Distinctive Functions of Syk and Lyn in Mediating Osmotic Stress- and Ultraviolet C Irradiation-induced Apoptosis in Chicken B Cells*

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By taking advantage of the established chicken B cell line, DT40 cells, which do not express tyrosine kinase Syk or Lyn, functional roles of Syk and Lyn in apoptotic response elicited by cellular stress were investigated. DT40 cells underwent apoptosis after hyperosmotic stress. In Syk-deficient DT40 cells, this apoptotic process was significantly enhanced. Ectopic expression of wild type, but not kinase-inactive, porcine Syk in Syk-deficient cells rescued cells from osmotic stress-induced apoptosis, demonstrating that the presence of functionally active Syk is necessary to protect cells from osmotic stress-induced apoptosis. In comparison, there was no effect on osmotic stress-induced apoptosis in Lyn-deficient DT40 cells. Interestingly, while Syk was not involved in ultraviolet C (UVC)-induced apoptosis, a deficiency of Lyn rendered cells resistant to UVC irradiation. These observations defined Syk and Lyn as important mediators of apoptosis in DT40 cells in response to osmotic stress and UVC irradiation, respectively. Furthermore, osmotic stress, but not UVC irradiation, could activate c-Jun N-terminal kinase (JNK) in DT40 cells. A deficiency in either Syk or Lyn did not affect the osmotic stress-induced activation of JNK. We, therefore, concluded that Syk and Lyn regulate the apoptotic responses to osmotic stress and UVC irradiation independently of the JNK pathway in DT40 cells.

Apoptosis, which is widely observed in different cells of various organisms, is the unique morphological pattern of cell death characterized by chromatin condensation and membrane blebbing. The most prominent event in the early stages of apoptosis is internucleosomal DNA cleavage by undefined endonuclease activities, which is widely used as a biochemical marker of apoptosis. It is generally believed that apoptosis plays important roles in developmental processes, maintenance of homeostasis, and elimination of damaged cells (1, 2). Cells usually undergo apoptosis when they suffer from cellular stress. The molecular mechanisms by which cellular stress regulates cell apoptosis are still poorly understood.

A growing body of evidence demonstrates that several protein kinases participate in regulating stress-triggered apoptosis. Abl, a nonreceptor protein-tyrosine kinase (PTK), which is localized to the nucleus and the cytoplasm and shares structural features with Src family PTKs (3, 4), has been identified as a negative regulator of apoptosis. Constitutive expression of the p210 Bcr-Abl proteins in chronic myelogenous leukemia progenitor cells confers resistance to apoptosis upon interleukin-3 withdrawal (5). Moreover, the down-regulation of Bcr-Abl protein levels by antisense oligonucleotides has been shown to render K562 cells susceptible to apoptosis (6). More recently, Btk has also been demonstrated as a mediator of radiation-induced apoptosis of DT40 cells (7). In addition to the PTKs, c-Jun N-terminal kinases (JNKs) have an unusually high affinity for their substrate, c-Jun, and phosphorylate it on specific N-terminal serine residues at positions 63 and 73, leading to enhanced c-Jun transactivation potential (8). JNKs are strongly activated by stimuli other than growth factors, including signals as diverse as UV irradiation (9), osmotic shock (10, 11), protein synthesis inhibitors (12), and tumor necrosis factor α (TNF-α) stimulation (13). The pathways of JNK activation have been partially delineated and Rac/Cdc42-MEKK1-JNKK protein kinases have been shown to be upstream of JNK (14, 15). Furthermore, Pyk2 and c-Abl have recently been identified as the upstream regulators of JNK activation in response to certain cellular stresses although the convergence point of both these PTKs into the JNK pathway is unclear (16–18). The growing evidence, which indicates various cellular stresses as the potential activators of JNK, suggest that JNK activation may play an important role in mediating cell death or cell survival in cells exposed to various stresses. In fact, JNK activation has been demonstrated to correlate with cell apoptosis triggered by cellular stress (19, 20). Ectopic expression of a dominant-negative c-Jun mutant lacking the N terminus or a dominant-negative kinaseinactive JNKK abolishes cellular stress-induced cell apoptosis (19). In PC12 cells, ectopic expression of various mutants, which either activate or inhibit the JNK signaling pathway, also enhance or inhibit nerve growth factor withdrawal-induced apoptosis (20). In comparison, expression of human wild-type, but not kinase-negative, JNK in yeast lacking the protein kinase Hog1 is able to promote growth on hyperosmolar media (10), which under normal conditions inhibits growth of these cells, suggesting that JNK activation delivers a signal for cell survival.

