The Adaptor Protein HSH2 Attenuates Apoptosis in Response to Ligation of the B Cell Antigen Receptor Complex on the B Lymphoma Cell Line, WEHI-231*

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Signals transduced by the B cell antigen receptor (BCR) play a central role in regulating the functional response of the cell to antigen. Depending on the nature of the antigenic signal and the developmental or differentiation state of the B cell, antigen receptor signaling can promote either apoptosis or survival and activation. Understanding the molecular mechanisms underlying BCR-mediated apoptosis constitutes an important area of research because aberrations in programmed cell death can result in the development of autoimmunity or cancer. Expression of the adaptor protein hematopoietic Src homology 2 (HSH2) was found to significantly decrease BCR-mediated apoptosis in the murine WEHI-231 cell line. Analysis of signal transduction pathways activated in response to BCR ligation revealed that HSH2 does not significantly alter total protein tyrosine phosphorylation or Ca2+ mobilization. HSH2 does not potentiate the activation-dependent phosphorylation of Akt either. With respect to MAPK activation, HSH2 was not observed to alter the activation of ERK or p38 in response to BCR ligation, but it does significantly potentiate JNK activation. Analysis of processes directly associated with apoptosis revealed that HSH2 inhibits mitochondrial depolarization to a significant degree, whereas it has only a slight effect on caspase activation and poly ADP-ribose polymerase cleavage. BCR-induced apoptosis of WEHI-231 cells is associated with the loss of endogenous HSH2 expression within 12 h, whereas inhibition of apoptosis in response to CD40-mediated signaling leads to stabilization of HSH2 expression. Thus, endogenous HSH2 expression correlates directly with survival of WEHI-231 cells, which supports the hypothesis that HSH2 modulates the apoptotic response through its ability to directly or indirectly promote mitochondrial stability.

Normal B lymphocyte homeostasis and immune function are critically dependent on regulatory pathways that control programmed cell death (apoptosis) (1–4). Aberrant function of apoptotic pathways can lead to numerous life-threatening problems including the development of immunodeficiency, autoimmunity, or cancer. Therefore, it is essential to develop a complete understanding of the pathways that control apoptosis of B lymphocytes. The B cell antigen receptor complex (BCR) regulates the development, homeostasis, and function of B cells through its ability to transduce signals that promote either apoptosis or survival and activation, depending on the developmental stage of the cell and the nature of the antigenic stimulus (4). Because BCR-mediated signal transduction plays a central role in regulating B lymphocyte apoptosis, understanding the molecular mechanism by which it does so constitutes an important area of investigation with a high degree of relevance for understanding numerous immunologic disease processes. Nevertheless, significant questions remain concerning the molecular linkage between antigen receptor signaling and apoptosis.

Ligation of the BCR leads to the activation of several distinct yet interacting signal transduction pathways that ultimately control the functional response of the cell (4–7). The initiation of signal transduction via the BCR involves the activation of protein tyrosine kinases, which then phosphorylate downstream effector proteins, leading to activation of phospholipase Cγ (PLCγ) and the production of second messengers that promote protein kinase C- and Ca2+-dependent signal transduction processes. Additionally, BCR ligation leads to activation of phosphatidylinositol 3-kinase (PI3K) and the downstream serine/threonine kinase AKT, which can promote survival of the cell (8–10). Finally, signaling through the BCR has been shown to regulate the function of mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (4, 11). These distinct pathways in turn promote activation of numerous transcriptional regulatory proteins including NFAT, NF-κB, and the AP-1 complex that act in concert to regulate gene transcription and the functional response of the B cell (4, 11). How BCR-mediated signaling ultimately determines the fate of the cell in terms of whether it proliferates or undergoes apoptosis is in part due to inherent differences in the strength of the antigenic signal, which in turn can affect the activation of pro-survival effector proteins (4). However, additional factors including developmental differences in partitioning of BCR-associated signaling components (12, 13), basal expression levels of BCL-2 family proteins (14, 15), expression/function of

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1 The abbreviations used are: BCR, B cell antigen receptor; PLCγ, phospholipase Cγ; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; HSH2, hematopoietic Src homology 2; PI, phosphatidylinositol; ANV, annexin V; PARP, poly ADP-ribose polymerase; NFAT, nuclear factor of activated T cells; Ab, antibody; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TBST, Tris-buffered saline containing Tween; DiOC6, 3,3′-dihexyl oxacarbocyanine iodide; HRP, horseradish peroxidase.
kinases, and phosphatases and the potential for differential expression of adaptor proteins are likely to play a significant role in determining the functional outcome of signaling via the BCR (4, 16–18).

