Role of Tyrosine Phosphorylation of HS1 in B Cell Antigen Receptor-mediated Apoptosis

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Summary
The 75-kD HS1 protein is highly tyrosine-phosphorylated during B cell antigen receptor (BCR)-mediated signaling. Owing to low expression of HS1, WEHI-231-derived M1 cells, unlike the parental cells, are insensitive to BCR-mediated apoptosis. Here, we show that BCR-associated tyrosine kinases Lyn and Syk synergistically phosphorylate HS1, and that Tyr-378 and Tyr-397 of HS1 are the critical residues for its BCR-induced phosphorylation. In addition, unlike wild-type HS1, a mutant HS1 carrying the mutations Phe-378 and Phe-397 was unable to render M1 cells sensitive to apoptosis. Wild-type HS1, but not the mutant, localized to the nucleus under the synergy of Lyn and Syk. Thus, tyrosine phosphorylation of HS1 is required for BCR-induced apoptosis and nuclear translocation of HS1 may be a prerequisite for B cell apoptosis.

Materials and Methods
Cell Culture and Antibodies. Retrovirus producer oCRE cells, CV-1 monkey kidney fibroblasts, and COS7 cells, a derivative of CV-1 cells expressing SV40 large T antigen, were maintained in Dulbecco’s modified Eagle medium containing 10% calf serum. WEHI-231 cells and its variant M1 cells (10) were maintained in RPMI-1640 as described (9). A mouse monoclonal antibody (mAb) specific to human HS1 was raised against 15 amino acids from Val-306 to Ser-320 of human HS1 (11). Specificity of the mAb was confirmed by immunoblotting the lysates of parental and human HS1-transfected WEHI-231 cells. Rabbit anti-human HS1 sera were as described (12). Affinity-purified goat Ab to mouse IgM (Fab')2 was from Southern Biotechnology (Birmingham, AL), FITC-labeled goat Ab to mouse IgG was from PharMingen (San Diego, CA), and an anti-phosphotyrosine mAb PY-20 was from ICN (Irvine, CA).

Plasmid Construction and Preparation of Recombinant Viruses. The cDNA fragments encoding human Lyn and porcine Syk were inserted into the expression vector pME-18S (13), to generate pME-Lyn and pME-Syk, respectively. The human HS1 cDNA and its mutant cDNA, encoding a product that lacks 23 amino acids from Tyr-378 to Val-400, were inserted into pME-18S to generate pME-HS1 and pME-HS1-ΔYY, respectively. The cDNA fragments encoding human HS1 mutants HS1-FY, HS1-YF, and HS1-FF, which carry Phe-378, Phe-397, and Phe-378/Phe-397, were also inserted into pME-18S to generate pME-HS1-FY, pME-HS1-YF, and pME-HS1-FF, respectively. The site-directed mutagenesis was performed as described (14). The retroviral vectors were constructed by cloning the cDNAs of human HS1 and its mutants into the pM5-neo plasmid as described (9).

DNA Transfection and Viral Infection. CV-1 cells were trans-
fectected with various combinations of pM E-Lyn, pM E-Syk, pM E-HS1, and pM E-HS1-DYY by the standard calcium-phosphate method. 2 d after transfection, cells were washed with serum-free medium and lysed with TNE (1% N-P-40, 50 mM Tris pH 8, 20 mM EDTA, 0.2 mM sodium orthovanadate with aprothin at 10 μg/ml buffer) (7). To obtain high titer retroviruses carrying the sequences for the HS1 and mutant proteins, U26 ERE helper cells (9) were transfected with the vector plasmids. The WEHI-231 cells seeded onto cover slides and M1 cells were infected by the recombinant viruses expressing the highest amount of exogenous HS1 or its mutants were cloned as described (9).

Immunoprecipitation and Immunoblotting. The proteins in the cell lysates were subjected to immunoprecipitation with Abs to the human HS1 protein as described (7). The proteins in the lysates were subjected to immunoprecipitation and immunoblotting. To examine the degree of the apoptotic death, cells were incubated with or without 4 μg/ml of anti-IgM for 48 h and the DNA content of the cells was measured as described (9).

