Syk-dependent and -independent Signaling Cascades in B Cells Elicited by Osmotic and Oxidative Stress*

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It was found that Syk protein-tyrosine kinase is rapidly activated in B cells after H₂O₂ treatment (oxidative stress) or increased extracellular NaCl concentration (osmotic stress) as well as in response to B cell receptor activation. In this study we examined the involvement of Syk in responses elicited by these types of extracellular stress, particularly Ca²⁺ responses and c-Jun amino-terminal kinase (JNK) activation, using a chicken B cell line, DT40, as well as the DT40-derived mutant DT40/Syk(−), which does not express Syk. Osmotic stress evokes increases in [Ca²⁺]i, by stimulating an extracellular Ca²⁺ influx in both DT40 and DT40/Syk(−) cells. In comparison, oxidative stress elicits an increase in [Ca²⁺]i, by stimulating both an extracellular Ca²⁺ influx and Ca²⁺ release from internal stores in DT40 cells, but this Ca²⁺ response is partially abolished in DT40/Syk(−) cells, indicating that the oxidative stress-induced Ca²⁺ response is at least partly dependent on Syk. Interestingly, the depletion of Ca²⁺ results in a significantly decreased level of Syk activation in DT40 cells stimulated by oxidative but not osmotic stress. Furthermore, JNK is activated to different extents by these two types of stress. The extent of JNK activation in DT40/Syk(−) cells in response to osmotic stress is comparable to that observed in DT40 cells. Intriguingly, oxidative stress-induced JNK activation is significantly compromised in DT40/Syk(−) cells. Collectively, these results indicate that both the Ca²⁺ response and JNK activity induced by oxidative stress are partly dependent on Syk, whereas those induced by osmotic stress are independent of Syk.

Protein-tyrosine kinases (PTKs) play crucial roles in a wide variety of cellular responses, including cellular activation, proliferation, and differentiation (1). The binding of growth factors and cytokines to their cognate receptors stimulates intrinsic tyrosine kinase activities associated with these receptors or nonreceptor PTKs that couple to these receptors, thereby triggering downstream signaling events (2). It has also been reported that extracellular stress, ionizing radiation, UV irradiation, and H₂O₂ or genotoxic agents activate the nonreceptor PTKs of the Src- and/or Syk/ZAP-70 family (3–7), although the exact roles of these nonreceptor PTKs in stress-activated signaling pathways remain to be elucidated. It has been well established that extracellular stimuli promoting cellular activation and proliferation induce the activation of receptor PTKs or nonreceptor PTKs, leading to the subsequent activation of the Ras-Raf-Mek-extracellular signal-regulated kinase (ERK) signaling cascade (8). Activated ERKs of the mitogen-activated protein kinase superfamily, in turn, regulate gene expression by phosphorylating transcription factors such as Elk1 (9).

Recently, novel members of the mitogen-activated protein kinase superfamily, now referred to as stress-activated protein kinases or the c-Jun amino-terminal kinases (JNKs), have been identified in yeast and mammalian cells. JNKs exhibit an extraordinarily high affinity for their substrate, c-Jun, and phosphorylate it on specific amino-terminal serine residues (Ser-63 and Ser-73), thereby augmenting the function of c-Jun as a transcriptional activator (10). Stimuli that primarily activate Raf-Mek-ERK can only poorly activate the JNK cascade (11). Unlike ERKs, JNKs are strongly activated by a variety of extracellular stress (12–16). It has been recently reported that Rac/Oct2-MEKK1-SEK protein kinases act upstream of JNK (17, 18). Although the participation of PTKs in stress signaling has been indicated (19), the identities and exact roles of such PTKs are largely unknown. Interestingly, recent studies reveal that c-Abl, a nonreceptor PTK localized in both the cytoplasm and the nucleus (20, 21), positively regulates the activities of JNKs in response to extracellular stress that selectively induces DNA damage (22, 23).

