A deficiency in Syk enhances ceramide-induced apoptosis in DT40 lymphoma B cells

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Abstract Syk deficiency significantly enhanced ceramide-induced apoptosis. Ectopic expression of wild-type or kinase-inactive Syk rendered Syk-negative cells resistant to ceramide-induced apoptosis. Furthermore, ceramide could not activate Syk, indicating that Syk protected DT40 cells from ceramide-induced apoptosis, via a mechanism independent of its activity. In addition, a deficiency in Lyn also resulted in the cells becoming susceptible to ceramide-induced apoptosis. However, no difference of Ara-C-induced apoptosis between wild-type and mutant cells was observed. c-Jun N-terminal kinases appeared not to be important in mediating the enhanced apoptosis, as they were still activated in mutant cells following ceramide treatment.

Key words: Apoptosis; Ceramide; c-Jun N-terminal kinase; Lyn; Syk

1. Introduction

Apoptosis is a unique morphological pattern of cell death characterized by chromatin condensation and membrane blebbing. The most prominent event, also a biochemical marker in the early stages, of apoptosis is the internucleosomal DNA cleavage by undefined endonuclease activities. It is generally believed that apoptosis plays important roles in developmental processes, maintenance of homeostasis, and elimination of cells that have suffered serious damage [1,2]. The mechanisms by which cells undergo apoptosis in response to various changes of environments are gradually becoming understood with a growing body of evidence demonstrating that protein kinases have important roles to play in cell apoptosis. Several non-receptor protein tyrosine kinases (PTKs), including Abl [3], Jak2 [4], Btk [5], Lyn [6] and Syk [7], have been shown to be either positive or negative regulators of apoptosis in response to cellular stresses. Furthermore, Syk is also involved in the regulation of receptor engagement-induced apoptosis in immature B cells [8] and of growth factor withdrawal-triggered apoptosis in eosinophils [9]. In addition to PTKs, c-Jun N-terminal kinases (JNKs) are strongly activated by stimuli other than growth factors, including signals as diverse as UV irradiation [10], osmotic shock [11,12], protein synthesis inhibitors [13], and tumor necrosis factor α stimulation [14]. Various cellular stresses as the best activators of JNK indicate that JNK activation may be critical for regulating cell survival. In fact, JNK activation is required for cell apoptotic responses triggered by growth factor withdrawal [15] or environmental stresses [16]. JNK activation, however, seems also likely to be involved in promoting cell survival. Blocking JNK activation correlates well with the inhibition of IgM-induced apoptosis in immature B cells and of CD95- or CD3-induced apoptosis in thymocytes [17,18]. Moreover, recent studies have revealed that PTKs including Abl [19,20], Pyk2 [21] and Syk [22] are upstream mediators of JNK activation in response to certain types of cellular stresses, suggesting a possibility that PTKs and JNKs may work in concert in the regulation of some cell responses in certain cell systems.

Ceramide, a product of sphingomyelin hydrolysis, has been implicated as a second messenger in apoptotic response triggered by various cellular stresses, such as heat shock, ultraviolet light, oxidative stress, ionizing radiation, tumor necrosis factor α, and Fas ligand [16,22–26]. Substantial lines of evidence are derived from studies showing that rapid ceramide generation by stresses correlates well with the subsequent apoptotic response [16,27]. Recently, a direct demonstration of ceramide as a primary mediator of the apoptotic response has been observed by investigating radiation-induced apoptosis in lymphoblasts from Niemann-Pick patients lacking expression of acid sphingomyelinase and from acid sphingomyelinase knockout mice [28]. A few effectors for ceramide have been identified, including JNKs [16], ceramide-activated protein kinase [29] and c-Raf [30]; however, the mechanisms by which ceramide regulates apoptotic response are elusive.

Considering the results showing that Syk plays critical roles in the regulation of apoptosis [7–9], we investigated the involvement of Syk in ceramide-induced apoptosis utilizing Syk-negative mutants. We report here that Syk protected DT40 cells from ceramide-induced apoptosis, via a mechanism independent of its activity and JNK activation.

