Differential Regulation of Oxidative and Osmotic Stress Induced Syk Activation by both Autophosphorylation and SH2 Domains†

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ABSTRACT: Syk, a nonreceptor protein-tyrosine kinase, is activated by both oxidative and osmotic stress and plays different roles in the transduction of stress signals. In this study, the regulation of oxidative and osmotic stress induced Syk activation was investigated utilizing Syk-negative DT40 cells, expressing various Syk mutants. Phosphorylation of Y518Y519 was demonstrated to be required for both oxidative and osmotic stress induced Syk activation. Syk activation by these two types of stress stimuli was a combination of both autophosphorylation and the activities of additional tyrosine kinases. Oxidative stress induced Syk tyrosine phosphorylation was almost completely attributed to autophosphorylation, whereas other tyrosine kinases were largely responsible for osmotic stress induced Syk tyrosine phosphorylation. Moreover, the Src homology 2 (SH2) domains of Syk differentially regulated Syk activation. Both mSH2-(N) Syk and mSH2(C) Syk, in which the phosphotyrosine-dependent binding motif within the SH2 domains contained point mutations, showed a significantly higher activity than that observed in wild-type Syk, following osmotic stress treatment. In comparison, in response to oxidative stress, only mSH2(N) Syk demonstrated a stronger activation than wild-type Syk. Therefore, differential activation and regulation of Syk may give an insight into the distinctive functions of Syk in oxidative and osmotic stress signaling.

Protein-tyrosine kinases (PTKs)[1] play crucial roles in a wide variety of cellular responses, including cell activation, proliferation, and differentiation (*1*). In addition to growth factors and cytokines, which stimulate intrinsic PTK activities associated with either receptor or nonreceptor PTKs, which couple to receptors following binding to their cognate receptors (*2*), extracellular stress stimuli such as ionizing radiation, ultraviolet radiation, hydrogen peroxide, and genotoxic agents can also activate PTKs, including Src- and Syk-family PTKs (*3*−*7*). Here, we are particularly interested in Syk, a nonreceptor PTK which was previously cloned in our laboratory (*8*). Syk is strongly activated following B cell receptor engagement (*9, 10*), by hydrogen peroxide (*11*−*13*) and by osmotic stress (*13, 14*). Both oxidative and osmotic stress also elicit increased tyrosine phosphorylation of cellular proteins, activation of c-Jun N-terminal kinases (JNKs), calcium mobilization, and cell apoptosis in a chicken B cell line, DT40 (*13, 14*). Genetic studies utilizing Syk-negative cells revealed, with respect to the involvement of Syk, that oxidative and osmotic stress signaling clearly differed in the following ways. (1) Oxidative stress, but not osmotic stress, induces a Syk-dependent calcium release from intracellular calcium stores. Vice versa, an increase in $[Ca^{2+}]_i$ is required for maximal activation of Syk induced by oxidative stress, but not osmotic stress. (2) Oxidative, but not osmotic, stress induced activation of JNKs is dependent on Syk (*13*). (3) Syk appears to function as an inhibitor of osmotic stress induced apoptosis, but has little effect on oxidative stress induced apoptosis (*14*). Although the molecular bases that could explain the distinctive functions of Syk in oxidative and osmotic stress signaling are not available at present, it is conceivable that intramolecular and intermolecular interaction of Syk with other signal transducers may be one of the key factors in determining the specificity described. Very recently, studies of the three-dimensional structures of Src and Hck demonstrated that the interaction of the tyrosine-phosphorylated carboxy-terminal tail with the Src homology 2 (SH2) domain, located on the opposite site of the molecule from the kinase site, made the peptide sequence that links the SH2 and kinase domains able to adopt a PP-II helix. This PP-II helix structure enables the SH3 domain able to bind the SH2-kinase linker (*15, 16*). In addition, Itk, which lacks the corresponding carboxy-terminal negative regulatory tyrosine residue, also interacts intramolecularly using an alternative mechanism (*17*). Interactions among these domains position the molecule in such a conformation that simultaneously disrupts the kinase activity and sequestrates SH2 and SH3 domain binding abilities (*17, 18*). Replacement of the SH2 and SH3 domains

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[1] Abbreviations: PTK, protein-tyrosine kinase; SH2, Src homology 2; SH3, Src homology 3; JNKs, c-Jun N-terminal kinases.