It has been shown that extracellular stress induces the activation of the Syk/ZAP-70 family of PTKs and Ca²⁺ mobilization in T and B lymphocytes, identical to those observed after antigen receptor activation (24, 25). Unlike the Src family of PTKs, the Syk/ZAP-70 family of PTKs, Syk and ZAP-70, contain two SH2 domains, no myristylation site, and no carboxyl-terminal negative regulatory tyrosine residues (26, 27). Syk is expressed in a wide range of hematopoietic cells, including T cells, B cells, myeloid cells, and platelets, whereas ZAP-70 expression is restricted to T cells and natural killer cells (28). Previous studies have demonstrated that Syk plays a crucial role in B cell receptor (BCR)-mediated signaling (29, 30). Syk-negative mutants of the chicken B cell line DT40 could be readily obtained by using the high frequency of homologous recombination (31). Thus, DT40 cells and the DT40-derived mutant lacking Syk (DT40/Syk(−)) have provided a powerful tool
to examine the role of Syk in BCR-mediated signaling (32). BCR activation in wild-type DT40 cells resulted in a rapid activation of Syk as well as a rapid increase in [Ca\(^{2+}\)]. It was found that the BCR-mediated Ca\(^{2+}\) response is almost completely abolished in DT40/Syk(-) cells (32). There is growing evidence that strongly suggests that upon BCR-activation, Syk mediates the activation of phospholipase C\(_y\), resulting in the subsequent production of inositol 1,4,5-trisphosphate, thereby inducing an elevation in [Ca\(^{2+}\)]. (32). Interestingly, H\(_2\)O\(_2\) treatment (oxidative stress) also induces a rapid activation of Syk as well as a rapid increase in [Ca\(^{2+}\)], in DT40 cells, and this Ca\(^{2+}\) response is drastically reduced in DT40/Syk(-) cells (33).

Here we report that osmotic as well as oxidative stress induces Syk activation, increases intracellular calcium concentration, and activates JNK in a chicken B cell line, DT40. In this study we examined the roles of Syk in the observed Ca\(^{2+}\) response and JNK activity induced by the respective stress, using DT40 and DT40/Syk(-) cells. Interestingly, it was found that both the Ca\(^{2+}\) response and JNK activation induced by oxidative stress were partly dependent on Syk, yet those induced by osmotic stress were independent of Syk. We will discuss the significance of our findings in respect to the role of Syk in different cellular stress responses.

MATERIALS AND METHODS

Materials—Acetoxyethyl esters of bis-(o-aminophenyl) ethane-N,N,N',N'-tetracetic acid (BAPTA-AM) were purchased from Life Technologies, Inc. Protein A was from Calbiochem. Hydrogen peroxide and Fura 2-AM were from Wako Pure Chemicals. Anti-phosphotyrosine antibody (4G10), mouse anti-human JNK1 monoclonal antibody, and polyclonal anti-ERK/mitogen-activated protein kinase antisera were from Upstate Biotechnology Inc., Pharmingen (San Diego, CA), and Santa Cruz Biotechnology, respectively. Enhanced chemiluminescence reagents were from DuPont. Glutathione-S-Phosphoesterase 4B was from Pharmacia Biotech Inc.

Cell Culture and Harvest—Establishment of DT40/Syk(-) and DT40/Syk(-) expressing porcine Syk was performed as described previously (32). DT40 and DT40-derived cells as well as the human B cell line Raji were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin in a humidified 95% air, 5% CO\(_2\) atmosphere. For experiments, cells were collected by centrifugation as described previously (33). For experiments requiring the depletion of calcium, cells were resuspended in calcium-free Hanks’ balanced salt solution buffer, and EGTA (final concentration, 1 mM) was added 2 min before stimulation. Cells were stimulated by hydrogen peroxide (oxidative stress) or sodium chloride (osmotic stress) at 37 °C.

Preparation of Glutathione S-Transferase Fusion Protein—pGEXSK-X-c-Jun (amino acids 1–79) glutathione S-transferase fusion protein expression vector was transfected into Escherichia coli XL1Blue. Proteins were purified following the protocol recommended by the manufacturer (Pharmacia).

Measurement of [Ca\(^{2+}\)]e—Calcium mobilization was measured using a fluorescent indicator Fura-2 as described previously (33).

Preparation of Cell Extracts—Stimulated cells (1 x 10\(^6\) cells/ml) were lysed in ice-cold lysis buffer (5 mM EDTA, 150 mM NaCl, 2% Triton X-100, 100 \(\mu\)M NaVO\(_4\), 2 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml leupeptin, and 50 mM Tris, pH 7.4) after a short centrifugation step. Lysates were clarified by centrifugation at 16,000 \(\times\) g for 15 min at 4 °C.

Immunoblot Analysis—Cell extracts or immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis, transferred electrophoretically onto polyvinylidene difluoride membranes, and then immunoblotted with the indicated antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence.