We and others have demonstrated that certain cellular stresses such as oxidative stress are potent activators of the Syk family PTKs, Syk and ZAP70, in lymphocytes (21–24). Recently, we found that osmotic stress can also activate Syk in human and chicken B cells (25). Thus, it is of interest to further investigate the functional roles of PTKs in the stress response.
Roles of Syk and Lyn in Stress Signaling

and the possible mechanisms by which PTKs execute their function in stress signaling. Mammalian cells are exposed to hyperosmotic conditions in the distal tubule of the kidney, during hemo or peritoneal dialysis, when the concentration of serum sodium rises as a consequence of dehydration or due to an infusion of hypertonic saline (26). Oxidative stress may occur in response to inflammation due to the production of superoxide anion and hydrogen peroxide by neutrophils and monocytes. Inflammatory cytokines such as TNF-α and interleukin-1 can also stimulate the production of hydrogen peroxide and reactive oxygen intermediates, thus leading to cells being exposed to oxidant stress (27). Under physiological conditions, lymphocytes are rarely exposed to these types of stresses, yet elucidating the functional roles of PTKs in stress-induced responses in vitro would provide a better understanding of the pathogenesis in response to these pathophysiological stresses in vivo. By taking advantage of established Syk- or Lyn-deficient cells, we investigated the functional roles of Syk and Lyn (in particular Syk) in the apoptotic response triggered by osmotic stress or ultraviolet C (UVC) irradiation. Here, we report that a deficiency of Syk, but not Lyn, results in a drastically enhanced apoptotic response to osmotic stress when compared with wild type DT40. Ectopic expression of wild type, but not the kinase-inactive, porcine Syk in Syk-deficient cells significantly promoted cell survival in response to osmotic stress. In contrast, Lyn is a positive mediator of the apoptotic response elicited by UVC irradiation, whereas Syk does not appear to participate in this apoptotic process. These results demonstrate that in DT40 cells, Syk may function as a specific inhibitor of osmotic-stress-induced apoptosis while Lyn acts as a positive mediator of UVC irradiation-induced apoptosis.

EXPERIMENTAL PROCEDURES

Materials—The generation of DT40/Lyn−, DT40/Syk−, DT40/Syk+/Syk, and DT40/Syk−/Syk(ΔK) cells and antisera against Lyn or Syk was carried out as described previously (28). RPMI 1640 was purchased from ICN Biomedicals Inc. Fetal bovine serum was from Life Technologies. Inc. Protein A was from Calbiochem Corp. Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology Inc. Mouse anti-human JNK1 monoclonal antibody was purchased from Pharmingen (San Diego, CA). Enhanced chemiluminescence reagents were from Amer sham Corp. Glutathione-Sepharose 4B was from Pharmacia Biotech Inc. GST expression vector containing the N-terminal fragment (amino acids 1 to 79) of c-Jun was a gift from Dr. Hibi (Osaka University, Japan).