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by high-affinity ligands, or disruption of intramolecular interaction by either point mutation or domain deletion, stimulates the kinase activity and restores the ability of kinase to recruit its substrates (*17, 18*).

Syk does not contain both a carboxy-terminal negative regulatory tyrosine residue and an SH3 domain (*8*). Extensive investigations have revealed that the intermolecular interaction of tyrosine-phosphorylated receptor subunits with the tandem SH2 domains of Syk is a major mechanism for Syk activation in B cell and Fc receptor signaling (*19*−*22*). Intramolecular interactions in Syk are as yet demonstrated; however, the importance of both SH2 domains and tyrosine phosphorylation within the activation loop of Syk is well documented in B cell and Fc receptor signaling. For example, binding of the SH2 domain to the subunits of B cell and Fc receptors and phosphorylation of Y518Y519 within the activation loop, are essential for Syk activation and antigen receptor functions (*23*−*25*). The patterns of oxidative stress induced tyrosine phosphorylation of cellular proteins in T- and B-lymphocytes are identical to those observed following antigen receptor engagement (*11, 26*). In addition, Syk regulates oxidative stress induced calcium release in the same way as Syk does in B cell receptor signaling, where Syk mediates the activation of phospholipase Cγ, resulting in the subsequent production of inositol 1,4,5-trisphosphate, thereby inducing an elevation in $[Ca^{2+}]_i$ (*12, 27, 28*). Accordingly, it is conceivable that Syk activation induced by antigens and stress stimuli may share some similarity. We therefore express wild-type and the various point mutations of Syk, K395R (ATP binding site), Y518FY519F (activation loop, designated autoP$^-$), R37GQ38GS39I [N-terminal SH2 domain, designated mSH2(N)], and R190GR192L [C-terminal SH2 domain, designated mSH2-(C)], in Syk-negative DT40 cells, respectively, to evaluate their roles in the regulation of oxidative and osmotic stress induced Syk activation.

Here, we demonstrate the requirement of functionally active Syk for oxidative, but not osmotic, stress stimulated tyrosine phosphorylation of cellular proteins. The functional differences of Syk in oxidative and osmotic stress signaling may be accounted for, at least in part, by differential regulation of Syk activation by autophosphorylation and the SH2 domains.

MATERIALS AND METHODS

Materials. Protein A was from Calbiochem Corp. Hydrogen peroxide and sodium chloride were from Wako Pure Chemicals. Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology Inc. Enhanced chemiluminescence reagents were from Dupont. Protein A−Sepharose 4B was from Pharmacia.

DNA Transfection, Cell Culture, and Harvest. Mutant Syk cDNAs (ATP binding site mutant, SH2 mutants, and autophosphorylation site mutant) were created using the polymerase chain reaction, and cloned into an *Eco*RI site of the pApuro vector, harboring the chicken actin promotor and puromycin-resistant gene (*23, 27*). These cDNAs were transfected into Syk-negative cells by electroporation using Gene pulser apparatus (Bio-Rad Laboratories) at 550 V, 25 μF and selected in the presence of 0.5 μg/mL puromycin. Expression of mutated Syk was assessed by immunoblot analysis. Clones expressing comparable levels of porcine Syk protein were chosen for experiments. The selected Syk-negative DT40 cells, expressing various mutated Syk, 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% CO_2 atmosphere. For experimental use, cells were collected by centrifugation as previously described (*12*). Cells were suspended in Hank's balanced salt solution buffer at the concentration of 10×10^6 cells/mL and then were stimulated by hydrogen peroxide (oxidative stress) or sodium chloride (osmotic stress) at 37 °C.

Preparation of Cell Extracts. Stimulated cells (1×10^7 cells/mL) were lysed in ice-cold lysis buffer (5 mM EDTA, 150 mM NaCl, 2% Triton X-100, 100 μM Na_3VO_4, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, 50 mM Tris-HCl, pH 7.4) following a short centrifugation step. Lysates were clarified by centrifugation at 16000*g* for 15 min at 4 °C.

Immunoblot Analysis. Cell extracts or immunoprecipitates were resolved on SDS−PAGE, transferred electrophoretically onto PVDF 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 as previously described (*8*). Autoradiography was carried out and quantitated using a phosphoImager.

