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Distinctive Functions of Syk N-Terminal and C-Terminal SH2 Domains in the Signaling Cascade Elicited by Oxidative Stress in B Cells[1]

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Syk plays a crucial role in the transduction of oxidative stress signaling. In this paper, we investigated the roles of Src homology 2 (SH2) domains of Syk in oxidative stress signaling, using Syk-negative DT40 cells expressing the N- or C-terminal SH2 domain mutant [mSH2(N) or mSH2(C)] of Syk. Tyrosine phosphorylation of Syk in cells expressing mSH2(N) Syk after H_2O_2 treatment was higher than that in cells expressing wild-type Syk or mSH2(C) Syk. The tyrosine phosphorylation of wild-type Syk and mSH2(C) Syk, but not that of mSH2(N), was sensitive to PP2, a specific inhibitor of Src-family protein-tyrosine kinase. In oxidative stress, the C-terminal SH2 domain of Syk was demonstrated to be required for induction of tyrosine phosphorylation of cellular proteins, phospholipase C (PLC)-γ2 phosphorylation, inositol 1,4,5-triphosphate (IP_3) generation, Ca^{2+} release from intracellular stores, and c-Jun N-terminal kinase activation. In contrast, in mSH2(N) Syk-expressing cells, tyrosine phosphorylation of intracellular proteins including PLC-γ2 was markedly induced in oxidative stress. The enhanced phosphorylation of mSH2(N) Syk and PLC-γ2, however, did not link to Ca^{2+} mobilization from intracellular pools and IP_3 generation. Thus, the N- and C-terminal SH2 domains of Syk possess distinctive functions in oxidative stress signaling.

Key words: oxidative stress, protein-tyrosine kinase, SH2 domain, Syk.

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, H_2O_2 treatment, osmotic shock, and genotoxic agents have also been reported to activate a set of non-receptor PTKs, for example, those of the Src family or the Syk/ZAP-70 family (*2–6*). In this study, we were particularly interested in Syk, which plays a crucial role in B cell receptor (BCR) and Fc receptor–mediated signaling (*7–9*). Oxidative and osmotic stresses induce tyrosine phosphorylation of Syk in the same manner as that observed after antigen receptor activation (*10–13*). Oxidative and osmotic stresses also elicit an increase in tyrosine phosphorylation of intracellular proteins, rapid calcium mobilization, activation of c-Jun N-terminal kinase (JNK), and finally apoptosis in the chicken B cell line DT40. Interestingly, genetic studies using Syk-negative cells revealed that the increased tyrosine phosphorylation of cellular proteins, as well as the Ca^{2+} release from intracellular stores and the activation of JNK by oxidative stress, but not by osmotic stress, are partly dependent on Syk (*11–13*).

Syk bears two Src homology 2 (SH2) domains (*14*). In BCR signaling, both SH2 domains are absolutely required for recruiting Syk to the phosphorylated immuno-receptor tyrosine-based activation motif (ITAMs), and they are crucial for BCR-mediated Syk activation and its subsequent downstream signaling pathway (*15*). In osmotic and oxidative stress, however, unlike in BCR signaling, Syk having mutations in its N-terminal SH2 domain demonstrates a stronger tyrosine phosphorylation than wild-type Syk (*16*). However, the exact roles of the two SH2 domains of Syk in these stress-signaling pathways are still largely unknown.

Herein, we studied the contribution of the two SH2 domains of Syk to the tyrosine phosphorylation of intracellular proteins, intracellular Ca^{2+} mobilization, inositol 1,4,5-triphosphate (IP_3) generation, phospholipase C (PLC)-γ2 phosphorylation, and the activation of JNK. We observed that while both SH2 domains are necessary for the rapid release of Ca^{2+} from intracellular stores and IP_3 generation in oxidative stress, they possess some distinctive functions: the C-terminal SH2 domain is required for the tyrosine phosphorylation of PLC-γ2 and the activation of JNK, while the N-terminal SH2 domain appears to play a key role in the regulation of tyrosine phosphorylation of cellular proteins and Src-family PTK-dependent phosphorylation of Syk.