Cell Culture and Harvest—DT40 (chicken B cells) and Raji (human B cells) cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified 95% air, 5% CO2 atmosphere. The parent culture was maintained in continuous logarithmic growth between (5-10) x 106 cells/ml. For experiment use, cells were collected by centrifugation, washed once in NaCl/P, buffered (136.8 mM NaCl, 2.68 mM KCl, 8.04 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.4), and then suspended at a density of 1 x 107 cells/ml in Hanks' balanced salt solution (136.7 mM NaCl, 5.4 mM KCl, 0.81 mM MgSO4, 1.3 mM CaCl2, 0.33 mM Na2HPO4, 0.44 mM KH2PO4, 5.6 mM dextrose, 4.2 mM NaHCO3, pH 7.4). Osmotic stress was achieved by the addition of a concentrated sodium chloride solution. Cells were stimulated at 37°C under gentle agitation.

Preparation of GST Fusion Protein—pGEXSX-c-Jun (amino acids 1 to 79) encodes a GST-fusion protein containing the JNK binding domain and the serine residues (at positions 63 and 73), the phosphorylation of which correlates well with the increased transcriptional activity of c-Jun. Escherichia coli XL1Blue were transfected with this glutathione S-transferase fusion protein expression vector. Proteins were purified following the protocol recommended by the manufacturer (Pharmacia). The amounts of purified proteins were estimated by SDS-polyacrylamide gel electrophoresis and subsequent staining with Coomassie Blue.

Preparation of Cell Extracts—Stimulated cells (1 x 107 cells) were lysed in ice-cold lysis buffer (5 mM EDTA, 150 mM NaCl, 2% Triton X-100, 100 μM vanadate, 2 mM phenylmethylsulfonfluoride, 10 μM leupeptin, 50 mM Tris, pH 7.4). Lysates were clarified by centrifugation at 16,000 x g for 15 min at 4°C.

Immunoblot Analysis—Cell extracts were immunoprecipitated with 0.3 μg of anti-Syk antibody, 1 μg of anti-JNK1 antibody, or 3 μl of anti-Lyn antisera with 40 μl of protein A-Sepharose 4B (50% slurry) for 1 h at 4°C. Immunoprecipitates were washed three times with lysis buffer, once with 10 mM Hepes, pH 8.0, buffer containing 500 mM NaCl, and once with the same Hepes buffer without NaCl. The washed immunoprecipitates were boiled with SDS sample buffer for 3 min, resolved on a 10% SDS-polyacrylamide gel electrophoresis, transferred electrically to polyvinylidene difluoride membranes, and then immunoprobed with 4G10 to detect tyrosine phosphorylation. The corresponding antibody was used to detect the protein levels of Syk, JNK, or Lyn. Immunoreactive proteins were visualized using enhanced chemiluminescence.

Assays for JNK Activity—Cell extracts were immunoprecipitated with 1 μl of anti-JNK1 with 40 μl of protein A-Sepharose 4B (50% slurry) for 1 h at 4°C. Anti-JNK immunoprecipitates were washed three times with lysis buffer, once with washing buffer (50 mM Hepes and 10 mM MgCl2, pH 7.6), and once with kinase assay buffer (10 mM Hepes, 10 mM MgCl2, 10 μM cold ATP, and 10 μM vanadate, pH 7.6). Immune complex kinase assays were performed in 30 μl of kinase assay buffer containing 1 μCi of [γ-32P]ATP and 5 μl of GST-c-Jun as a substrate. After a 20-min incubation at 30°C, the reaction was terminated by the addition of SDS sample buffer followed by boiling for 5 min. The samples were separated by SDS-polyacrylamide gel electrophoresis. Autoradiography was carried out utilizing a phosphorymager (Fuji BAS 2000).

DNA Fragmentation Analysis—Cells (5 x 106/ml) were treated for the time stated with the indicated concentration of sodium chloride dissolved in RPMI 1640 media. UV irradiation was performed using a model 1800 Stratalinker UV cross-linker (Stratagene, La Jolla, CA) with 254-nm lamps. Cells (5 x 106/ml) in RPMI 1640 media were irradiated in an open tissue culture dish and then cultured for the indicated time. 5 x 106 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 proteinase 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 on a 2% agarose gel in the presence of 0.5 μg/ml ethidium bromide.