RESULTS

Requirement of Syk for Oxidative Stress, but Not Osmotic Stress, Induced Tyrosine Phosphorylation of Cellular Proteins. In B cells, hydrogen peroxide (oxidative stress) or sodium chloride (osmotic stress) treatment induced rapid activation of Syk and tyrosine phosphorylation of cellular proteins (*11, 13*). Syk has been implicated to play distinctive roles in oxidative and osmotic stress signaling (*13, 14*). Here, we further evaluated the contribution of Syk to the overall tyrosine phosphorylation of cellular proteins elicited by these two stress stimuli. Lysates from both control and stimulated DT40 cells, were subjected to anti-phosphotyrosine immunoblotting analysis. As shown in Figure 1A, exposure of DT40 cells to both oxidative stress and osmotic stress stimulated the rapid tyrosine phosphorylation of cellular proteins. The phosphorylation profiles were distinctive, yet overlapping. In Syk-negative cells, oxidative stress induced tyrosine phosphorylation was drastically reduced, while osmotic stress induced tyrosine phosphorylation was marginally affected (Figure 1A). To confirm the importance of Syk for oxidative stress induced tyrosine phosphorylation, we introduced wild-type Syk into Syk-negative cells. Ectopic expression of the wild-type porcine Syk in Syk-negative cells fully restored the ability of oxidative stress to stimulate a similar, but somewhat stronger tyrosine phosphorylation of cellular proteins to that observed in wild-type cells (Figure 1B). However, expression of the K395R Syk failed to compensate the loss of oxidative stress induced tyrosine phosphorylation in Syk-negative cells, showing that functionally active Syk was critical for oxidative stress induced tyrosine phosphorylation.

Phosphorylation of Y518Y519 Is Critical for Oxidative and Osmotic Stress Induced Syk Activation. Phosphorylation of

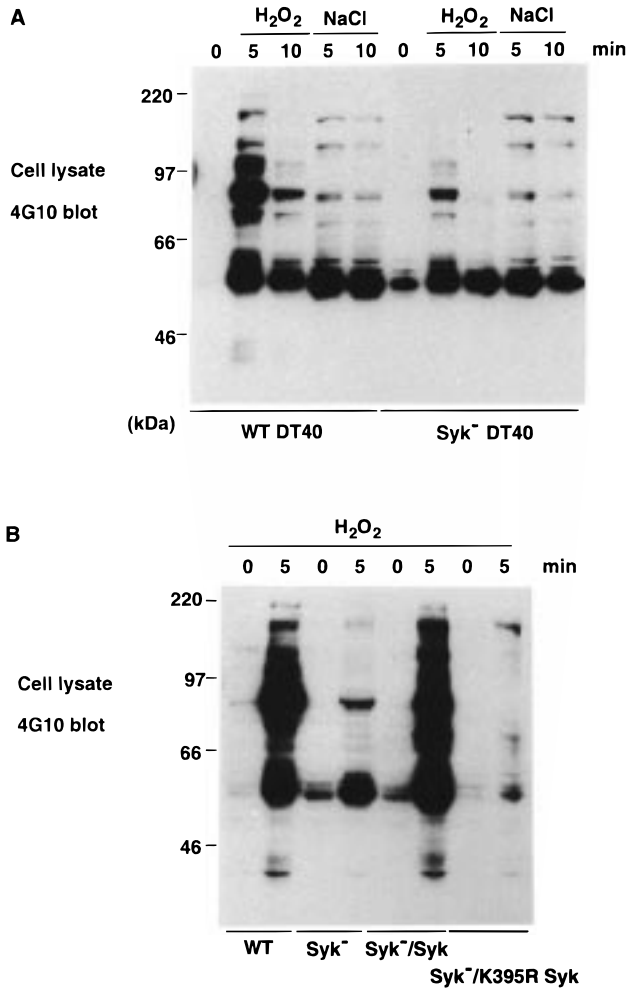

FIGURE 1: Requirement of functionally active Syk for oxidative stress stimulated tyrosine phosphorylation of cellular proteins in DT40 cells. (A) Anti-phosphotyrosine immunoblot of cell lysates from wild-type and Syk-negative DT40 cells following oxidative stress (2 mM hydrogen peroxide) and osmotic stress (an additional 0.3 M sodium chloride) treatment for the indicated time points. (B) Anti-phosphotyrosine immunoblot of cell lysates from wild-type (WT), Syk-negative, and Syk-negative DT40 cells expressing wild-type or kinase-inactive (K395R) porcine Syk following oxidative stress treatment. The positions of the molecular mass markers are shown on the left (kDa).