2. Materials and methods

2.1. Materials and chemicals

Generation of Syk^-, Lyn^-, Syk^-/Syk cells was done as described previously [31]. RPMI 1640 was purchased from ICN Biomedicals. Fetal bovine serum was from Gibco. Protein A was from Calbiochem. Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology. Mouse anti-human JNK1 monoclonal antibody was purchased from Pharmingen (San Diego, CA, USA). Enhanced chemiluminescence (ECL) reagents were from Amersham. Glutathione Sepharose 4B was from Pharmacia. C_2-ceramide was from Sigma. GST expression vector containing the N-terminal fragment (amino acids 1–79) of c-Jun was a gift from Dr. Hibi (Osaka University, Japan).

2.2. Cell culture and harvesting

DT40 cells were maintained in RPMI 1640 medium, supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 95% air/5% CO_2 atmosphere. The parent culture was maintained in continuous logarithmic growth at $(5–10)\times10^5$ cells/ml. For experiment use, cells were collected by centrifugation, washed once with NaCl/P_i buffer (136.8 mM NaCl, 2.68 mM KCl, 8.04 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) and then

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suspended (1×10^7 cells/ml) in Hanks' balanced salt solution (HBSS: 136.7 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM dextrose, 4.2 mM $NaHCO_3$, pH 7.4). Cells were stimulated at 37°C with gentle stirring.

2.3. Preparation of GST fusion protein and of cell extracts

Purification of GST-c-Jun fusion protein and cell lysate preparation were carried out as described previously [22].

2.4. Immunoblot analysis and JNK assay

Western blot and kinase assay for JNK were performed following the described procedures [22].

2.5. DNA fragmentation analysis

Cells (5×10^5/ml) were treated with ceramide in RPMI 1640 medium for the indicated times. 5×10^6 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. The DNA fragmentation was analyzed on a 2% agarose gel in the presence of 0.5 μg/ml ethidium bromide.

3. Results

3.1. Inhibition of ceramide-induced apoptosis by Syk

The capacity of ceramide to induce apoptosis in DT40 cells was first examined, as assessed by DNA fragmentation, a typical biochemical marker of apoptosis. After exposure of DT40 cells to various doses of ceramide for 16 h, DNA was extracted and run in a 2% agarose gel containing ethidium bromide. Ceramide less than 10 μM was unable to elicit an apoptotic response (Fig. 1). When the ceramide concentration was over 25 μM, in particular at a dose of 50 μM, cells subjected to ceramide treatment underwent apparent apoptosis. In following experiments, 50 μM ceramide was therefore used for the studies of apoptosis. To explore whether Syk played a role in regulating ceramide-induced apoptosis, wild-type and Syk-negative DT40 cells were treated with ceramide. As shown in Fig. 1, the nucleosomal DNA ladders appeared 16 h after ceramide treatment in wild-type cells. In Syk-negative cells, however, apparent DNA fragmentation occurred at 8 h, and the magnitude of fragmented DNA was continuously increased, indicating the progression of massive fragmentation of chromosomal DNA. To demonstrate more definitely a role of Syk in ceramide-induced apoptosis in DT40 cells, we introduced a porcine *syk* cDNA into Syk-negative cells. Expression of porcine Syk in Syk-negative cells rendered cells resistant to ceramide-induced apoptosis, providing clear evidence that Syk appeared to inhibit ceramide-induced apoptosis.

3.2. Syk activity was not critical for the protection of cells from ceramide-induced apoptosis

To evaluate the importance of Syk activity in ceramide-induced apoptosis, we transfected kinase-inactive porcine *syk* cDNA into Syk-negative cells. The lack of Syk activity was demonstrated by an in vitro kinase assay, which showed there was no detectable autophosphorylation, which is seen under normal conditions (Fig. 2A, top). Expression levels of wild-type and kinase-inactive porcine Syk were comparable, as revealed by immunoblotting (Fig. 2A, bottom). As in the case of Syk-negative cells expressing wild-type Syk, ectopic expression of kinase-inactive Syk in Syk-negative cells converted the cells resistant to ceramide (Fig. 2B). Consistent with this was the observation that ceramide could not activate Syk in DT40 cells under our experimental condition (Fig. 2C).