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Abbreviations: PTK, protein-tyrosine kinase, SH2, Src homology 2; IP_3, inositol 1,4,5-triphosphate, JNK, c-Jun amino-terminal kinase; BCR, B cell receptor; PLC, phospholipase C; ERK, extracellular-signal regulated kinase; ITAMs, immuno-receptor tyrosine based activation motifs.

MATERIALS AND METHODS

Materials—RPMI 1640 was purchased from ICN Biomedicals. Fetal bovine serum was from Gibco and Sigma. Protein A-Sepharose CL-4B was from Amersham Pharmacia Biotech AB. Hydrogen peroxide and Fura 2-AM were from Wako Pure Chemicals. Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology, Pharmingen (San Diego, CA). Anti–PLC-γ2 polyclonal antibody and anti-Syk polyclonal antibodies (C-20 and N-19) were purchased from Santa Cruz Biotechnology. Mouse anti–human JNK1 antibody was purchased from Pharmingen.

Cell Culture and Harvest—Establishment of DT40/Syk$^-$ and DT40/Syk$^-$ expressing porcine Syk or mutants [Syk/Syk$^-$, mSH2(N), and mSH2(C)] was performed as described previously (*16*). DT40 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml kanamycin in a humidified 95% air/5% CO_2 atmosphere. Cells collected by centrifugation at 400 ×*g* for 5 min were washed with PBS and resuspended (1×10^7 cells/ml) in Hank's balanced salt solution (HBSS). To deplete extracellular calcium, cells were resuspended in calcium-free HBSS containing 0.5 mM EGTA. Cells were stimulated with hydrogen peroxide at 37°C with gentle stirring.

Measurement of $[Ca^{2+}]_i$—Calcium mobilization was performed using Fura 2-AM. Cells were washed once and loaded with 5 μM Fura 2-AM in HBSS. After incubation for 30 min at 37°C, cells were washed and diluted to 2×10^6 cells/ml. The fluorescence of cell suspensions was continuously monitored with a Fluorescence Spectrophotometer (model F4500, Hitachi Limited, Tokyo) with standard monitor settings at 340 and 380 nm excitation wavelengths and 510 nm emission.

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

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

Assays for JNK Activity—pGEX3X-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). Immunoprecipitated proteins with anti-JNK1 antibody and protein A-Sepharose CL-4B (as described above) were washed five times: three times with lysis buffer, once with washing buffer (50 mM HEPES, pH 7.6, and 10 mM $MgCl_2$), and finally with kinase assay buffer (10 mM HEPES, pH 7.6, 10 mM $MgCl_2$, 10 μM ATP, and 10 μM vanadate). Immunocomplex kinase assays were performed in 30 μl of kinase assay buffer containing 1 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and 5 μg of glutathione S-transferase-c-Jun (GSTcJun) as a substrate. After a 20-min incubation at 30°C, reactions were terminated by the addition of electrophoresis sample buffer and 5 min of boiling. Autoradiography of SDS-PAGE was carried out and quantitated using a phosphoimager (Fuji BAS 2000).