A synthesis of HS1 Subcellular Localization. COS-7 cells were transfected with various combinations of pM E-Lyn, pM E-Syk, pM E-HS1, and pM E-HS1-FF. The cells seeded onto cover slides were fixed and permeabilized as described (15). The cells on cover slides were incubated with FITC-labeled goat anti-mouse IgG and observed using fluorescence microscopy (Zeiss). To examine the amount of nuclear HS1 in the BCR-stimulated WEHI-231 cells, cells were incubated with 20 μg/ml of anti-IgM Ab for the indicated time. Then, nuclei were separated from the cell homogenates by centrifugation and solubilized as described (7).

Results and Discussion

The HS1 protein is highly tyrosine-phosphorylated upon BCR cross-linking (7), probably by Lyn and/or Syk. Because tyrosine phosphorylation of various cellular proteins was greatly enhanced in fibroblasts transfected with both Lyn and Syk expression plasmids as compared with those transfected with either the Lyn or Syk plasmid alone (6), we examined whether Lyn and Syk can synergistically phosphorylate HS1 in the cells. The results showed that HS1 was highly tyrosine-phosphorylated only when both Lyn and Syk were coexpressed (Fig. 1). This is consistent with our previous observation that another Src family member, Fyn, cooperates with ZAP-70, an analogue of Syk, in phosphorylating HS1 in T cells (12). It has been shown that Lyn activates Syk when they are coexpressed in fibroblasts (6) and that BCR cross-linking induces little activation of Syk in the splenic B cells (Nishizumi, H., unpublished data) and mast cells (16) of lyn−/− mice. Accordingly, lyn−/− splenocytes failed to induce tyrosine phosphorylation of HS1 upon BCR cross-linking (4). In contrast, Lyn is not detectably activated by Syk (6). Therefore, our results suggest that Lyn activated by Syk phosphorylates HS1 directly in BCR-mediated signaling.

Of the 17 tyrosine residues in human HS1, Tyr-378 and Tyr-397 are preceded by acidic residues (EDY 378 and EGDY 397), which are characteristic of many tyrosine phosphorylation sites (17, 18). In fact, the EGDY 397EE sequence of HS1 is the best known substrate for Syk kinase (19). Because tyrosine-phosphorylated HS1 interacts with the SH2 domains of Src family kinases (7), and because a phosphorylated pYED/E sequence shows the highest affinity for these domains (20), we predicted that the relevant phosphorylation sites on HS1 would be followed by two acidic residues. Of all the tyrosine residues in HS1, only Tyr-378 and Tyr-397 match the consensus (Y 378ED and Y 397EE) (Fig. 2 A). These amino acids are also conserved in mouse HS1 (21). Thus, Tyr-378 and Tyr-397 of human HS1 are likely phosphorylated by the BCR-associated kinases. Indeed, when coexpressed with Lyn and Syk in CV-1 cells, a deletion mutant HSI-ΔYY, lacking 23 amino acids from Tyr-378 to Val-400, was tyrosine phosphorylated at a greatly reduced level compared with wild-type HS1 (Fig. 2 B).

To verify BCR-mediated phosphorylation on Tyr-378 and Tyr-397 of HS1, we generated three HS1 mutants, HS1-FY, HS1-YF, and HS1-FF, in which Tyr-378 and Tyr-397, and both have been substituted by phenylalanine, respectively. These mutants and wild-type HS1 were expressed in WEHI-231 cells by retroviral infection. By probing the anti-human HS1 immunoprecipitants with the anti-phosphotyrosine antibody, we showed that HS1-FF was not detectably tyrosine-phosphorylated upon BCR cross-linking, whereas wild-type HS1 was highly and rapidly tyrosine-phosphorylated (Fig. 2 C). Both HS1-FY and HS1-YF were phosphorylated at a very low level. Thus, Tyr-378 and Tyr-397 are important for tyrosine phosphorylation of HS1 upon BCR stimulation.