RESULTS

Activation of Syk by NaCl, but Not by UVC Irradiation, in DT40 Cells—To investigate the activation of Syk in response to low levels of hyperosmotic stress (0.2 M NaCl), anti-Syk immunoprecipitates from DT40 cell lysates, treated with or without sodium chloride, were subject to immunoblotting with an anti-phosphotyrosine antibody. As shown in Fig. 1A, top, exposure of DT40 cells to 0.2 M sodium chloride stimulated a rapid and sustained tyrosine phosphorylation of Syk. This increase in tyrosine phosphorylation reached a maximum at 1 min of exposure and remained elevated over a 15-min incubation time. The observed tyrosine phosphorylation of Syk was dependent on the concentration of sodium chloride used. Exposure of cells to 0.1 M sodium chloride for 5 min induced a significant increase in Syk tyrosine phosphorylation. Immunoblot analysis with an anti-Syk antibody revealed that the amounts of Syk immunoprecipitated from treated or untreated DT40 cells were comparable (Fig. 1A, bottom). Therefore, the elevated tyrosine phosphorylation was a specific response to sodium chloride treatment. Activation of Syk by osmotic stress was also observed in the human B cell line, Raji (Fig. 1B), indicating that osmotic stress-induced Syk activation was not unique to DT40 cells. In contrast, no detectable tyrosine phosphorylation of Syk was observed following UVC irradiation, up to 1,000 J/m2, under our experimental conditions (Fig. 1C, top); data not shown, although the amounts of Syk immunoprecipitated from untreated and treated DT40 cells were comparable (Fig. 1C, bottom). Thus, Syk was not activated by UVC irradiation in DT40 cells.

Inhibition of Osmotic Stress- but Not UVC-induced Apoptosis by Syk—To explore whether Syk plays a role in regulating osmotic stress-induced cell apoptosis, DT40 and DT40/Syk
cells were treated with 0.2 M sodium chloride. Cell apoptosis was assessed by DNA fragmentation, a typical biochemical marker of apoptosis, by running extracted DNA on a 2% ethidium bromide-containing agarose gel. As presented in Fig. 4A, exposure to sodium chloride induced apoptosis in DT40 cells, which was significant at a concentration of 0.2 M sodium chloride (A) or irradiated by 100 J/m² ultraviolet C (B) and collected by centrifugation at the indicated time points. Cellular DNA was extracted and analyzed on a 2% agarose gel, containing ethidium bromide, to detect DNA laddering. WT, wild type.

**Fig. 2.** **Syk inhibits osmotic stress-induced but not UVC-induced apoptosis in DT40 cells.** DT40, DT40/Syk−, and DT40/Syk+/Syk cells (5 × 10⁷/ml, 15 ml total) were exposed to 0.2 M sodium chloride (A) or irradiated by 100 J/m² ultraviolet C (B) and collected by centrifugation at the indicated time points. Cellular DNA was extracted and analyzed on a 2% agarose gel, containing ethidium bromide, to detect DNA laddering. WT, wild type.

**Fig. 1.** **Activation of Syk in B cells by osmotic stress but not by ultraviolet C.** A and B, osmotic stress activates Syk in both DT40 cells and Raji cells. Anti-phosphotyrosine immunoblot (top) and anti-Syk immunoblot (bottom) of anti-Syk immunoprecipitates from chicken B cells (DT40) after sodium chloride stimulation (A) or from human B cells (Raji) following 0.4 M respective hyperosmolarity-producing agent treatment (B). C, ultraviolet C irradiation does not activate Syk. Anti-phosphotyrosine immunoblot (top) and anti-Syk immunoblot (bottom) of anti-Syk immunoprecipitates from DT40 cells after ultraviolet C irradiation. IP, immunoprecipitation; IB, immunoblot. The positions of molecular mass markers are shown on the left (kDa). An arrow represents the position of Syk.