Immunoprecipitation and Kinase Assays—The immunoprecipitated kinase activity of Syk was measured in a 30-μl reaction mixture that included 10 μg of H2B histone as the exogenous substrate (27). Immunoprecipitates by anti-JNK1 antibody (1 μg) with 40 μl of protein A-Sepharose 4B were washed three times with lysis buffer, once with washing buffer (50 mM HEPES, pH 7.6, and 10 mM MgCl\(_2\)), and once with kinase assay buffer (10 mM HEPES, pH 7.6, 10 mM MgCl\(_2\), 10 μM of cold ATP, and 10 μM vanadate). Immune complex kinase assays were performed in a 30-μl kinase assay buffer containing 1 μCi of [γ-\(^{32}\)P]ATP (3,000 Ci/mmol) and 5 μg of glutathione S-transferase-c-Jun as the substrate. After a 20-min incubation at 30 °C, reactions were terminated by the addition of SDS sample buffer and 5 min of boiling. Autoradiography was carried out and quantitated using a phosphoimager (Fuji BAS 2000).

RESULTS

Osmotic Stress Induces Syk Activation in Cells of a B Cell Lineage—In our previous study we showed that H\(_2\)O\(_2\) treatment (oxidative stress) as well as BCR activation induced rapid tyrosine phosphorylation and activation of Syk in chicken B cell line DT40 (33, 34) (Fig. 1A). To investigate whether or not high osmolar exposure (osmotic stress), a distinct extracellular stress, can induce tyrosine phosphorylation of Syk in DT40 cells, anti-Syk immunoprecipitates of cell lysates from DT40 cells treated with or without sodium chloride were subjected to immunoblotting with anti-phosphotyrosine antibody. As shown in Fig. 1B (top), the exposure of DT40 cells to sodium chloride triggered a rapid and sustained tyrosine phosphorylation of Syk. This increase in tyrosine phosphorylation was almost maximal within 1 min and remained elevated throughout the 15-min incubation (Fig. 1B, top). This observed tyrosine phosphorylation of Syk was dependent on the concentration of sodium chloride used. When exposed to low concentrations of sodium chloride, such as 0.2 M, significant tyrosine phosphorylation of Syk was observed after a 5-min incubation (Fig. 1B). In parallel with Syk tyrosine phosphorylation, sodium chloride stimulated a rapid and transient increase in Syk activity (Fig. 1C). The ability of Syk to phosphorylate the exogenous substrate, H2B histone, was increased 2–3-fold upon treatment. Immunoblot analysis with an anti-Syk antibody revealed that the amounts of Syk immunoprecipitated from treated or untreated DT40 cells were comparable (Fig. 1, A and B, bottom), indicating that the result obtained reflects changes in the specific activity of Syk. Furthermore, it was shown that Syk activation was also induced by other high- osmolarity producers, such as potassium chloride, lithium chloride, and sorbitol (Fig. 1D). Like DT40 cells, tyrosine phosphorylation of Syk was observed in a human B cell line, Raji, upon exposure to the respective high-osmolarity producers (data not shown). These observations indicate that Syk activation by osmotic stress is not restricted to DT40 cells and may be a common biochemical event for cells of a B cell lineage.

Oxidative and Osmotic Stress Elicit Differential Ca\(^{2+}\) Responses via Syk-dependent and -independent Mechanisms—An increase in [Ca\(^{2+}\)], has been appreciated as one of the earliest events during lymphocyte activation. To address whether oxidative and osmotic stress can elicit a Ca\(^{2+}\) response, intracellular Ca\(^{2+}\) mobilization in DT40 cells was examined using a fluorescent indicator, Fura-2. A representative profile is shown in Fig. 2. Both oxidative and osmotic stress induced increases in [Ca\(^{2+}\)], however, the kinetics of the response differed. Oxidative stress triggered a rapid and sustained increase in [Ca\(^{2+}\)], that was maximal within 1 min and decreased only slightly over the duration of the experiment. In contrast, osmotic stress induced a relatively slow and sustained increase in [Ca\(^{2+}\)]. In the absence of calcium influx after EGTA chelated the extracellular calcium, oxidative stress was still capable of inducing an increase in [Ca\(^{2+}\)], but to a lesser extent, whereas osmotic stress failed to trigger an increase in [Ca\(^{2+}\)], under the same conditions. Hence, it became apparent that an increase in [Ca\(^{2+}\)], by oxidative stress was mediated by both release from intracellular calcium stores and extracellular calcium influx, but that the increase in [Ca\(^{2+}\)], observed for osmotic stress was solely dependent on extracellular calcium influx.