RESULTS

Tyrosine Phosphorylation of Cellular Proteins in Cells Expressing mSH2(N) Syk Was Stronger Than That of Wild-Type Cells—We previously reported that in oxidative stress, unlike in BCR signaling, the N-terminal SH2 domain mutant [mSH2(N)] of Syk, in which the phosphotyrosine-dependent binding motif within the N-terminal SH2 domain of Syk contained a point mutation, showed a particularly stronger tyrosine phosphorylation than both wild-type and the C-terminal SH2 domain mutant [mSH2(C)] of Syk (*16* and Fig. 1A). Tyrosine phosphorylation of Syk in mSH2(N) Syk or mSH2(C) Syk-expressing cells as well as that in wild-type Syk-expressing cells reached a peak at 2–5 min of H_2O_2 treatment (data not shown). One of the earliest events following oxidative stress treatment is the induction of tyrosine phosphorylation of cellular proteins. To elucidate the contribution of both Syk SH2 domains to this early tyrosine phosphorylation pattern of intracellular proteins, whole cell lysates from wild-type Syk, mSH2(N), and mSH2(C) Syk–expressing cells stimulated by H_2O_2 were subjected to anti-phosphotyrosine immunoblotting as shown in Fig. 1B. As expected, DT40 cells expressing wild-type or SH2 mutants of Syk exhibited many substrates that became rapidly phosphorylated on tyrosine residues after oxidative stress. However, cells expressing mSH2(N) Syk showed a stronger tyrosine phosphorylation pattern of cellular proteins than wild-type Syk–expressing cells. Conversely, mSH2(C) Syk–expressing cells demonstrated a weaker tyrosine phosphorylation of cellular proteins than wild-type cells (Fig. 1B). These results suggested that the N-terminal SH2 domain of Syk had a potential negative regulatory role in tyrosine phosphorylation of cellular proteins following oxidative stress, while the C-terminal SH2 domain played an important role in this phosphorylation.

Oxidative Stress–Induced mSH2(N) Syk Tyrosine Phosphorylation Does Not Depend on Src-Family PTK Activity—Upon oxidative stress stimulation, tyrosine phosphorylation of Syk has been extensively described to be dependent on the presence of Src-family PTK (*11*). Indeed, Lyn-deficient DT40 cells subjected to oxidative stress exhibited a complete absence of tyrosine phosphorylation on Syk (*11*). These observations led us to investigate the potential role of Src-family PTK in the enhanced tyrosine phosphorylation of mSH2(N) Syk after oxidative stress. To address this question, we used a selective inhibitor of Src-family PTK, namely, PP2 (*17*). Wild-type Syk, mSH2(N), and mSH2(C) Syk-expressing DT40 cells were stimulated with H_2O_2 in the presence of various concentrations of PP2. Interestingly, while wild-type Syk and mSH2(C) Syk tyrosine phosphorylation were severely inhibited in the presence of PP2 in a dose-dependent manner, the tyrosine phosphorylation

of mSH2(N) Syk was scarcely affected by increasing concentrations of PP2 (Fig. 2). These results suggested that the enhanced tyrosine phosphorylation of mSH2(N) Syk is not due to a high activity of Src-family PTK to phosphorylate Syk. The previously reported observation that mSH2-(N) Syk autophosphorylation activity following oxidative stress was significantly increased (*16*) supported the conjecture that the enhanced tyrosine phosphorylation of mSH2-(N) Syk is due to an uncontrolled over autophosphorylation activity of mSH2(N) Syk. The N-terminal SH2 domain mutation might induce structural alteration of Syk and thereby the enhanced tyrosine phosphorylation of mSH2(N) Syk upon oxidative stress.