A growing body of literature has begun to delineate the key role that adaptor proteins play in modulating signal transduction via lymphocyte antigen receptors. Adaptor proteins have the ability to quantitatively and/or qualitatively change the nature of antigen receptor signaling by generating diverse multimolecular signaling complexes in unique spatial/temporal contexts (16–18). In the B cell, studies have shown that BLNK (SLP-65) is involved in coupling BCR proximal protein tyrosine kinases to PLCγ1, thereby promoting its activation, as well as recruiting and promoting activation of Vav and Nck (19–21). Recently, Bam32 has been shown to regulate the activation of MAPKs ERK and JNK through a novel pathway that involves its interaction with HPK1 (22–24). Similarly, GRP1/Gads and NTAL/LAB are thought to regulate MAPK activation (16–18). In the B cell, studies have shown that BLNK (SLP-65) is involved in coupling BCR proximal protein tyrosine kinases to PLCG1, thereby promoting its activation, as well as recruiting and promoting activation of Vav and Nck (19–21).

HSH2 was observed to decrease in response to pro-apoptotic caspase activation. A key finding is that the endogenous level of HSH2 contains a single SH2 domain, though HSH2 was not observed to cause global changes in lineage but can be detected in cells of the myelomonocytic lineage (28, 29). HSH2 was expressed predominantly in cells of the lymphoid lineage as well as the myelomonocytic lineage (28, 29). HSH2 contains a single SH2 domain, three conserved proline-rich regions and two tyrosine residues that are potential sites of phosphorylation. Expression of HSH2 in the WEHI-231 B cell line was found to protect these cells from undergoing apoptosis in response to BCR ligation. Although HSH2 was not observed to cause global changes in BCR-mediated signal transduction, it did potentiate JNK activation. Moreover, HSH2 was observed to prevent mitochondrial destabilization, whereas it exerted only a modest effect on caspase activation. A key finding is that the endogenous level of HSH2 was observed to decrease in response to pro-apoptotic BCR signaling, whereas it was maintained by anti-apoptotic CD40-mediated signaling. These findings support the conclusion that HSH2 has the ability to modulate the apoptotic response to signals delivered through the BCR complex.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture—**WEHI-231 murine B lymphoma cells and Phoenix gp retroviral packaging cells (provided by Gary Nolan, Department of Microbiology and Immunology, Stanford University, Palo Alto, CA) were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 2 μM l-glutamine, 50 μM 2-mercapto-ethanol, 100 μg/ml streptomycin-penicillin, and 50 μg/ml gentamicin (Sigma) at 37 °C under 5% CO2.

**Antibodies—**WEHI-231 cells were stimulated with polyclonal goat anti-mouse IgM (Fab′2) Ab purchased from BIOScience International (Camarillo, CA). For Western blot analysis, the following antibodies were purchased: mouse anti-FLAG mAb conjugated to horseradish peroxidase (HRP) (Sigma); anti-phosphotyrosine mAb conjugated to HRP (4G10, Upstate Biotechnology Inc., Waltham, MA); mouse anti-Bcl-xL mAb, and mouse anti-Caspase-7 mAb (Biogenesis, San Diego, CA); mouse anti-actin mAb (AC-40; Sigma); rabbit polyclonal anti-phospho-ERK (19762, Thr-0292/Tyr-204), anti-ERK, anti-phospho-JNK (98F2, Thr-183/Tyr-185), anti-JNK, anti-phospho-p38 (3D7, Thr-180/Tyr-182), anti-p38, anti-phospho-AKT (244F9, Thr-308; 493H12, Ser-473), anti-AKT, anti-caspase-3, anti-caspase-9, and anti-poly ADP-ribose polymerase (PARP) Abs (Cell Signaling Technology, Beverly, MA). The hybridoma producing anti-CD40 mAb (1C10) was obtained from Dr. Frances Lund at the Trudeau Institute (Saranac Lake, NY). Rabbit polyclonal anti-HSH2 Ab was generated by immunizing rabbits with intact recombinant HSH2.