To support this observation that Syk may have a role in regulating apoptotic response triggered by osmotic stress, we made use of genetic approaches in which wild-type porcine syk cDNA was transfected into DT40/Syk− cells, and the selected clone was designated as DT40/Syk−/Syk cells. It is highly conceivable that DT40/Syk−/Syk cells would be resistant to osmotic stress if Syk, in fact, negatively regulates osmotic stress-induced apoptosis. As shown in Fig. 2A, expression of wild-type porcine Syk into DT40/Syk− cells protected Syk-deficient cells from osmotic stress-induced cell death, demonstrating that Syk may be an inhibitor of osmotic stress-induced apoptosis in DT40 cells. Interestingly, UVC irradiation, which was unable to activate Syk in DT40 cells (Fig. 1B), triggered rapid DNA fragmentation independently of Syk (Fig. 2B).

**Requirement for the Kinase Activity of Syk in the Protection of Cells from Osmotic Stress-induced Apoptosis**—To evaluate an important functional role of the kinase activity of Syk in mediating the enhanced DNA fragmentation induced by osmotic stress in DT40/Syk−/Syk cells, we transfected kinase-inactive porcine syk cDNA into DT40/Syk− cells. The lack of Syk kinase activity was demonstrated by an in vitro kinase assay that showed there was no detectable autophosphorylation, which is seen under normal conditions (Fig. 3A, top). Expression levels of wild-type and kinase-inactive porcine Syk were comparable, as revealed by immunoblotting (Fig. 3A, bottom). Expression of kinase-inactive porcine Syk in DT40/Syk− cells largely failed to block cell death (Fig. 3B, right) while expression of wild-type porcine Syk in DT40/Syk− cells was sufficient to elicit an anti-apoptotic response to osmotic stress (Fig. 3B, middle). This finding indicated that the kinase activity of Syk is required for the anti-apoptotic effect observed.

**Lyn, though Not Involved in Osmotic Stress-induced Apoptosis, Mediates UVC-induced Apoptosis**—Lyn is another major nonreceptor-type PTK predominantly expressed in B-lineage cells (28, 29). Lyn is physically and functionally associated with CD19 (29), and inhibition of Lyn activity by an anti-CD19-genistein immunoconjugate triggers rapid apoptotic cell death in Ramos Burkitt lymphoma cells, suggesting that Lyn in association with CD19 is an important regulator of apoptosis (30). To investigate the specificity or the functional redundancy of PTKs in mediating osmotic stress-induced apoptotic process in DT40 cells, the roles of Lyn in cell apoptosis were examined using established Lyn-deficient (DT40/Lyn−) cells. After a 12-h exposure to the indicated concentration of sodium chloride, the extracted DNA from untreated and treated cells was separated on 2% agarose gels. As presented in Fig. 4A, exposure to sodium chloride induced apoptosis in DT40 cells, which was significant at a concentration of 0.2 M sodium chloride. A deficiency of Syk produced a drastically enhanced, dose-dependent apoptotic response based on DNA fragmentation (Fig. 4A). In contrast, a deficiency of Lyn did not have any effect on osmotic stress-induced apoptosis when compared with that observed in wild-type cells. The extent of DNA fragmentation was comparable in DT40 and DT40/Lyn− cells. Therefore, in DT40 cells, Syk, but
not Lyn, appears to be a specific negative regulator of apoptosis in response to osmotic stress. To examine the role of Lyn in UVC-induced apoptosis, cells were irradiated by the indicated doses of UVC. Cells were harvested 12 h after irradiation, and apoptosis was analyzed by DNA laddering. Intriguingly, a deficiency of Lyn rendered cells resistant to UVC-induced apoptosis. The resistance of Lyn-deficient cells to UVC-induced apoptosis was observed from 100 up to 1,000 J/m² (Fig. 4).

Thus, it has become apparent that Lyn has distinct roles in the mediation of osmotic stress- or UVC irradiation-induced apoptosis. To further elucidate the possible functional role of Lyn in cell death signaling, the extent and kinetics of Lyn activation by these two different stresses were examined. As revealed by anti-phosphotyrosine immunoblotting analysis (Fig. 4C), both osmotic stress and UVC irradiation were able to activate Lyn to different degrees. Activation of Lyn by osmotic stress and UVC irradiation was rapid and sustained within the time examined.