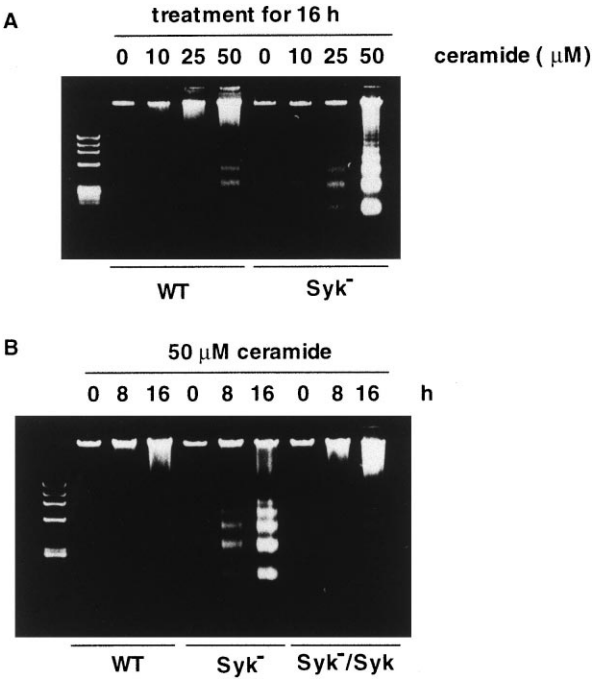

Fig. 1. Inhibition of ceramide-induced apoptosis by Syk in DT40 cells. A: Dose dependence. Wild-type and Syk$^-$ DT40 cells were treated with the indicated concentrations of ceramide for 16 h. Extracted DNA was run in 2% agarose gel to detect DNA laddering. B: Inhibition of ceramide-induced apoptosis by Syk. Wild-type, Syk$^-$ and Syk$^-$/Syk cells were exposed to 50 μM ceramide for the indicated time periods. Extracted DNA was run in 2% agarose gel to detect DNA laddering.

Together these findings indicate that Syk activity was not required for its anti-apoptotic effect observed.

3.3. Lyn was also involved in ceramide-induced apoptosis

Lyn, another predominantly expressed src-family PTK in B-lineage cells [6,31], has been indicated to be an important regulator of apoptosis [6,9] and of ligand-dependent Syk activation in hematopoietic cells [9,32]. To investigate the specificity or the functional redundancy of PTKs in mediating ceramide-induced apoptosis in DT40 cells, the roles of Lyn in cell apoptosis were examined, utilizing Lyn-negative cells established by having this gene knocked out [31]. Under the same experimental conditions as applied to Syk-negative cells, a deficiency in Lyn also rendered the cells susceptible to ceramide-induced apoptosis, when compared with wild-type cells (Fig. 3A), indicating that like Syk, Lyn has a role in the protection of cells from ceramide-induced apoptosis. To make sure that the observation was not due to clone alteration, we examined the effect of Syk and Lyn on apoptosis induced by the anti-cancer drug Ara-C in a clinical concentration. Treatment of DT40 cells with 10 μM Ara-C induced apparent cell apoptosis as revealed by DNA laddering (Fig. 3B). A deficiency in either Syk or Lyn did not alter Ara-C-induced apoptosis (Fig. 3B). Therefore, Syk and Lyn appeared to be specific inhibitors of ceramide-induced apoptosis in DT40 cells.