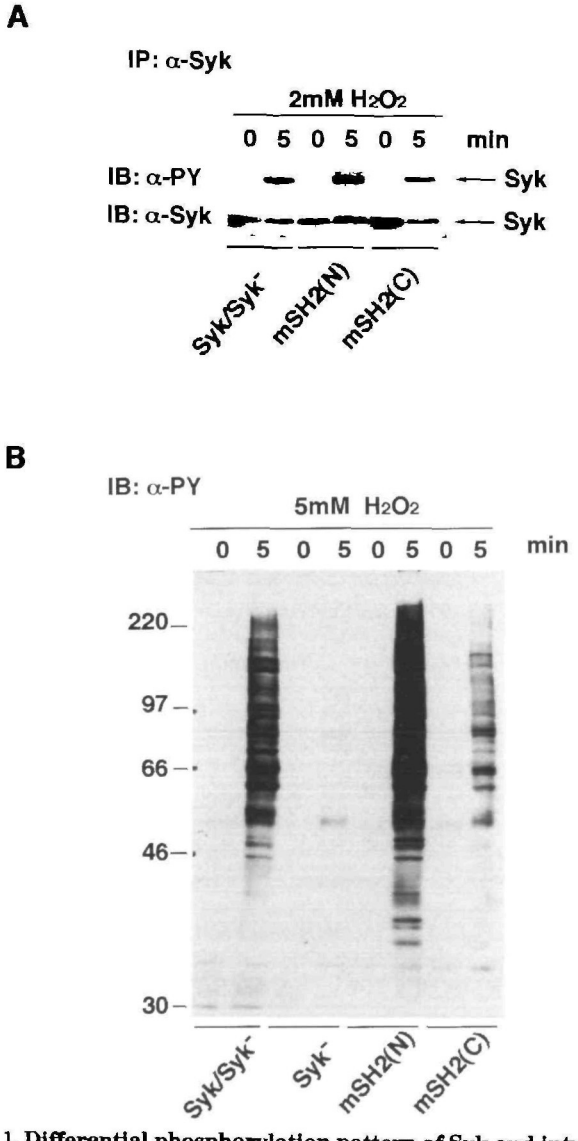

Fig 1. **Differential phosphorylation pattern of Syk and intracellular proteins following oxidative stress in DT40 cells expressing SH2 mutant of Syk.** DT40 cells deficient in Syk or transfected to constitutively express either wild-type Syk, mSH2(N) or mSH2(C) Syk were submitted to H_2O_2 treatment for the indicated period of time Syk immunoprecipitated from the total cell lysate using an anti-Syk antibody (A), or proteins from total cell lysate (B) were subjected to SDS-PAGE, then analyzed by immunoblotting using either an anti-phosphotyrosine antibody (4G10) (A, upper panel and B) or an anti-Syk antibody to visualize the equal loading of Syk protein in each lane (A, lower panel) Results are from one representative experiment out of four.

Intracellular Pool Ca^{2+} Mobilization Was Highly Dependent on Syk SH2 Domain Integrity—We previously showed that oxidative stress in DT40 cells induced an increase in cytoplasmic free calcium ($[Ca^{2+}]_i$), which was mediated by both a Ca^{2+} release from intracellular pools and an extracellular calcium influx (*11*). This previous study led us to conclude that only the Ca^{2+} release from intracellular pools was dependent on Syk activation following oxidative stress. Therefore, in the present work we investigated whether the Syk SH2 domains were involved in this oxidative stress-mediated Ca^{2+} mobilization.

As expected and described before (*11*), H_2O_2 treatment of wild-type cells induced a rapid increase of $[Ca^{2+}]_i$ followed by a slight decrease, whereas Syk-deficient cells or cells expressing either of the SH2 mutants of Syk showed a relatively slow but sustained increase in $[Ca^{2+}]_i$ following oxidative stress (Fig. 3A). However, it is striking to note that: (i) the kinetic of $[Ca^{2+}]_i$ increase in mSH2(N)-expressing cells is quicker than that in mSH2(C)-expressing cells or Syk-deficient cells (the $[Ca^{2+}]_i$ detected at each time point is 1.5–2 times higher than that of mSH2(C)-expressing cells or Syk-deficient cells); (ii) the $[Ca^{2+}]_i$ plateau reached at 5 min is higher in mSH2(N) Syk-expressing cells than any other types of tested cells including the wild-type DT40 cells. Identical results were obtained in five other experiments performed under the same conditions.

To determine the influence of Ca^{2+} release from intracellular pools during the above observations, another set of experiments was performed in the total absence of extracellular Ca^{2+}. Cells suspended in calcium-free HBSS/EGTA buffer were treated with H_2O_2 for 3 min, then $CaCl_2$ (final concentration 3 mM) was added to the cell suspension, and extracellular calcium influx was observed (Fig. 3B). As shown and already described (*11*), in the complete absence of extracellular Ca^{2+}, the rapid increase of $[Ca^{2+}]_i$ observed in wild-type DT40 cells after oxidative stress remained similar to that observed in the presence of extracellular Ca^{2+}. However, in Syk-deficient cells and the two SH2 mutants of