tyrosine residues within the activation loop of PTKs is a critical event for PTK activation and downstream signaling (*29*−*31*). Based on sequence homology, tyrosine 518 and/or tyrosine 519 of Syk are presumed to be the major phosphorylation sites within the so-called activation loop (*32*). To characterize the effect of phosphorylation of Y518Y519 on Syk activation following oxidative and osmotic stress treatment, we expressed Syk, containing tyrosine to phenylalanine substitutions at positions 518 and 519, in Syk-negative cells. Immunoblot analysis with antiphosphotyrosine antibody revealed that mutations of Y518Y519 to F518F519 (designated autop⁻) had little effect on the basal level tyrosine phosphorylation of Syk (Figure 2). Both oxidative and osmotic stress stimulated a significant increase in the tyrosine phosphorylation of wild-type Syk. However, tyrosine phosphorylation of autop⁻ Syk was drastically reduced compared with that of wild-type Syk (Figure 2). Consistent with this, cells expressing autop⁻ Syk showed a reduction in tyrosine phosphorylation of cellular

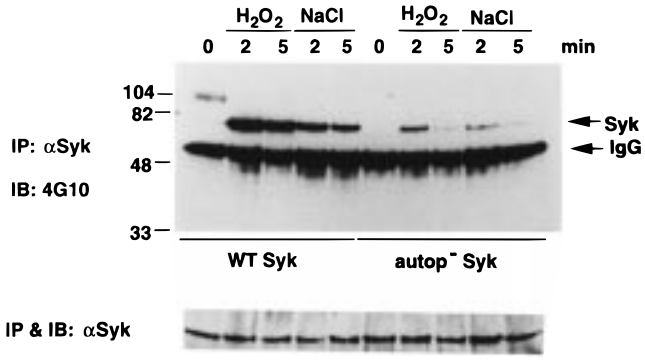

FIGURE 2: Phosphorylation of Y518Y519 is important for oxidative and osmotic stress induced Syk activation. Anti-phosphotyrosine immunoblot (top) and anti-Syk immunoblot (bottom) of anti-Syk immunoprecipitates from Syk-negative cells expressing wild-type or autop⁻ porcine Syk following oxidative stress (2 mM hydrogen peroxide) or osmotic stress (an additional 0.3 M sodium chloride) stimulation. IP, immunoprecipitation; IB, immunoblot. Arrows indicate the positions of Syk and IgG, respectively.

proteins (data not shown). These results demonstrated that tyrosines 518 and/or 519 also have an important impact on stress stimuli elicited Syk activation.

Differential Requirement of Autophosphorylation for Oxidative and Osmotic Stress Induced Syk Activation. Syk appears to have distinctive roles in oxidative and osmotic stress signaling (*13*, *14*, and Figure 1). A possible explanation is that the activation of Syk by these two stress stimuli is via different mechanisms. To test this hypothesis, we analyzed the effect of autophosphorylation on Syk activation by expressing the K395R mutant in Syk-negative cells, which bears a substitution of lysine by arginine in the ATP binding site, rendering Syk catalytically inactive. After exposure of Syk-negative cells expressing wild-type or K395R Syk to oxidative or osmotic stress, Syk was immunoprecipitated, separated on a SDS−polyacrylamide gel, transferred to a PVDF membrane, and immunoblotted with an anti-phosphotyrosine antibody, 4G10. Albeit the magnitude of phosphorylation was somewhat stronger in response to oxidative stress, both stress stimuli induced rapid tyrosine phosphorylation of wild-type Syk (Figure 3, upper panel). Intriguingly, in response to oxidative stress, there was a very low level of tyrosine phosphorylation of K395R Syk, whereas osmotic stress stimulated tyrosine phosphorylation of K395R Syk was largely unaffected compared to that of wild-type Syk. Densitometric analysis using NIH Image software (Version 1.60) revealed that tyrosine phosphorylation of K395R Syk was ∼10% and ∼65% of that of WT Syk following oxidative and osmotic stress treatment, respectively. Reprobing the same filter with an anti-Syk antibody revealed that the amount of Syk immunoprecipitated from all samples was comparable (Figure 3, bottom panel), indicating that oxidative stress stimulated tyrosine phosphorylation of Syk resulted mainly from autophosphorylation. In contrast, Syk was largely phosphorylated by other PTK(s) following osmotic stress treatment.