3.4. Differential regulation of ceramide-induced JNK activation by Syk and Lyn

To address the responses of JNKs to ceramide in DT40

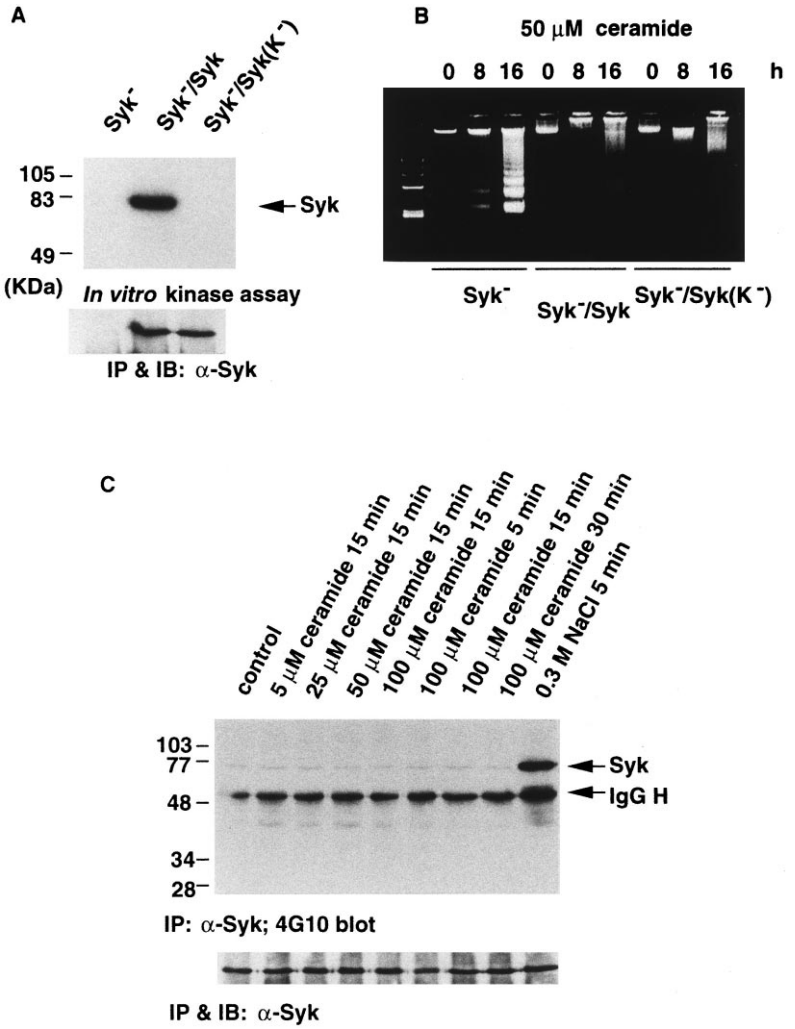

Fig. 2. Syk activity was not required for protecting cells from ceramide-induced apoptosis. A: Kinase activity and protein expression of porcine wild-type and kinase-inactive Syk in Syk$^-$ cells. Lysates from Syk$^-$, Syk$^-$/Syk, and Syk$^-$/Syk(K$^-$) cells were immunoprecipitated with anti-Syk antibody and divided into two portions. One was used for the Syk immunocomplex kinase assay (top), the other 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. C: Ceramide did not activate Syk. Anti-phosphotyrosine immunoblot (top) and anti-Syk immunoblot (bottom) of anti-Syk immunoprecipitates from DT40 cells after ceramide treatment. IP: immunoprecipitation; IB: immunoblot. The positions of molecular mass marker are shown on the left (kDa). An arrow represents the position of Syk.

cells, the N-terminal domain (amino acids 1–79) of c-Jun fused to GST was used as a probe to monitor JNK activity before and after stimulation. Ceramide treatment activated JNK in a time- and dose-dependent manner (Fig. 4A). Exposure of cells to 100 μM ceramide stimulated a 10-fold increase in JNK activity. JNK activity reached a peak at 15 min incubation and decreased thereafter.

Abl, Pyk2 and Syk have recently been shown to positively regulate the activation of JNKs in response to certain types of cellular stress [19–22]. Further, JNK activation has been demonstrated to correlate with apoptosis induced by cellular stress [16]. These observations led us to analyze whether Syk or Lyn regulated ceramide-induced apoptosis in DT40 cells via the JNK pathway. As shown in Fig. 4B, in Syk-negative cells, JNK was activated to a similar extent as observed in wild-type cells, though somewhat different in kinetics. In Lyn-negative cells, JNK was still responsive to ceramide treatment. However, the magnitude of JNK activation was two-fold reduced when compared to that observed in wild-type cells (Fig. 4C). Immunoblot analysis with an anti-JNK antibody showed that the same amount of JNK in each sample was immunoprecipitated (Fig. 4). A deficiency in either Syk or Lyn, therefore, did not abolish ceramide-induced JNK activation.