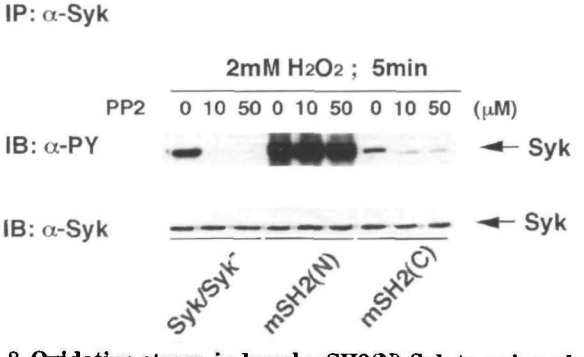

Fig. 2. **Oxidative stress–induced mSH2(N) Syk tyrosine phosphorylation does not depend on Src-family PTK activity.** DT40 cells expressing wild-type or mSH2 Syk were preincubated for 15 min in the presence of the indicated concentrations of PP2, then stimulated for 5 min with 5 mM H_2O_2 at 37°C Anti-Syk immunoprecipitates from cell lysates were immunoblotted with either an anti-phosphotyrosine antibody (upper panel) or an anti-Syk antibody (lower panel) Shown is one representative experiment out of three.

Syk-expressing cells, the measured $[Ca^{2+}]_i$ varied only slightly from the baseline in the complete absence of extracellular Ca^{2+}, while a Ca^{2+} influx from extracellular environment was detected after the addition of calcium to the cell suspension. These observations indicated that both SH2 domains of Syk were essential for the mobilization of Ca^{2+} from intracellular storage during oxidative stress.

Mutations of Syk SH2 Domains Abolished IP_3 Generation Following Oxidative Stress—Previous studies have suggested that some of the Ca^{2+} release from intracellular pools after oxidative stress is derived from IP_3-dependent release. In contrast to wild-type cells, IP_3 generation following oxidative stress was abolished in Syk-deficient cells, which was consistent with the loss of Ca^{2+} release from intracellular pools (*11*). To test the involvement of Syk SH2 domains in IP_3 generation, we assayed IP_3 production following oxidative stress in DT40 cells expressing mSH2 Syk. As expected, oxidative stress led to a rapid and high IP_3 generation in wild-type cells, while 85–90% of the IP_3 generation following oxidative stress was abolished in both mSH2(N) and mSH2(C) (Fig. 4A). However, the remaining IP_3 generation in the two SH2 mutants might account for the slight rise of $[Ca^{2+}]_i$, when H_2O_2 stimulation was performed in the complete absence of extracellular Ca^{2+} (Fig. 3B).

Since IP_3 generation is known to be totally dependent on PLC-γ2 activity in DT40 cells (*18*), we examined whether the inhibition of IP_3 generation in mSH2(N) and mSH2(C) Syk-expressing cells was directly related to a lack of PLC-γ2 tyrosine phosphorylation following H_2O_2 treatment. To this end, anti–PLC-γ2 immunoprecipitates were prepared from H_2O_2-treated cells and immunoblotted with anti-phosphotyrosine antibody. Figure 4B shows that the tyrosine phosphorylation of PLC-γ2 following oxidative stress occurred at a similar level in mSH2(N) Syk-expressing cells to that in wild-type Syk–expressing cells, while in Syk-deficient cells and mSH2(C) Syk-expressing cells the oxidative treatment did not lead to PLC-γ2 tyrosine phosphorylation. The time course of tyrosine phosphorylation of PLC-γ2 in mSH2(N) Syk–expressing cells following oxidative stress was similar to that in wild-type Syk–expressing cells (data not shown). Thus, the absence of PLC-γ2 tyrosine phosphorylation in mSH2(C) Syk–expressing cells may explain the lack of IP_3 generation in response to H_2O_2 treatment. This is not the case however, in mSH2(N) Syk–expressing cells, and thus we examined the subcellular localization of PLC-γ2 upon H_2O_2 stimulation in these cells. Tyrosine