Syk plays a critical role in regulating an increase in [Ca\(^{2+}\)], during anti-IgM-induced B cell activation (32). To investigate whether the Ca\(^{2+}\) responses induced by extracellular stress...
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were dependent on Syk, [Ca\(^{2+}\)]

under the same conditions (data not shown). Interestingly, the depletion of intracellular/extracellular free calcium resulted in a drastic decrease in tyrosine phosphorylation of Syk, as well as other cellular proteins, in response to oxidative stress (Fig. 3B and data not shown). Collectively, these observations revealed that an increase in [Ca\(^{2+}\)], was required for full-scale activation of Syk by oxidative but not osmotic stress.

Differential Regulation of JNKs and ERKs by Oxidative and Osmotic Stress—Previous studies demonstrate that a variety of extracellular stimuli activate members of the mitogen-activated protein kinase superfamily, such as JNKs and ERKs, to different extents (35–37). Hence, we address the question of whether JNKs as well as ERKs are activated in DT40 cells by oxidative and osmotic stress. JNK activity was drastically increased (∼9-fold increase compared to basal) by oxidative stress (1 mM H\(_2\)O\(_2\) as measured by the phosphorylation of glutathione S-transferase-c-Jun (Fig. 4A). JNKs were also activated (approximately 33-fold compared to basal) by osmotic stress (0.3 M NaCl). As shown in Fig. 4A, JNK activation by both oxidative and osmotic stress occurred in a dose-dependent manner.

ERKs have been extensively studied in the context of growth factor signal transmission (38). However, it has been recently reported that in addition to JNKs, ERKs are also activated by some extracellular stress (39–41). Accordingly, we examined whether ERKs were also activated in DT40 cells by oxidative and osmotic stress. Elevated ERK activity correlates well with some extracellular stress (39–41). JNK activity was drastically increased (∼9-fold increase compared to basal) by oxidative stress (1 mM H\(_2\)O\(_2\) as measured by the phosphorylation of glutathione S-transferase-c-Jun (Fig. 4A). JNKs were also activated (approximately 33-fold compared to basal) by osmotic stress (0.3 M NaCl). As shown in Fig. 4A, JNK activation by both oxidative and osmotic stress occurred in a dose-dependent manner.

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**FIG. 1.** Syk activation in DT40 cells after oxidative and osmotic stress. A, anti-phosphotyrosine immunoblot (top) and anti-Syk immunoprecipitates from DT40 cells after hydrogen peroxide stimulation (oxidative stress). IP, immunoprecipitation; IB, immunoblot. B, anti-phosphotyrosine immunoblot (top) and anti-Syk immunoprecipitates from DT40 cells after hydrogen peroxide stimulation (oxidative stress). C, in vitro kinase assay of anti-Syk immunoprecipitates from DT40 cells after 0.4 M sodium chloride or 1 mM hydrogen peroxide stimulation, using H\(_2\)B histone as an exogenous substrate. D, anti-phosphotyrosine immunoblot of anti-Syk immunoprecipitates from DT40 cells after 0.8 M sodium chloride (lanes 2 and 3), 0.8 M potassium chloride (lanes 4 and 5), 0.8 M lithium chloride (lanes 6 and 7), and 0.8 M sorbitol (lanes 8 and 9) stimulation. Left, positions of the molecular mass markers (in kDa). Arrows, the positions of Syk. An asterisk indicates the position of H\(_2\)B histone.

Increase in [Ca\(^{2+}\)], is Required for the Full-Scale Activation of Syk by Oxidative but not Osmotic Stress—The result showing a partially impaired Ca\(^{2+}\) response in DT40/Syk(−) cells subjected to oxidative stress suggests that Syk is one of the upstream signaling molecules required for mediating the Ca\(^{2+}\) response in oxidative stress signaling. Next we examined whether an increase in [Ca\(^{2+}\)], could, in turn, modulate the activity of Syk in oxidative stress- and osmotic stress-induced signaling.

