activation was considerably reduced. Pretreatment with wortmannin, a PI 3-kinase-specific inhibitor, completely blocked the Syk-dependent Akt activation. Following stimulation by low doses of H₂O₂, a significant increase in PI 3-kinase activity was found in wild-type but not in Syk-deficient cells. These findings suggest that PI 3-kinase mediates Syk-dependent Akt activation pathway. Furthermore, viability of Syk-deficient cells, after exposure to H₂O₂, was dramatically decreased and caspase-9 activity was greatly increased compared with that of the wild-type cells. These results suggest that Syk is essential for the Akt survival pathway in B cells and enhances cellular resistance to oxidative stress-induced apoptosis.

Protein-tyrosine kinases (PTKs) play crucial roles in a wide variety of cellular responses including cell proliferation, differentiation, and apoptosis (1). Extracellular stresses such as ionizing radiation, H₂O₂ treatment, osmotic shock, or genotoxic agents have also been reported to activate various PTKs (2–5). Syk, which belongs to nonreceptor PTKs and plays a crucial role in B cell receptor-mediated signaling, is also activated by oxidative and osmotic stress (6–10). Genetic studies using Syk-deficient B cells have revealed that Syk is essential for the increased tyrosine phosphorylation of cellular proteins, Ca²⁺ release from intracellular stores, and c-Jun N-terminal kinase (JNK) activation following oxidative stress (9, 10). Thus, it has been obvious that Syk plays a crucial role in the transduction of oxidative stress signaling in B cells.

It has been demonstrated that oxidative stress induces apoptotic and necrotic cell death in many cell types (11, 12). Treatment of cells in vitro with H₂O₂ causes DNA strand breaks, oxidation of lipids and proteins, activation of poly-(ADP)-riboseylation, and depletion of cellular energy stores (13). On the other hand, oxidative stress can trigger the activation of some signaling pathways that are involved in cell survival including the phosphorylation cascades leading to the activation of mitogen-activated protein kinase, nuclear factor-κB (NF-κB), and the serine-threonine kinase Akt (10, 14, 15). Akt becomes activated in a phosphatidylinositol (PI) 3-kinase-dependent manner not only in response to insulin and various growth factors but also in response to extracellular stresses such as H₂O₂ treatment and heat shock (15–19). Furthermore, it has been obvious that activation of Akt is necessary for cell survival and the prevention of apoptosis, which occur by phosphorylation of the Bcl-x inhibitor BAD or caspase-9 and by regulation of signaling via transcription factors such as NF-κB (20–22). However, the role of oxidative stress-induced protein-tyrosine phosphorylation in the activation of Akt survival pathway is not clear.

In this paper, we report on the role of Syk in the activation of Akt survival pathway in B cells exposed to oxidative stress. We have found that Syk is required for the activation of Akt survival pathway and enhances cellular resistance to cell death in B cells exposed to oxidative stress. Our data provide the first genetic evidence for the mechanism of regulation of Akt by oxidative stress-activated PTKs.

**EXPERIMENTAL PROCEDURES**

**Materials**—The RPMI 1640 medium was purchased from ICN Biomedicals. Fetal bovine serum was from Life Technologies, Inc. and Sigma. Protein A-Sepharose CL-4B was from Amersham Pharmacia Biotech AB. wortmannin was from Sigma. Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology, Inc. Anti-Syk antibodies (C20 and N19) were purchased from Santa Cruz Biotechnology, Inc. Anti-Akt and anti-phospho-Akt (Ser-473) antibodies were purchased from New England Biolabs, Inc.

**Cell Culture**—Establishment of Syk-deficient DT40 cells (Syk⁻) and Syk⁻ cells expressing porcine Syk (Syk/Syk⁻) or its mutants (KD and Autop) was performed as described previously (23). DT40 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml kanamycin in a humidified 95% air, 5% CO₂ atmosphere. Cells, collected by centrifugation at 400 × g for 5 min, were washed with phosphate-buffered saline and resuspended (1 × 10⁸ cells/ml) in Hanks’ balanced salt solution. Cells were stimulated with H₂O₂ at 37 °C with gentle stirring at concentrations described in the figure legends.

**Immunoprecipitation and Immunoblotting**—Stimulated cells (1 × 10⁷ cells/ml) were lysed in ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 100 μM NaVO₄, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotonin). Lysates were clarified by centrifugation at 12,000 × g for 10 min at 4 °C. The supernatants were incubated sequentially (1 h for each incu-
**Fig. 1.** Syk-dependent Akt activation by treatment with low doses of H$_2$O$_2$. A, wild-type (WT) or Syk-deficient (Syk$^-$) DT40 cells were treated with 100 μM H$_2$O$_2$ for the indicated times, and whole cell lysates were subjected to immunoblotting (IB) analysis using anti-phospho-Akt or anti-Akt antibodies. B, wild-type and Syk-deficient cells were treated with the indicated doses of H$_2$O$_2$, and whole cell lysates were subjected to immunoblotting analysis. C, anti-Akt immunoprecipitates (IP) were subjected to in vitro kinase assay (IVK) using histone 2B as a substrate. The results shown are from one representative experiment that was replicated four times.

**RESULTS**

**Akt Is Activated in a Syk-dependent Manner by Treatment with Low Doses of H$_2$O$_2$**—We have previously reported that Syk is activated by oxidative stress and plays a crucial role in Ca$^{2+}$ release from intracellular stores and JNK activation in B cells, suggesting that Syk is a key molecule in the transduction of oxidative stress signals (9, 10). It has been reported that cells exhibit resistance to oxidative stress-induced apoptosis via the activation of Akt survival pathway (19). In this study, we have examined the role of Syk in the activation of Akt survival pathway in DT40 B cells exposed to oxidative stress. Wild-type or Syk-deficient cells were treated with various concentrations of H$_2$O$_2$, and the whole cell lysates were subjected to immunoblotting analysis using anti-phospho-Akt antibody, which recognizes phosphorylated Ser-473 of Akt, an active form of Akt.