Differential Roles of the SH2(N) and SH2(C) Domains in Oxidative and Osmotic Stress Induced Syk Activation. It has been suggested that SH2 domains are crucial in regulating kinase activity and mediating their interaction with up- and downstream signal tranducers (*17*, *18*, *23*, *24*). Therefore, we investigated whether the Syk SH2 domains are involved

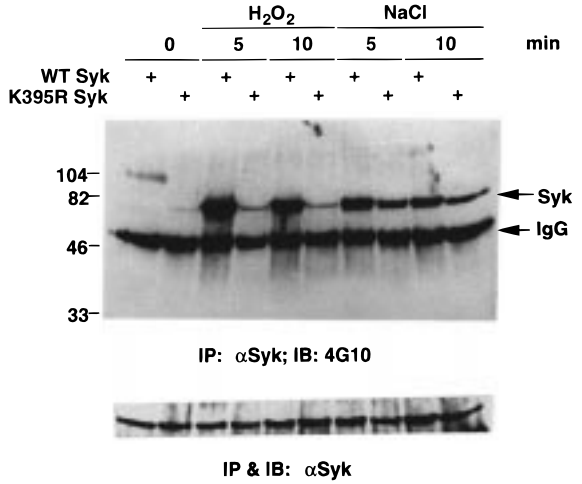

FIGURE 3: Differential contribution of autophosphorylation to oxidative and osmotic stress induced Syk activation. Anti-phosphotyrosine immunoblot (top) and anti-Syk immunoblot (bottom) of anti-Syk immunoprecipitates from Syk-negative cells expressing wild-type or K395R porcine Syk following oxidative stress (2 mM hydrogen peroxide) or osmotic stress (an additional 0.3 M sodium chloride) stimulation.

in Syk activation in response to oxidative and osmotic stress. We constructed the mSH2(N) and mSH2(C) Syk mutants, R37GQ38GS39I and R190GR192L, which are found within the phosphotyrosine-dependent binding motif within the N- and C-terminal SH2 domain of Syk, respectively. These mutations within the SH2 domains did not alter basal level of Syk tyrosine phosphorylation (Figure 4). However, the point mutation in the phosphotyrosine-dependent binding motif of the N-terminal SH2 domain led to enhanced tyrosine phosphorylation of mSH2(N) Syk compared to wild-type Syk after exposure of cells to both oxidative and osmotic stress (Figure 4A). Interestingly, compared to wild-type Syk, the point mutation within the phosphotyrosine-dependent binding motif in the C-terminal SH2 domain resulted in an increase in osmotic stress induced tyrosine phosphorylation of mSH2-(C) Syk, without significantly altering oxidative stress induced tyrosine phosphorylation of mSH2(C) Syk (Figure 4B). Importantly, the enhanced tyrosine phosphorylation of mSH2 Syk is unlikely to be a general phenotype, as the mutation of the phosphotyrosine-dependent binding motif in the SH2(N) domain reduced the anti-IgM-stimulated tyrosine phosphorylation of mSH2(N) Syk (Figure 4A). The critical roles of SH2 domains for Syk activation and antigen receptor function following B cell receptor cross-linking were already described elsewhere (*23*). In addition, in response to only osmotic stress, two tyrosine-phosphorylated proteins of approximately 36/38 kDa molecular mass were specifically coimmunoprecipitated with mSH2(N) Syk. The identities of these two tyrosine-phosphorylated proteins remain to be clarified.