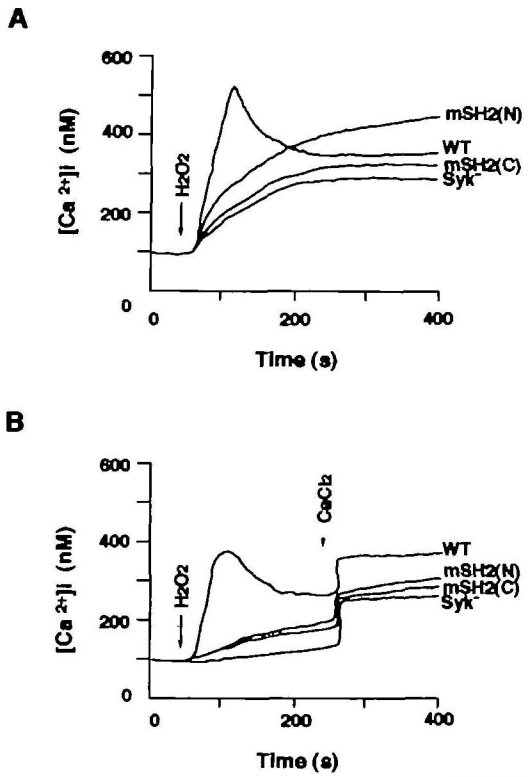

Fig. 3 **Calcium mobilization in wild-type or mSH2 Syk–expressing DT40 cells subjected to oxidative stress.** DT40 cells (2×10^6 cells/ml) loaded with Fura 2-AM and resuspended in either HBSS (A) or calcium-free HBSS/EGTA (B) were stimulated with 5 mM H_2O_2. The arrows and the arrowhead on the graphs indicate the start of the stimulation and the addition of calcium chloride (final concentration 3 mM) respectively. These figures show typical data of six independent experiments.

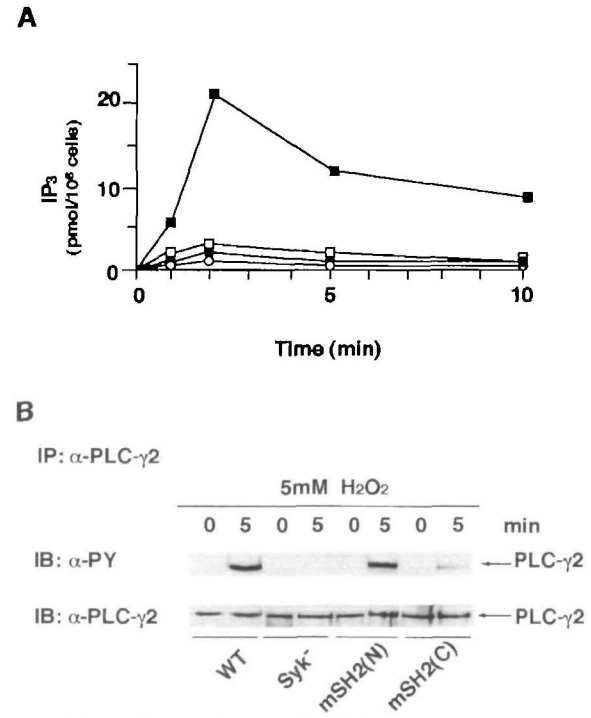

Fig 4 **Effect of mutation in the SH2 domain of Syk on IP_3 generation and tyrosine phosphorylation of PLC-γ2 following oxidative stress.** (A) IP_3 quantification in wild-type or mSH2 Syk–expressing DT40 cells. Cells (1×10^7 cells/ml) were stimulated with 5 mM H_2O_2 at 37°C for the indicated times. IP_3 generation in the different types of cells was then estimated as described in "MATERIALS AND METHODS." Shown is one representative graph out of four obtained under the same conditions. ■, wild-type Syk; □, mSH2(N)Syk; ●, mSH2(C)Syk; ○, Syk^- (B) Tyrosine phosphorylation pattern of PLC-γ2 in wild-type Syk, Syk^-, mSH2(N), or mSH2(C) Syk–expressing DT40 cells after oxidative stress. Cells (1×10^7 cells/ml) treated with H_2O_2 for the indicated times were lysed. Anti–PLC-γ2 immunoprecipitates were immunoblotted with either an anti-phosphotyrosine antibody (B, upper panel) or the anti–PLC-γ2 antibody (B, lower panel). An identical phosphorylation pattern was observed in four other experiments performed in the same way.