**Differential Responses of JNK to Osmotic Stress and UVC Irradiation**—Relaying stress signals to the JNK pathway remains poorly understood. However, Pyk2 and c-Abl have recently been shown to positively regulate the activation of JNKs in response to osmotic stress (16) and genotoxic stress (UVC irradiation or ara-C treatment) (17, 18). Further, JNK activation has been shown to correlate with apoptosis induced by certain forms of extracellular stress (19). These observations lead us to analyze whether Syk and/or Lyn regulate apoptosis in DT40 cells via the JNK pathway. As revealed by phospho-tyrosylation of an exogenous substrate, GST-c-Jun(1–79), exposure of DT40 cells to 0.2 M sodium chloride induced a 7–9-fold increase in JNK activity over the control (Fig. 5A, top). A deficiency in either Syk or Lyn had a marginal effect, if any, on JNK activation since JNK activity in DT40, DT40/Syk⁻, and DT40/Lyn⁻ cells under the experimental conditions employed although DT40/Lyn⁻ cells displayed an ∼2-fold higher basal activity compared with wild-type cells (Fig. 5B, and data not shown).

**DISCUSSION**

Cellular stress, including ionizing irradiation, hydrogen peroxide, sodium chloride, and low energy electromagnetic fields, activates several nonreceptor PTKs such as Btk, Syk, Lyn, and ZAP70 (7, 21, 22, 24, 25, 31, 32), which are predominantly expressed in lymphocytes. In addition, cellular stress usually damages cells, thereby resulting in elimination of injured cells by apoptosis (19, 20). The mechanisms by which extracellular stimuli trigger cell apoptosis are not well understood, yet PTKs have been indicated to play an important role in mediating cell apoptosis in response to extracellular stimuli. Constitutive expression of Bcr-Abl confers resistance to interleukin-3 withdrawal-induced apoptosis in leukemia progenitor cells while down-regulation of Bcr-Abl renders K562 cells susceptible to apoptosis (5, 6). Immature B cells undergo apoptosis when activated through the B cell receptor, and a Syk deficiency blocks this apoptotic response (33).

DT40 cells that lack the expression of either Syk or Lyn provide a powerful tool to study the exact role of the respective components in stress-induced cell death. In this study, we further examined the possible functional role of Lyn through the JNK signaling pathway using DT40 cells that lack the expression of either Syk or Lyn. As revealed by anti-phosphotyrosine immunoblotting analysis (Fig. 4A), Lyn was not involved in osmotic stress-induced apoptosis. cows milk induced a 7–9-fold increase in JNK activity over the control (Fig. 5A, top). A deficiency in either Syk or Lyn had a marginal effect, if any, on JNK activation since JNK activity in DT40, DT40/Syk⁻, and DT40/Lyn⁻ cells under the experimental conditions employed although DT40/Lyn⁻ cells displayed an ∼2-fold higher basal activity compared with wild-type cells (Fig. 5B, and data not shown).

**FIG. 3. Requirement of the kinase activity of Syk for protecting cells from osmotic stress-induced apoptosis.** A, tyrosine kinase activity and protein expression of porcine wild-type and kinase-inactive Syk in DT40/Syk⁻ cells. Lysates from DT40/Syk⁻, DT40/Syk⁻/Syk, and DT40/Syk⁻/Syk(K²) cells were immunoprecipitated with anti-Syk antibody and divided into two portions. One was used for the Syk immunocomplex kinase assay (top), and the other was used for immunoblot analysis using an anti-Syk antibody (bottom). B, DNA fragmentation assay. Extracted DNA was analyzed on a 2% agarose gel, containing ethidium bromide, to detect DNA laddering. IP, immunoprecipitation; IB, immunoblot.