Significant Increase in mSH2 Syk Activities following Oxidative and Osmotic Stress Treatment. Syk can be targeted for tyrosine phosphorylation by autophosphorylation and/or other PTK(s). To address the mechanism by which mSH2 Syk had enhanced tyrosine phosphorylation compared to wild-type Syk, under the stressed conditions studied, we examined the kinase activity of wild-type and mSH2 Syk in an *in vitro* kinase assay system. Both oxidative and osmotic stress stimulated a moderate increase in the activity of wild-

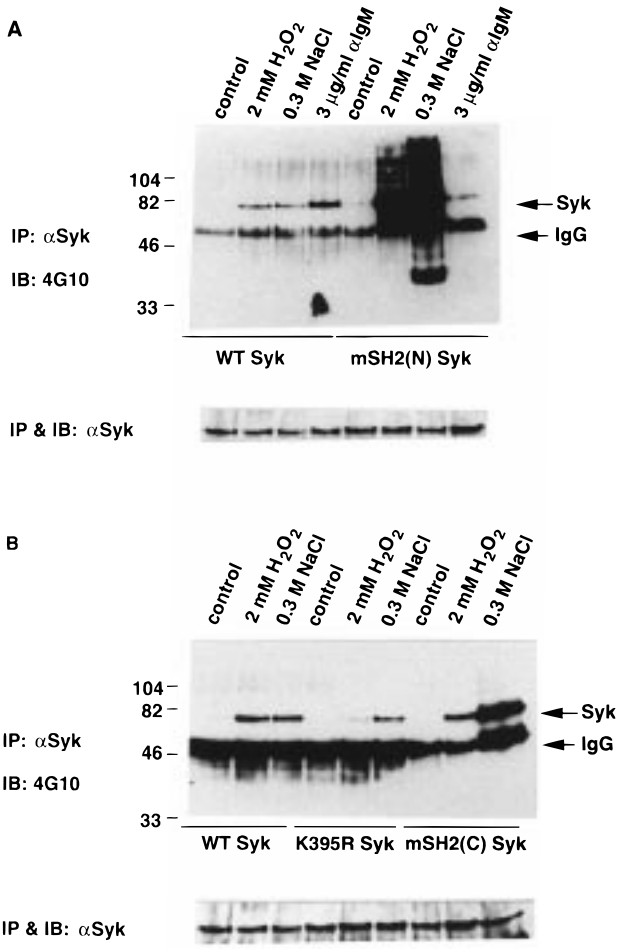

FIGURE 4: Negative regulation of oxidative or osmotic stress induced Syk activation by its SH2 domains. (A) Anti-phosphotyrosine immunoblot (top) and anti-Syk immunoblot (bottom) of anti-Syk immunoprecipitates from Syk-negative cells expressing wild-type or mSH2(N) porcine Syk following oxidative or osmotic stress stimulation. (B) Anti-phosphotyrosine immunoblot (top) and anti-Syk immunoblot (bottom) of anti-Syk immunoprecipitates from Syk-negative cells expressing wild-type, K395R, or mSH2(C) porcine Syk following oxidative or osmotic stress stimulation.

type Syk (Figure 5) (note: the less significant increase in Syk activity following stimulation might be due to the relative high basal level in the overexpressed conditions). The increase in Syk activity following stress treatment was further confirmed by the increased ability of Syk to phosphorylate exogenous substrate H2B histones (*13*). Interestingly, oxidative stress only produced a higher increase in the kinase activity of mSH2(N) Syk, compared to that of wild-type Syk. In comparison, following osmotic stress, there was an increase in the kinase activity of both mSH2-(N) and mSH2(C) Syk, significantly above that observed for wild-type Syk (Figure 5). The magnitude of the kinase activities of various point-mutated Syks correlated well with their respective levels of tyrosine phosphorylation, indicating that the enhanced tyrosine phosphorylation of mSH2 Syk was mainly attributed to an increase in their autophosphorylation activities. In conjunction with Syk tyrosine phosphorylation and Syk activity (Figures 4 and 5), a strong induction of overall tyrosine phosphorylation on cellular proteins, mainly in the change of intensity, was detected in cells expressing mSH2 Syk compared to cells expressing wild-type Syk, with the exception of mSH2(C) Syk in