**Plasmids—**The cDNA encoding full-length HSH2 and Bcl-xL were PCR-amplified from total murine splenocyte cDNA using KOD high fidelity polymerase from Novagen (Madison, WI). For HSH2, a BglII site was included in the forward primer (5′-GAGGACATCCTCGGCA-TGGCAGAAGCC-3′), and a FLAG tag and HpaI site were included in the reverse primer (5′-GAGACGTCTAGTCGAGTTAATTCTGCTGTC-3′). The HSH2 PCR product was digested with BglII and HpaI restriction enzymes and then ligated into the BglII and HpaI sites of the pMSCV-puro vector from Clontech. For Bcl-xL, an XhoI site was included in the forward primer (5′-GAGACGTCTAGTCGAGTTAATTCTGCTGTC-3′) and the HSH2 PCR product was digested with XhoI and EcoRI restriction enzymes and ligated into the XhoI and EcoRI sites of pMSCV-puro.

**Transfection and Transduction—**HSH2:pMSCV-puro, Bcl-xL:pMSCV-puro, or empty pMSCV-puro plasmids were co-transfected with the pcLE-ECO plasmid encoding the ectropic receptor envelope into Phoenix gp retroviral packaging cells using Lipofectamine 2000 from Invitrogen, according to the manufacturer’s instructions. Viral supernatant was collected from transfectants 36 h after transfection. The viral supernatant was incubated with WEHI-231 cells for 12 h in the presence of polybrene (2.5 μg/ml). After transduction, cells were incubated with (1.5 μg/ml) puromycin (Mediatek, Inc., Herndon, VA) for 48 h to select against nontransduced cells.

**DNA Content Analysis—**WEHI-231 cells (1 × 10⁶ cells/ml) were stimulated with 1 μg/ml polyclonal anti-IgM (Fab′2) Ab for up to 48 h. After stimulation, cells were washed with PBS and fixed in 70% ethanol. The fixed cells were washed with a solution containing 0.1% sodium citrate and 0.1% Triton X-100 to remove nucleotide fragments. Following the wash step, cells were incubated with 10 μg/ml RNase A and 50 μg/ml propidium iodide (PI) for 15 min at 37 °C. Samples were then analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences). Events with sub-G0/G1 DNA content were scored as apoptotic.

**Annexin V Staining of Cell Surface Phosphatidylserine—**WEHI-231 cells (1 × 10⁶ cells/ml) were stimulated with 1 μg/ml polyclonal anti-IgM (Fab′2) Ab for up to 48 h. After stimulation, cells were washed with PBS and resuspended in 100 μl of annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂, pH 7.4). The cells were then incubated with 5 μl of annexin V (AVN) conjugated with Alexa-488 (Molecular Probes, Eugene, OR) and 50 μg/ml RNase A (30). The incubated cells were protected from light at room temperature for 15 min and then immediately analyzed using a FACScan flow cytometer (BD Biosciences). Two-color AVNP1 staining was analyzed to discriminate between three distinct population of cells. Cells that were AVN+PI− were scored as viable cells, AVN−PI− cells were scored as early apoptotic cells, and AVN−PI+ cells were scored as late apoptotic or necrotic cells. An assay could not differentiate between the two possibilities.

**Western Blot Analysis—**WEHI-231 cells (2 × 10⁶ cells/sample) were stimulated with 1 μg/ml polyclonal anti-IgM (Fab′2) Ab in the presence or absence of anti-CD40 mAb (1C10, 1.5 μg/ml) for the time points indicated. After stimulation, cells were immediately washed in ice-cold PBS to stop the reaction. Next, the cells were washed twice with ice-cold PBS and resuspended in 0.5 ml of lysis buffer (25 mM HEPES, 50 mM NaCl, 10 mM EDTA, 1 mg/ml EGTA, 0.1 mM NaVO₃, 50 mM NaF, and 1% Nonidet P-40). Phenylmethylsulfonyl fluoride and a protease inhibitor mixture (Sigma) were added to the lysis buffer just before use. Cell lysates were incubated for 1 h on ice and then centrifuged at 13,000 × g for 15 min at 4 °C. Detergent-soluble lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked in TBST containing 3% nonfat milk (or TBST with 3% bovine serum albumin for 4G10 blotting) overnight at 4 °C and then washed four times with TBST. Next, the membranes were incubated with primary antibodies for 1 h at room temperature and then washed four times with TBST. For primary antibodies not directly conjugated to HRP, secondary goat anti-mouse Ig or goat anti-rabbit Ig Abs conjugated to HRP (BIOSOURCE International) were incubated with the membranes for 1 h at room temperature and then washed four times with TBST. Finally, proteins of interest were visualized using ECL West-Pico chemiluminescent substrate (Pierce) and subsequent exposure to autoradiographic film (Eastman Kodak Co.).