For this purpose, tyrosine phosphorylation of Syk was slightly affected by the depletion of intracellular/extracellular free calcium. Consistent with this result, the tyrosine phosphorylation of cellular proteins by osmotic stress was marginally affected
JNK activation was compromised in DT40/Syk(−) cells observed in response to 0.5 and 1 mM hydrogen peroxide. However, in the case of oxidative stress, DT40 cells was not a consistent observation when stimulated by 0.1M NaCl. This finding led us to analyze whether oxidative but not osmotic stress can activate ERK in DT40 cells.

**Differential Requirement of Syk for Activation of JNKs and ERKs by Oxidative and Osmotic Stress—**ERKs are well characterized downstream effectors of receptor or nonreceptor PTKs. JNKs are shown to be regulated by distinct protein kinases in a similar cascade, however, the roles of PTKs involved in the regulation of JNKs remain largely unknown. Recently, c-Abl was shown to regulate the activation of JNKs in response to cellular stress that selectively damages DNA (22, 23). This finding led us to analyze whether Syk plays a similar role in regulating JNK activation in response to oxidative and osmotic stress. As shown in Fig. 5A, JNKs were activated by osmotic stress to comparable levels in both DT40 and DT40/Syk(−) cells. The increase in JNK activity was 2.4- and 18.0-fold in DT40 cells and 4.6- and 16.0-fold in DT40/Syk(−) cells (Fig. 5A) cell lysates were comparable (data not shown).

**DISCUSSION**

Recent studies have indicated that different types of extracellular stress, including ionizing radiation, UV irradiation, and H2O2, activate a set of nonreceptor PTKs, such as the Src- and Syk/ZAP-70 family of PTKs (3–7), although the functional roles of these PTKs in stress-induced signaling pathways remain unclear. It has also been well documented that such stress can elicit other signaling events, including the activation of JNKs (and ERKs), and increase intracellular Ca2+ concentrations (12, 13, 24, 25, 39–41, 45). Here, we show that Syk is activated in a B cell line (DT40 cells) after treatment with H2O2 (oxidative stress) and with NaCl (osmotic stress), although the kinetics of Syk activation are different (Fig. 1). Furthermore, it was found that both oxidative and osmotic stress induce a Ca2+ response and activation of JNKs (and ERKs) (Figs. 2 and 4). In this study, we have focused on the possible role(s) of Syk in regulating the Ca2+ response as well as the activation of JNKs (and ERKs) induced by oxidative and osmotic stress.

Previous studies have demonstrated that Syk plays a crucial role in platelet activation, B cell development, and B cell activation (29, 30, 46–48). An important role of Syk in BCR-mediated signaling was further elucidated by using a chicken B cell line, DT40, and the DT40-derived mutant DT40/Syk(−), which does not express Syk (32). In fact, the BCR-mediated Ca2+ response was almost completely abolished in...
oxidative stress is partly dependent on Syk. The dependency of an increase in \([	ext{Ca}^{2+}]_i\) and the subsequent production of inositol 1,4,5-trisphosphate, thereby inducing the release of \(\text{Ca}^{2+}\) from intracellular stores. However, it remains unclear how osmotic as well as oxidative stress induces extracellular calcium influx. In contrast to hyperosmolar stress, it was reported that the \(\text{Ca}^{2+}\) response induced by oxidative stress was mediated by a release from intracellular calcium stores (50).

DT40/Syk(−) cells, indicating the crucial role of Syk in the \(\text{Ca}^{2+}\) response upon BCR activation (43).

DT40 as well as DT40/Syk(−) cells have provided a powerful tool to dissect signaling cascades triggered by oxidative and osmotic stress. We have shown that both oxidative and osmotic stress elicit a \(\text{Ca}^{2+}\) response in DT40 cells, although the oxidative stress- but not osmotic stress-induced \(\text{Ca}^{2+}\) response was delayed and lower in magnitude in DT40/Syk(−) cells (Fig. 2). Thus, it became evident that the osmotic stress-induced \(\text{Ca}^{2+}\) response is independent of Syk, whereas that induced by oxidative stress is partly dependent on Syk. The dependency of the oxidative stress-induced \(\text{Ca}^{2+}\) response on Syk was further emphasized when the expression of porcine Syk in DT40/Syk(−) cells resulted in a calcium response very similar to that observed in DT40 cells. In addition, experiments utilizing calcium chelators highlighted that the requirement for calcium exhibited by Syk differed between oxidative and osmotic stress (Fig. 3). Oxidative but not osmotic stress-induced tyrosine phosphorylation of Syk (a surrogate marker for Syk activation) was reported that the \(\text{Ca}^{2+}\) response induced by oxidative stress was impaired in DT40/Syk(−) cells (33) and that oxidative stress induced tyrosine phosphorylation of phospholipase Cγ1 as well as promoting the association of Syk with phospholipase Cγ1 in porcine peripheral blood lymphocytes (49), it is likely that oxidative stress-induced Syk activity results in the tyrosine phosphorylation of phospholipase Cγ and the subsequent production of inositol 1,4,5-trisphosphate, thereby inducing the release of \(\text{Ca}^{2+}\) from intracellular stores. However, it remains unclear how osmotic as well as oxidative stress induces extracellular calcium influx. In contrast to hyperosmolar stress, it was reported that the \(\text{Ca}^{2+}\) response induced by hypoosmolar stress was mediated by a release from intracellular calcium stores (50).