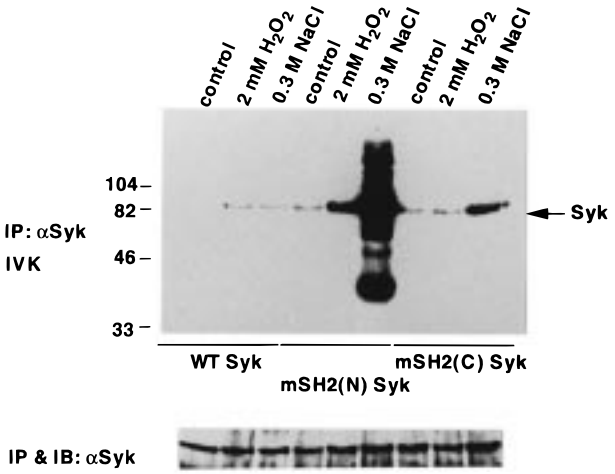

FIGURE 5: Alteration of wild-type and mSH2 Syk activities following oxidative or osmotic stress stimulation. Syk was immunoprecipitated from Syk-negative cells expressing wild-type, mSH2-(N), or mSH2(C) porcine Syk following 5 min of oxidative or osmotic stress. Anti-Syk immunoprecipitates were subject to *in vitro* kinase assay as described under Materials and Methods. IVK, *in vitro* kinase assay.

response to oxidative stress (data not shown). Moreover, the 36/38 kDa proteins observed previously were again detected in the immunocomplex with mSH2(N) Syk isolated from osmotic stress treated cells, suggesting either that these 36/38 kDa proteins could be phosphorylated *in vitro* by Syk or that they could autophosphorylate.

DISCUSSION

Syk plays a crucial role in platelet activation, B cell development, and hematopoietic cell signaling (*9, 10, 22, 33*−*35*). In addition, two stress stimuli, oxidative and osmotic stress, strongly activated Syk in chicken and human B cells (*11, 13*). Genetic studies utilizing Syk-negative cells demonstrated that Syk plays distinctive functions in oxidative and osmotic stress signaling. In oxidative stress signaling, Syk works upstream to stimulate calcium release and JNK activation (*12, 13*). In comparison, Syk is a negative regulator of osmotic stress induced apoptosis, without any apparent role in the mediation of calcium mobilization or JNK activation (*13, 14*). In this study, different roles of Syk in oxidative and osmotic stress signaling were further deduced by demonstrating that a functionally active Syk was essential for oxidative stress stimulated tyrosine phosphorylation of cellular proteins, while Syk had only a marginal effect on osmotic stress induced tyrosine phosphorylation of cellular proteins. The functional differences of Syk may be partially accounted for by differential regulation of oxidative and osmotic stress induced Syk activation by autophosphorylation and its SH2 domains (see discussion below).

A number of PTKs are activated by the phosphorylation of tyrosine residues within the activation loop of the kinase domain (*29*−*31*). In the case of Syk, tyrosines at positions 518 and 519 have been supposed to be the major phosphorylation sites within the so-called activation loop (*32*). Phosphorylation of Y518Y519 is, in fact, essential for stimulating the catalytic activity of Syk, which has been shown to play a major role in both B cell and Fc receptor functions (*23, 25*). This is also the case in oxidative and osmotic stress signaling with respect to Syk activation. Mutation of tyrosine to phenylalanine of Y518Y519 reduced oxidative and osmotic stress induced Syk activation, as monitored by Syk tyrosine phosphorylation (Figure 2). Phosphorylation of the tyrosine residues within the activation loop appears to be the general requirement for PTK activation in response to a variety of agonists.

The availability of the kinase-inactive Syk, created by the K395R mutation in the ATP binding site, enables us able to differentiate between whether Syk is phosphorylated by other tyrosine kinases whereby K395R Syk is phosphorylated, or autophosphorylates in which case K395R Syk is not phosphorylated. Investigation using Syk-negative cells expressing wild-type and K395R Syk demonstrated the differential regulation of Syk activation by oxidative and osmotic stress. Oxidative stress induced tyrosine phosphorylation of Syk was generated by the combination of both Syk activity and additional PTK activity, with the Syk activity being responsible for the majority of this phosphorylation. In comparison, additional PTK, rather than Syk itself, was the main contributor of osmotic stress induced Syk tyrosine phosphorylation, as the tyrosine phosphorylation of K595R Syk remained largely intact (Figure 3). After osmotic stress stimulation, additional PTK phosphorylating K395R Syk seemed unlikely to form a stable association with K395R Syk as no apparent signal was detected in the K395R Syk immunocomplex following *in vitro* kinase assay (data not shown). At present, the PTK(s) involved in oxidative and osmotic stress induced Syk phosphorylation in DT40 cells remain(s) to be elucidated.