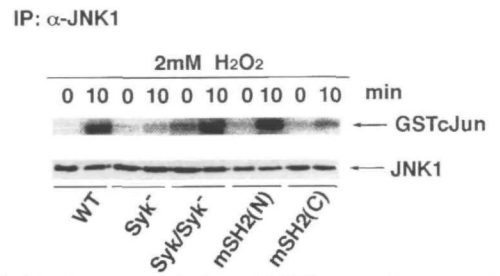

Fig 5 **Oxidative stress–induced JNK activation remained effective in N-terminal SH2 mutated Syk expressing cells.** Wild-type Syk, Syk⁻, mSH2(N), or mSH2(C) Syk–expressing DT40 cells (1×10^7 cells/ml) were stimulated for the indicated times in the presence of 2 mM H_2O_2. JNK protein was immunoprecipitated from the total cell lysates using an anti-JNK antibody, and JNK activity in the immunoprecipitate was determined using a specific *in vitro* kinase assay as described in "MATERIALS AND METHODS" (upper panel). The equal amounts of JNK used in the *in vitro* kinase assay were further controlled in an immunoblotting experiment using the anti-JNK antibody for detection (lower panel). Results shown are from one representative experiment out of four.

phosphorylated PLC-γ2 was translocated to the particulate fraction in H_2O_2-treated mSH2(N) Syk–expressing cells as well as in wild-type cells (data not shown). This suggested that the subcellular localization of PLC-γ2 did not explain the lack of IP_3 generation in H_2O_2-treated mSH2(N) Syk–expressing cells.

Mutations of Syk SH2 Domains Differentially Affect JNK Activation Following Oxidative Stress—We previously demonstrated that MAPKs such as JNK and ERK were activated during oxidative stress, and oxidative stress-induced JNK activation was partly dependent on Syk (*12*). Therefore we examined whether mutations of Syk in either of its SH2 domains affected JNK activation by oxidative stress. In mSH2(C) Syk–expressing cells, JNK activity was almost totally abolished following oxidative stress, but in mSH2(N) Syk–expressing cells, as in wild-type cells, it was increased by oxidative stress (Fig. 5) and the maximum activity appeared at 10 min (data not shown). These findings showed the distinctive roles of the Syk SH2 domains in oxidative stress–induced JNK activation: the C-terminal SH2 domain seemed to play an important role in JNK activation, whereas mutation of Syk SH2(N) domain had no effect on JNK activation by oxidative stress.

DISCUSSION

In this paper, we explored the roles of Syk SH2 domains in oxidative stress signaling. In BCR-mediated Syk activation and downstream signaling, both SH2 domains of Syk are crucial. They are necessary for Syk binding to the ITAMs, and this binding is necessary for Syk activation after BCR ligation (*8*). However, in oxidative stress stimulation, mSH2(N) Syk demonstrated a stronger tyrosine phosphorylation than wild-type Syk (Fig. 1A). This suggested that recruitment of Syk to ITAMs is not necessary for tyrosine phosphorylation of Syk by oxidative stress. These findings illustrated that the mechanisms of Syk tyrosine phosphorylation by oxidative stress were distinct from those induced by antigen receptor crosslinking. However, the mechanism of Syk activation following oxidative stress remains unknown.