4. Discussion

An increase in intracellular ceramide both in vivo and in vitro results in cell apoptosis [16,27,28]. Several effectors of ceramide, such as JNK [16], ceramide-activated protein kinase [29] and c-Raf [30], have been identified, but how the death signals elicited by ceramide are relayed to the nuclei is poorly defined. In this study, taking advantage of tyrosine kinase-deficient mutants, we provide genetic evidence to show that a deficiency in Syk or Lyn speeds up cell apoptosis in response to ceramide treatment. The importance of Syk for ceramide-induced apoptosis is further highlighted by showing that overexpression of porcine Syk in Syk-negative cells makes cells resistant to ceramide treatment. These results suggest a critical

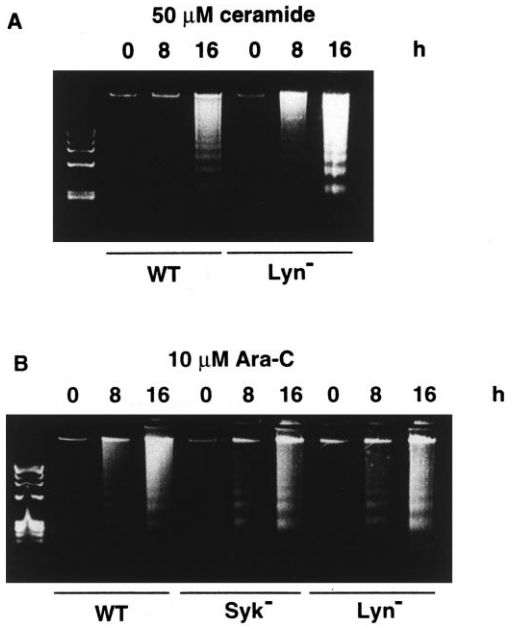

Fig. 3. Indispensability of Lyn for ceramide-induced apoptosis in DT40 cells. A: Enhanced cell apoptosis in Lyn$^-$ DT40 cells. Wild-type and Lyn$^-$ DT40 cells (5×10^5/ml) were exposed to 50 μM ceramide, and cells were collected at indicated time points. Cellular DNA was extracted and analyzed on 2% agarose gel to detect DNA laddering. B: Dispensability of Syk and Lyn for Ara-C-induced apoptosis. Wild-type, Syk$^-$ and Lyn$^-$ DT40 cells (5×10^5/ml) were exposed to 10 μM Ara-C, and cells were collected at indicated time points. Cellular DNA was extracted and analyzed on 2% agarose gel to detect DNA laddering.

role for Syk in the regulation of ceramide-induced apoptosis. We therefore asked whether ceramide treatment leads to Syk activation. Unexpectedly, exposure of cells to ceramide up to 100 μM for 30 min failed to trigger a detectable increase in the tyrosine phosphorylation of Syk, implying that Syk activity is not important for its anti-apoptotic effect against ceramide. This notion is supported by evidence showing that expression of kinase-inactive Syk in Syk-negative cells also renders cells resistant to ceramide treatment. These results are different from the observation showing that Syk activity is partly required for the protection of DT40 cells from osmotic stress-induced apoptosis [7]. Treatment of cells with various stress stimuli usually increases intracellular ceramide; however, the involvement of ceramide in stress-induced apoptosis is complex and still elusive. Ceramide production is not always required for stress-induced apoptosis [33–35] and is not always able to trigger apoptosis [36]. Together with the observation that osmotic stress, but not ceramide treatment, activates Syk, it is reasonable that Syk activity has a marginal effect on ceramide-induced apoptosis. While the mechanism by which Syk inhibits ceramide-induced apoptosis is not clear at present, one may assume that Syk may function as an adaptor to mediate the ceramide effect. In this respect, SH2 domains from a number of signaling molecules, including Src and Abl, have been demonstrated to prohibit apoptosis, depending on the specific interaction with endogenous tyrosine-phosphorylated ligands [37], while apoptosis was not affected by the treatment with tyrosine kinase inhibitors such as herbimycin A and genistein [37,38]. Evans et al. further identified the adaptor protein Crk as an important regulator of apoptosis through its SH2 and SH3 domains [39].