**FIG. 4. Lyn, though not involved in osmotic stress-induced apoptosis, mediates UVC-induced apoptosis.** A, after cells were treated with 0.1 M or 0.2 M sodium chloride for 12 h, cellular DNA was extracted and analyzed on a 2% agarose gel, containing ethidium bromide, to detect DNA laddering. B, after irradiation by various doses of UVC, DT40, DT40/Syk⁻, and DT40/Lyn⁻ cells were further maintained in culture medium for 12 h. Extracted DNA was run on a 2% agarose gel, containing ethidium bromide, to detect DNA laddering. C, activation of Lyn by osmotic stress and UVC irradiation. Anti-phosphotyrosine immunoblot (top) and anti-Lyn immunoblot (bottom) of anti-Lyn immunoprecipitates from chicken B cells (DT40) after sodium chloride stimulation (upper panel) or ultraviolet C irradiation (lower panel).
PTKs in stress signaling. In this study, we have focused on the functions of Syk and Lyn (in particular Syk) in the osmotic stress- and UVC irradiation-triggered apoptotic response in DT40 cells. We have observed in wild-type DT40 cells that, after a 16-h exposure to sodium chloride (osmotic stress), there is a significant induction of cell apoptosis. However, a deficiency in Syk results in a drastic enhancement of cell apoptosis, indicating that the presence of Syk inhibits osmotic stress-induced apoptosis in DT40 cells. The negative regulatory role of Syk in the osmotic stress-induced apoptotic response is further emphasized by the fact that ectopic expression of the wild-type porcine syk gene into DT40/Syk− cells leads to an apoptotic response very similar to that observed in wild-type DT40 cells. Enhanced apoptosis in DT40/Syk− cells is also observed, but to a much lesser extent, when cells are subject to oxidative stress using 1 mM hydrogen peroxide (data not shown), which is a stronger activator of Syk (21, 23–25). In addition, experiments utilizing kinase-inactive Syk mutant highlight that the kinase activity of Syk is required to render Syk-deficient cells resistant to osmotic stress since expression of the kinase-inactive form of Syk largely fails to protect Syk-deficient cells from osmotic stress-induced apoptosis. Consistent with the requirement for kinase activity, ultraviolet C irradiation, which fails to activate Syk in DT40 cells (Fig. 1B), induces a very similar DNA fragmentation pattern in both wild type and Syk-deficient cells (Fig. 2B). Lyn is expressed predominantly in B-lineage cells (28, 29), and Lyn in association with CD19 is an important mediator of apoptosis in Ramos Burkitt lymphoma cells (30). These findings led us to examine whether Lyn was involved in mediating the apoptotic response induced by osmotic stress. Unlike Syk-deficient cells, a deficiency of Lyn does not alter the sensitivity of cells to osmotic stress, when compared with wild type cells, although Lyn is activated when cells are exposed to osmotic stress. (Fig. 4, A and C). These results clearly indicate that Syk, but not Lyn, is an inhibitor of osmotic stress-induced apoptosis in DT40 cells. On the other hand, the functional roles of Syk and Lyn in UVC irradiation-induced apoptosis are quite different from those in osmotic stress-induced apoptosis. Upon UVC irradiation, Lyn acts as a positive mediator of apoptosis. In fact, abolishment of Lyn, but not Syk, blocks UVC irradiation-induced apoptosis (Fig. 4B). In B cells, Btk, Syk, and Lyn are abundantly expressed (28, 34, 35). All of them are activated following B-cell receptor engagement (28, 34, 35) and γ-ray irradiation (7). An interesting issue to be addressed is the functional roles of each PTK activated by a specific agonist. In the case of the apoptotic response, induction of apoptosis by B-cell receptor engagement is mediated by both Syk and Btk but not Lyn (7, 33). Btk, but not Syk and Lyn, is involved in γ irradiation-triggered apoptosis (7). Although Btk participates in both B-cell receptor engagement- and γ irradiation-induced apoptosis, phospholipase Cγ2, which is downstream of Btk, is only used in B-cell receptor signaling as a putative signal transducer to relay the death signal to the nucleus (7, 33). Both Syk and Btk positively mediate apoptosis induced by B-cell receptor engagement and γ irradiation (7, 33). Similarly, Lyn, but not Syk, functions as a positive mediator of UVC-induced apoptosis (Fig. 4B). In contrast, Syk, but not Lyn, functions as a negative mediator of osmotic stress-induced apoptosis (Figs. 2 and 4). In the same cell system, the apoptotic responses induced by these types of extracellular stress require the participation of different members of these three nonreceptor PTKs to relay the death signals to downstream effectors. Although the critical factors that determine the specificity of the PTK and the signaling pathways responsible for apoptosis are poorly defined at present, one can assume that both the docking sites provided by PTKs and the various sets of downstream signaling molecules utilized by them may be critical in determining the fate of the cell.