In investigating the roles of Syk SH2 domains in oxidative signaling, we found that SH2(N) and SH2(C) have distinctive functions. First, in cells expressing mSH2(N) Syk, tyrosine phosphorylation of whole-cell proteins following Syk activation by oxidative stress was enhanced compared to that in wild-type cells, whereas tyrosine phosphorylation of intracellular proteins in mSH2(C) Syk–expressing cells was considerably inhibited (Fig. 1B). This suggested that the C-terminal SH2 domain of Syk was required for tyrosine phosphorylation of Syk downstream proteins, while the N-terminal SH2 domain of Syk had a potential negative regulatory role in tyrosine phosphorylation of cellular proteins following oxidative stress. Second, the enhanced phosphorylation of mSH2(N) Syk following H_2O_2 treatment was not inhibited by PP2, while the remaining phosphorylation of mSH2(C) Syk and the wild-type Syk phosphorylation were inhibited by PP2 under the same conditions (Fig. 2). This suggests that the N-terminal SH2 domain is mainly responsible for the interaction with and phosphorylation by Src-family PTK of Syk. Third, in mSH2(C) Syk–expressing cells, oxidative stress–induced tyrosine phosphorylation of PLC-γ2 and subsequent production of IP_3 were inhibited; but in mSH2(N) Syk–expressing cells, IP_3 generation following oxidative stress was inhibited, while the extent of tyrosine phosphorylation of PLC-γ2 was similar to that observed in wild-type cells. Fourth, in mSH2(N) Syk–expressing cells, oxidative stress induced JNK activation in the same way as in wild-type cells; but in mSH2(C) Syk-expressing cells, oxidative stress-induced JNK activation was inhibited. Thus, the C-terminal SH2 domain of Syk played an important role in PLC-γ2 tyrosine phosphorylation and JNK activation in oxidative stress signaling, whereas the N-terminal SH2 domain of Syk had a potential negative regulatory role in tyrosine phosphorylation of cellular proteins.

At present, we can offer no reasonable explanation of how the N-terminal SH2 domain negatively regulated Syk activation during oxidative stress signaling. One conceivable mechanism involves the SH2 domain–dependent recruitment of inhibitory molecules to Syk. Several groups have reported a family of inhibitor proteins functioning in growth factor and cytokine receptor signaling that terminate the activation of PTKs when they are recruited to the PTK complex (*19*, *20*). Alternatively, an intramolecular interaction between the SH2 domain and the phosphotyrosine residue within Syk may give rise to structural hindrance that limits Syk activation. Further studies are required to clarify these possibilities.

mSH2(N) Syk also mediated tyrosine phosphorylation of PLC-γ2 upon oxidative stress, while impairing IP_3 generation and Ca^{2+} release from intracellular stores. The reasons for these observations are unknown. Translocation of tyrosine-phosphorylated PLC-γ2 to the particulate fraction in H_2O_2-treated mSH2(N) Syk–expressing cells was the same as in wild-type cells. One reason may be a difference in the tyrosine residues of PLC-γ2 phosphorylated by wild-type and mSH2(N) Syk following oxidative stress. In fact, PDGF-induced IP_3 generation requires PLC-γ1 phosphorylation on tyrosine residues 783 and 1254, whereas phosphorylation of only tyrosine 771 could not induce IP_3 generation (*21*). Although we could not determine the phosphorylated site of PLC-γ2 in wild-type and mSH2(N) Syk–expressing cells exposed to oxidative stress, their phospho-

rylation sites might be different. Another reason is that Syk SH2 domains may participate in the inositol phospholipid hydrolysis by PLC-γ during oxidative stress. Thus, Syk may not only induce the proper phosphorylation of PLC-γ2 but also directly support the inositol phospholipid hydrolysis by PLC-γ2 through its SH2 domains.

In summary, we demonstrated distinctive functions of the two SH2 domains of Syk in oxidative stress signaling. The C-terminal SH2 domain of Syk is essential for each downstream element of the oxidative stress signaling pathway (*i.e.*, tyrosine phosphorylation of intracellular proteins, Ca^{2+} release from intracellular pools, and JNK activation). On the other hand, the N-terminal SH2 domain is absolutely required for IP_3 generation and subsequent Ca^{2+} release from intracellular pools, but appears also to play an important role in the negative regulation of tyrosine phosphorylation of intracellular protein and Src-family PTK-dependent phosphorylation of Syk.

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