Recently, much attention has been paid to the role(s) of JNKs (and ERKs) in stress signaling pathways (51). In fact, a variety of extracellular stress have been shown to induce activation of JNKs (and ERKs) (12, 13, 39–41). To date, the role(s) of PTKs in stress-induced activation of JNKs has not been elucidated, with the exception of c-Abl. c-Abl is a nonreceptor PTK localized in both the cytoplasm and nucleus and is activated upon the exposure of cells (U-937 and NIH3T3 cells) to DNA-damaging agents (22, 23). Interestingly, it has been shown that cells deficient in c-Abl fail to activate JNKs after exposure to DNA-damaging agents and that activation of JNKs can be restored by an ectopic expression of c-Abl in these cells (22, 23). Collectively, these results suggest that c-Abl plays a crucial role in mediating the activation of JNKs upon exposure to DNA-damaging agents.

It was found that osmotic stress induced the activation of JNKs and ERKs by oxidative and osmotic stress in DT40 cells. A, dose-dependent JNK activation. DT40 cells were stimulated with 0.1 mM hydrogen peroxide (lane 2), 1 mM hydrogen peroxide (lane 3), 0.1 mM sodium chloride (lane 4), 0.3 mM sodium chloride (lane 5), or 0.5 mM sodium chloride (lane 6). In vitro kinase assay for JNK was performed as described under “Materials and Methods.” B, ERK activation examined by mobility shift assay. Cells were stimulated for 10 min with various doses of either hydrogen peroxide (lanes 2–4) or sodium chloride (lanes 5–7) or with 200 ng/ml 12-O-tetradecanoylphorbol-13-acetate (lane 8) as a positive control. Lysates from 2 × 10⁶ cells were separated by 12% SDS-polyacrylamide gel electrophoresis and then immunoblotted with anti-ERK antibody (K-23; Santa Cruz Biotechnology). p44 ERK, the mobility-shifted form of p44 ERK.