JNK is one of the ceramide effectors and activation of JNK pathway results in the inhibition of apoptosis induced by certain agonists [17,18,40]. The effects of Syk and Lyn on JNK activation by ceramide have been investigated. In DT40 cells, a deficiency in Lyn partly inhibits ceramide-induced JNK activation, whereas ceramide-triggered JNK activation remains intact in Syk-negative cells. Accordingly, JNK activation appears not to be important in mediating the enhanced cell apoptosis in Syk- or Lyn-negative cells following ceramide treatment, although we cannot completely deny the involvement of JNK in the enhancement of ceramide-induced apoptosis. One should be cautious in drawing conclusions regarding JNK function in cell apoptosis, as this issue is quite complex. JNK activation has also been reported to either lead to apoptosis [15,16,41] or have nothing to do with apop-

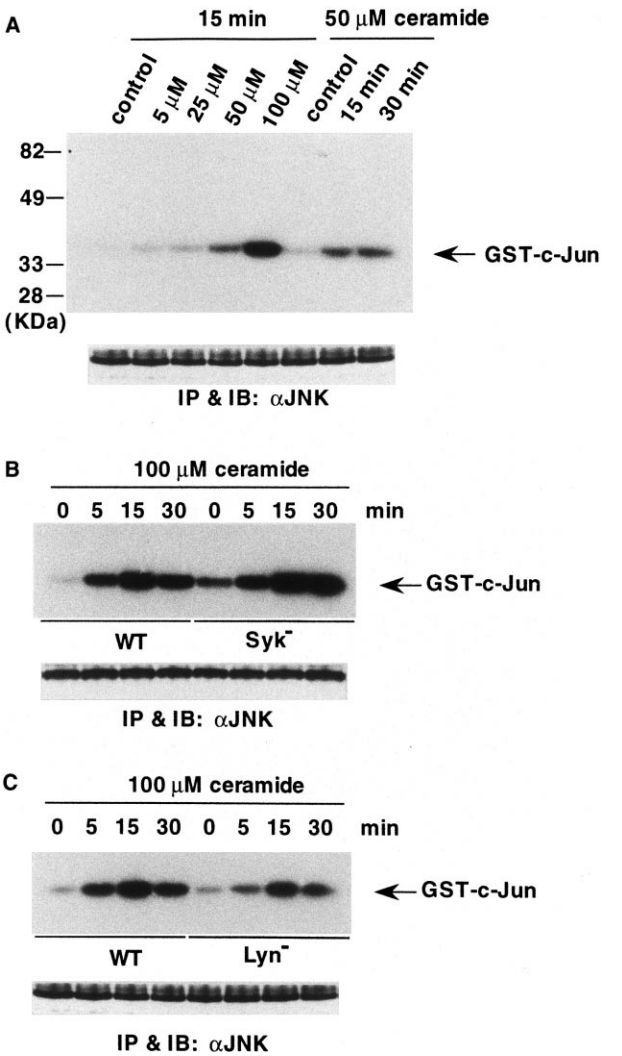

Fig. 4. Differential regulation of ceramide-induced JNK activation by Syk and Lyn. A: Activation of JNK by ceramide in a time- and dose-dependent manner. DT40 cells were stimulated with various doses of ceramide for 15 min or with 50 μM ceramide for the indicated time periods. B: Dispensability of Syk for JNK activation. Wild-type and Syk$^-$ DT40 cells were stimulated with 100 μM ceramide for the indicated time periods. C: Partial requirement of Lyn for ceramide-induced JNK activation. Wild-type and Lyn$^-$ DT40 cells were stimulated with 100 μM ceramide for the indicated-time periods. The JNK assay was performed as described in Section 2.

tosis [35] in response to various agonists. The real functions of JNK in ceramide-induced apoptosis cannot be addressed until JNK-null DT40 cells are available.

In conclusion, Syk is a specific inhibitor of ceramide-induced apoptosis in DT40 cells, as demonstrated by the combined results showing that a deficiency in Syk enhances ceramide-induced apoptosis, which is restored following ectopic expression of Syk in Syk-negative cells, and that a deficiency in Syk does not affect Ara-C-induced apoptosis.

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