Recently, researchers have paid much attention to the roles of the JNK pathway in the apoptotic response induced by cellular stress (19, 20). Blocking the JNK pathway abolishes apoptosis induced by extracellular stress, including hydrogen peroxide, heat shock, UVC irradiation, and γ radiation (19). Furthermore, it has been shown that c-Abl and Pyk2 work upstream of JNK activation in response to certain extracellular stresses (16–18). We therefore analyzed the activation of JNK in wild type and Syk- and Lyn-deficient DT40 cells after exposure to osmotic stress or UVC irradiation. The results show that JNK activation in these cells was comparable in response to osmotic stress (Fig. 5A), excluding the possibility that the susceptibility of Syk-deficient cells to osmotic stress-induced apoptosis is due to the altered JNK activity. The signaling pathways involved in cell death are not well understood. There are several pathways that may work either independently or interactively to execute signal transmission. For example, nerve growth factor withdrawal activates JNK and induces apoptosis in PC12 cells. Following nerve growth factor withdrawal, some survival-promoting agents, such as Bcl2 and N-acetylcysteine, promote cell survival and block JNK activation simultaneously, whereas others significantly promote cell survival without affecting JNK activity (36). The idea that JNK activation and c-Jun are crucial mediators of apoptosis in response to tumor necrosis factor (19, 37) has been challenged by recent findings that demonstrate that cell apoptosis, elicited by tumor necrosis factor, is not linked to JNK activation (38). With respect to cell death signaling in response to various types of extracellular stress, many questions remain to be answered. What determines Btk as the mediator of γ radiation-induced apoptosis among activated nonreceptor PTKs? Why is it that Syk func-

Fig. 5. Differential responses of JNK to osmotic stress and UVC irradiation. A, cells were treated with 0.2 M sodium chloride for the indicated time points, and JNK was immunoprecipitated by anti-JNK1 antibodies. B, cells were harvested at the indicated time points after 100 J/m2 UVC irradiation. JNK activity was examined by the in vitro phosphorylation of GST-c-Jun (top). Protein levels of JNK immunoprecipitated from various cell lysates were estimated by anti-JNK immunoblotting analysis (bottom). GST-cJun, GST-c-Jun; IP, immunoprecipitation; IB, immunoblot.
Roles of Syk and Lyn in Stress Signaling

Preparations as an inhibitor of apoptosis in response to osmotic stress but Lyn positively mediates UVC irradiation-induced apoptosis? To fully address these issues further investigation is warranted.

In summary, our present studies demonstrate that a deficiency in Syk selectively confers a strong susceptibility to osmotic stress from the combined results of three different experimental approaches. First, DNA laddering, a hallmark of lymphocyte apoptosis, is enhanced in DT40/Syk−/− cells under the same condition. Second, overexpression of porcine Syk in DT40/Syk−/− cells renders cells more resistant to apoptosis. In contrast, a deficiency of Lyn, but not Syk, blocks the apoptotic response induced by UVC irradiation. These results indicate that, in DT40 lymphoma cells, Syk is a negative mediator of osmotic stress-induced apoptosis while Lyn is a positive mediator of UVC irradiation-induced apoptosis.

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