In addition to tyrosine phosphorylation of the putative activation loop, the SH2 domain is another critical motif in the phosphotyrosine-dependent regulation of PTK activation. Mutation of phosphotyrosine-dependent binding sites within the SH2 domains blocks activation of both PTK(s) (*19, 20*) and antigen receptor functions (*23, 24*). Studies with Syk-negative cells expressing mSH2(N) Syk or mSH2(C) Syk showed that Syk SH2 domains, in general, attenuated Syk activation induced by oxidative and osmotic stress, since point mutation of the phosphotyrosine-dependent binding site within the SH2 domain enhanced mSH2(N) or mSH2(C) Syk activation compared to wild-type Syk (Figure 4). Consistent with this observation, mSH2 Syk showed a stronger increase in the activity than did wild-type Syk (Figure 5). Therefore, in the intact cells, stimulation of wild-type and point-mutated Syk activity correlates well with their levels of tyrosine phosphorylation, suggesting that the enhanced tyrosine phosphorylation of mSH2 Syk may mainly be accounted for by the increased autophosphorylation ability, and that the tyrosine phosphorylation level of Syk is representative of its activity. In addition, the effect of the SH2 mutations on oxidative and osmotic stress induced Syk activation clearly differed. Mutation of either SH2(N) or SH2(C) enhanced osmotic stress induced Syk activation, but only the mutation of SH2(N) enhanced oxidative stress induced Syk activation, with the SH2(C) mutation having no effect. Furthermore, following osmotic stress but not oxidative stress, only mSH2-(N) Syk was capable of coimmunoprecipitating two tyrosine-phosphorylated proteins of approximately 36/38 kDa molecular mass. Together with the differential contribution of autophosphorylation to Syk activation, these differences may give insight into understanding the different roles of Syk in

oxidative and osmotic stress signaling. At this time, we are unable to offer a reasonable explanation as to how the SH2 domains negatively regulated Syk activation in oxidative and osmotic stress signaling. However, one can assume that the SH2 domain-dependent recruitment of inhibitory molecules to Syk is a potential mechanism. Several groups have recently cloned a family of inhibitor proteins that terminate the activation of PTKs when they were recruited to the PTK complex, in growth factor and cytokine receptor signaling (*36*, *37*). Alternatively, the putative intramolecular interaction between the SH2 domain and the phosphotyrosine residue within Syk provides a structural hindrance that may limit Syk activation. With respect to this, Syk has a polypeptide with an amino acid sequence of Tyr-Ala-Lys-Ile, which resembles the preferred binding motif (phospho-Tyr-hydrophilic-hydophilic-Ile/Leu) for the SH2 domains of Syk (*8*, *38*). However, Tyr-Ala-Lys-Ile is not the best binding motif for the C-terminal SH2 domain of Syk which selects for Leu rather than Ile at the +3 position (*38*), and, as observed in this study, may indicate the lesser importance of the C-terminal SH2 domain in the regulation of Syk activation by stress stimuli as observed (Figures 4 and 5). Further studies are required to clarify these possibilities. However, it should be noted that the negative regulation of Syk activation by the SH2 domains appeared to be restricted in oxidative and osmotic stress signaling, as mutation of the phosphotyrosine-dependent binding motif in the SH2 domains abrogated B cell receptor-induced Syk activation and B cell receptor functions (Figure 4 and *23*). These results illustrated that the mechanisms of Syk activation by stress stimuli were distinctive from those induced by receptor cross-linking. This finding is particularly interesting and deserves further detailed investigation.

In conclusion, utilizing Syk-negative cells expressing various point-mutated Syk, we demonstrated that Syk was differentially regulated by autophosphorylation and SH2 domains in response to oxidative and osmotic stress. These differences provided us with the molecular basis for understanding the different functions of Syk in regulation of cell responses due to oxidative and osmotic stress.

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