**PI 3-Kinase Assay**—PI 3-kinase assay was performed by the method of Aagaard-Tillery et al. (25). Briefly, 20 μl of phosphatidylinositol 4,5-diphosphate at 1 mg/ml were added to immunoprecipitates obtained from cell lysates using anti-phosphotyrosine antibody. Kinase reactions were initiated by the addition of 20 μl of kinase buffer (20 mM HEPES, pH 8.0, 50 mM MgCl$_2$, 40 μM ATP, 200 μM adenosine, and 20 μM of $[γ$-32P]ATP (3000 Ci/mmol) and incubated for 20 min at 30 °C. Reactions were stopped by the addition of the appropriate volume of 3× Laemmli sample buffer and boiled for 5 min. Samples were resolved by 15% SDS-polyacrylamide gel electrophoresis and evaluated by autoradiography.

**Cell Viability**—Wild-type DT40 and mutant cells (5 × 10$^5$ cells/ml) were stimulated using the indicated concentrations of H$_2$O$_2$. After 22 h, cell viability was determined by the trypan blue dye exclusion method.

**DNA Fragmentation Analysis**—Wild-type and Syk-deficient DT40 cells (5 × 10$^5$ cells/ml) were stimulated with the indicated concentration of H$_2$O$_2$ for 22 h. 5 × 10$^5$ cells were lysed in 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 200 mM NaCl, 0.4% Triton X-100, and 0.1 mg/ml protein K) for 20 min at room temperature followed by a 30-min incubation with 0.1 mg/ml RNase A at 50 °C. DNA fragmentation was analyzed using a 2.5% agarose gel in the presence of 0.5 μg/ml ethidium bromide.

**Detection of Caspase-9 Activity**—After stimulation by H$_2$O$_2$, cell lysates were tested for protease activity by the addition of a caspase-9-specific peptide that is conjugated to the color reporter molecule p-nitroanilide (LEHD-pNA substrate) using the Caspase-9 Colorimetric Assay (R&D Systems Inc.) according to the manufacturer’s instructions.

**RESULTS**

**Akt Is Activated in a Syk-dependent Manner by Treatment with Low Doses of H$_2$O$_2$**—We have previously reported that Syk is activated by oxidative stress and plays a crucial role in Ca$^{2+}$ release from intracellular stores and JNK activation in B cells, suggesting that Syk is a key molecule in the transduction of oxidative stress signals (9, 10). It has been reported that cells exhibit resistance to oxidative stress-induced apoptosis via the activation of Akt survival pathway (19). In this study, we have examined the role of Syk in the activation of Akt survival pathway in DT40 B cells exposed to oxidative stress. Wild-type or Syk-deficient cells were treated with various concentrations of H$_2$O$_2$, and the whole cell lysates were subjected to immunoblotting analysis using anti-phospho-Akt antibody, which recognizes phosphorylated Ser-473 of Akt, an active form of Akt.

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**Cell Viability**—Wild-type DT40 and mutant cells (5 × 10$^5$ cells/ml) were stimulated using the indicated concentrations of H$_2$O$_2$. After 22 h, cell viability was determined by the trypan blue dye exclusion method.

**DNA Fragmentation Analysis**—Wild-type and Syk-deficient DT40 cells (5 × 10$^5$ cells/ml) were stimulated with the indicated concentration of H$_2$O$_2$ for 22 h. 5 × 10$^5$ cells were lysed in 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 200 mM NaCl, 0.4% Triton X-100, and 0.1 mg/ml protein K) for 20 min at room temperature followed by a 30-min incubation with 0.1 mg/ml RNase A at 50 °C. DNA fragmentation was analyzed using a 2.5% agarose gel in the presence of 0.5 μg/ml ethidium bromide.

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Syk Is Required for Akt Survival Pathway

**DISCUSSION**

It has been well established that oxidative stress induces the activation of various PTKs, subsequently leading to an increase
Syk Is Required for Akt Survival Pathway

We have previously demonstrated that in B cells exposed to oxidative stress, Syk plays a crucial role in signal transduction, including Ca²⁺ release from intracellular pools and the activation of JNK (9, 10). In this study, we have found that Syk also induces PI 3-kinase activation mediated by Akt activation in B cells following oxidative stress. It has been reported that Syk phosphorylates caspase-9 and inhibits its protease activity (21), so we suggest that Akt, which is activated in a Syk-dependent manner following oxidative stress, inhibits caspase-9 activity. However, we could not determine whether caspase-9 was the substrate for Akt in B cells following oxidative stress. There is another possibility, that Syk modulates caspase-9 activity mediated by cytochrome c. Further studies are required to clarify the anti-apoptotic function of Syk in oxidative stress.

We have previously reported that Akt activation in B cells stimulated with high doses of H₂O₂ (0.5–1 mM) (10). It has also been shown that JNK is activated by stressful stimuli, and a high level of JNK activity is correlated in many instances with the induction of apoptosis (32). However, in B cells stimulated with a low dose of H₂O₂ (100 μM), JNK is hardly activated (10). Therefore, we suspect that JNK has little if any role in B cell apoptosis induced by low doses of H₂O₂.

In summary, our findings provide another physiological role for Syk in oxidative stress signaling. Syk induces the activation of the PI 3-kinase-Akt survival pathway following oxidative stress, thereby enhancing cell survival against oxidative stress.

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