**FIG. 4.** Differential regulation of JNKs and ERKs by oxidative and osmotic stress in DT40 cells. A, dose-dependent JNK activation. DT40 cells were stimulated with 0.1 mM hydrogen peroxide (lane 2), 1 mM hydrogen peroxide (lane 3), 0.1 mM sodium chloride (lane 4), 0.3 mM sodium chloride (lane 5), or 0.5 mM sodium chloride (lane 6). B, ERK activation examined by mobility shift assay. Cells were stimulated for 10 min with various doses of either hydrogen peroxide (lanes 2–4) or sodium chloride (lanes 5–7) or with 200 ng/ml 12-O-tetradecanoylphorbol-13-acetate (lane 8) as a positive control. Lanes 2–4, 0.1 mM hydrogen peroxide (lanes 2–4), 0.3 mM sodium chloride (lane 5), or 0.5 mM sodium chloride (lane 6). In vitro kinase assay for JNK was performed as described under “Materials and Methods.” B, Syk is not required for ERK activation induced by oxidative stress. DT40 and DT40/Syk(−) cells were stimulated for 10 min with 0.5 mM hydrogen peroxide (lanes 2 and 7), 0.1 mM sodium chloride (lanes 4 and 9), or 0.3 mM sodium chloride (lanes 5 and 10). In vitro kinase assay for JNK was performed as described under “Materials and Methods.” B, Syk is not required for ERK activation induced by oxidative stress. DT40 and DT40/Syk(−) cells were stimulated with 0.1 mM hydrogen peroxide (lanes 2 and 7), 0.5 mM hydrogen peroxide (lanes 3 and 8), 1 mM hydrogen peroxide (lanes 4 and 9), or 200 ng/ml 12-O-tetradecanoylphorbol-13-acetate (lanes 5 and 10) for 10 min. Mobility shift assay was performed as described in the legend to Fig. 4.
JNKs, whereas oxidative stress induced the activation of both JNKs and ERKs in DT40 cells as assessed by an in vitro kinase assay (for JNKs) and by SDS-polyacrylamide gel electrophoresis mobility shift analysis (for ERKs) (Fig. 4). Interestingly, oxidative but not osmotic stress-induced activation of JNKs was compromised in DT40/Syk(−) cells, indicating that Syk was required for oxidative stress-induced activation of JNKs (Fig. 5). In contrast, oxidative stress-induced activation of ERKs was not affected by the absence of Syk (Fig. 5). Accordingly, it became obvious that Syk played an important role in the oxidative stress-induced signaling cascades, particularly in the case of the Ca²⁺ response and the activation of JNKs in the B cell line DT40. However, Syk alone is not sufficient to mediate the signaling cascades elicited by oxidative stress because both Ca²⁺ response and JNK activation are observed in DT40/Syk(−) cells, although to a lesser degree. It is of importance to identify the additional upstream signaling molecule(s) that mediates the oxidative stress-induced Ca²⁺ response as well as the activation of JNKs. Because it was reported that Rae1/Cdc42-MEK1-SEK protein kinases work upstream of JNKs under certain conditions, it is possible that oxidative stress may also activate this cascade. Further study will be required to clarify such a possibility. On the other hand, Syk is not required for the osmotic stress-induced Ca²⁺ response or the activation of JNKs, although Syk is apparently activated in response to osmotic stress. What is the exact role(s) of Syk in osmotic stress-induced signaling? Is the activation of Syk induced by osmotic stress an epiphenomenon? At present, the functional role(s) of Syk in osmotic stress-induced signaling remains unclear. However, it is likely that Syk plays an important role in osmotic stress-induced signaling cascades other than the Ca²⁺ response and JNK activation. In fact, Syk-negative mutants of DT40 cells are more susceptible to osmotic stress-induced apoptosis than the wild-type cells, and ectopic expression of porcine Syk in Syk-negative mutants renders cells resistant to osmotic stress-induced apoptosis.²

An important finding made in this study is that the functional role(s) of Syk differs between oxidative and osmotic stress-induced signaling pathways. Although both stresses can activate Syk, this activation is qualitatively different. At the present time, we do not know the molecular basis that could explain the qualitative difference observed in Syk activation. Further study will be required to clarify this issue. The results presented in this study also reveal that the mechanism for activating (or regulating) Syk by different stresses may be distinct. As shown, there are differences in the kinetics of Syk activation as well as the calcium requirement for Syk activation stimulated by oxidative and osmotic stress.

Syk is a 72-kDa nonreceptor PTK that is widely expressed in immunohematopoietic cells (27). Our results clearly demonstrate an important role for Syk in stress-induced signaling cascades in B cells. ZAP-70, another member of the Syk/ZAP-70 family of PTKs, is mainly expressed in mature T cells and natural killer cells (26) and is also activated by oxidative stress (25). Therefore, it will be of interest to examine whether or not ZAP-70 plays a role similar to that of Syk in stress-induced signaling cascades in both T cells and natural killer cells.

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REFERENCES

1. Ulrich, A., and Schlesinger, J. (1990) Cell 61, 203–212
2. Taniguchi, T. (1985) Science 230, 251–252
3. Brunell, J. H., Burkhartd, A. L., Bolen, J. B., and Grinstein, S. (1996) J. Biol.
4. Hardwick, J. S., and Selton, B. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4527–4531
5. Kharbanda, S., Yuan, Z.-M., Rubin, E., Weichselbaum, R., and Kufe, D. (1994) J. Biol. Chem. 269, 20739–20743
6. Kharbanda, S., Yuan, Z. M., Taneja, N., Weichselbaum, R., and Kufe, D. W. (1995) Oncogene 9, 3033–3034
7. Schieve, G. L., Kirihara, J. M., Myers, D. E., and Shaw, P. E. (1992) Nature 358, 414–417
8. S. Qin, Y. Minami, T. Kurosaki, and H. Yamamura, unpublished observations.