WEHI-231-derived M1 cells are resistant to mlgM-induced apoptosis, unlike their parental cells. This resistibility is due to very low expression of HS1 in the cells (9). M1 cells are rendered sensitive to BCR-mediated apoptosis by the exogenous expression of wild-type HS1 (9). Furthermore, peritoneal B cells from HSI−/− mice do not undergo apoptosis upon BCR cross-linking (8). These data indicate that HS1 is a critical molecule for BCR-mediated apoptosis.
apoptosis. However, unlike wild-type HS1, the exogenously introduced HS1-FF protein failed to restore the sensitivity of M1 cells to BCR-mediated apoptosis (Fig. 3A). This was not due to differences in the expression levels of these proteins. In addition, the HS1-FF protein in M1 cells, as in WEHI-231 cells, was not tyrosine-phosphorylated by BCR cross-linking (Fig. 3B). Therefore, tyrosine phosphorylation of HS1 is essential for BCR-mediated apoptosis.

Despite the presence of a putative nuclear localization signal, HS1 localizes mainly in the cytoplasm of resting B cells (7). Consistently, HS1 expressed in CO S7 cells was present in the cytoplasm. However, HS1 coexpressed together with Lyn and Syk was mostly in the nucleus. In contrast, HS1-FF remained in the cytoplasm in the presence of Lyn and Syk (Fig. 4A). Thus, tyrosine phosphorylation of HS1 appears to be required for its own translocation from the cytoplasm to the nucleus. These data suggest that BCR cross-linking causes a significant fraction of tyrosine-phosphorylated HS1 to localize to the nucleus. Consistently, subcellular localization experiments showed that the amount of HS1 in the nucleus is increased after BCR cross-linking (Fig. 4B). Similarly, the amount of wild-type human HS1 but not HS1-FF mutant expressed in M1 cells was increased in the nuclei upon BCR stimulation (Nishizumi, H., unpublished data). Tyrosine phosphorylation on HS1 may trigger its conformational alteration that allows its nuclear translocation and thereby signaling to its downstream targets.

Because de novo protein synthesis is required for BCR-mediated apoptosis of WEHI-231 cells (22), the transcriptional and/or translational regulation of an as yet unidentified gene(s) is involved in the process. HS1 possesses motifs characteristic of transcription factors (11). Therefore, it may regulate gene expression as a transcription factor following translocation into the nucleus, as is proposed for the STAT family of proteins. Alternatively, HS1 may interact with the other transcription factors or may transport a protein(s) critical for apoptosis into the nucleus. Molecules that may interact with the other motifs, such as the SH3 domain, of HS1 have yet to be determined.

Basing on the present data we propose a model that, upon BCR cross-linking, Syk becomes fully activated by interaction with the other motifs, such as the SH3 domain, of HS1. This interaction would then allow Lyn to interact with the other motifs, such as the SH3 domain, of HS1. Therefore, it may regulate gene expression as a transcription factor following translocation into the nucleus, as is proposed for the STAT family of proteins. Alternatively, HS1 may interact with the other transcription factors or may transport a protein(s) critical for apoptosis into the nucleus. Molecules that may interact with the other motifs, such as the SH3 domain, of HS1 have yet to be determined.
is consistent with the sequential phosphorylation model in which the primary kinase phosphorylates a residue that is directly recognized by the secondary phosphate-directed kinase. The secondary kinase then phosphorylates another residue nearby (23). A similar mechanism is proposed for tyrosine phosphorylation of p130Cas by Abl (24). Accordingly, not only wild-type HS1 but also HS1-YF and HS1-FY would be phosphorylated upon BCR stimulation on Tyr-378 and Tyr-397, respectively, and thereby would interact with Lyn, allowing their further phosphorylation. However, HS1-YF and HS1-FY were much less tyrosine phosphorylated than wild-type HS1 (Fig. 2 C), as though no secondary kinases were available. It should be noted that Syk can interact with unphosphorylated HS1 and that the interaction terminates once the HS1 protein becomes tyrosine-phosphorylated at appropriate sites (Fukuda, T., un-
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