For Western blot analysis of caspase cleavage, WEHI-231 cells (1 × 10⁶ cells/m) were stimulated with 1 μg/ml polyclonal anti-IgM (Fab′2) Ab or PBS for 6 h in 10 ml of culture medium. Four hours after stimulation, cells were pelleted and lysed in 0.1 ml of radioimmunoprecipitation buffer (25 mM HEPES (pH 7.8), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture, which were added just before use. The lysates were incubated on ice for 1 h and then centrifuged at 13,000 × g for 15 min at 4 °C. The total protein content of the detergent-soluble lysate was quantitated using the BCA protein assay.
4 °C to remove nuclei and cell debris. The supernatant was collected. WEHI-231 cells to express these proteins. Bcl-xL overexpression was induced Apoptosis—Oda et al. (28) first identified human HSH2 after searching existing databases for genes that encode proteins with regions homologous to SH2 domains. The hsh2 gene encodes a 352-amino-acid protein with expression of transcripts restricted to cells of the hematopoietic lineage, including B and T lymphocytes. Based on a search of expressed sequence tag databases using the BLAST algorithm, we identified the mouse and rat homologues of human HSH2 consisting of 334 and 335 amino acids, respectively. Recently, another group has cloned the mouse homologue of HSH2 referred to as ALX (29). Primary amino acid sequence analysis of mouse HSH2 revealed an N-terminal SH2 domain and three conserved PXPF motifs that are likely to facilitate protein-protein interactions; however, no domains associated with catalytic activity were detected. Based on this sequence analysis, it was logical to hypothesize that HSH2 functions as an adaptor protein. Moreover, the expression of HSH2 in lymphocytes raised the possibility that it might function as a regulator of antigen receptor-mediated signal transduction.

To determine whether HSH2 is capable of regulating antigen receptor signal transduction, the WEHI-231 B lymphoma cell line was chosen. Cross-linking of membrane IgM on WEHI-231 cells results in growth arrest and the induction of apoptosis (31–33). Because of this functional response, WEHI-231 cells have been used extensively as a model for immature B cell negative selection. Additionally, stimulation of WEHI-231 cells through the BCR initiates a tyrosine phosphorylation-based signaling cascade, which makes them a useful model for studying BCR-mediated signal transduction. Finally, WEHI-231 cells can be efficiently transduced by retroviruses, thus facilitating their genetic manipulation (34). We utilized these properties of WEHI-231 cells to examine the functional and biochemical consequences of HSH2 expression on antigen receptor signaling following surface immunoglobulin M cross-linking.

Retrovirus encoding full-length FLAG-tagged HSH2, Bcl-xL, and the empty pMSCV-puro vector were used to transduce WEHI-231 cells to express these proteins. Bcl-xL overexpression was used as a control for the apoptosis assays that follow because it has been demonstrated to function as a potent inhibitor of antigen-receptor-induced apoptosis in WEHI-231 cells (35, 36). Cells were transduced with retrovirus encoding empty pMSCV-puro to control for any effects that retroviral transduction might have on WEHI-231 function. After transduction, nontransduced cells were eliminated using puromycin drug selection. Even before drug selection, transduction efficiencies of 70–80% were achieved as determined by the percentage of enhanced green fluorescent protein-positive WEHI-231 cells after transduction with an enhanced green fluorescent protein-expressing retrovirus (data not shown). As expected, HSH2 was detected by Western blotting with anti-FLAG mAb only in HSH2-transduced WEHI-231 cells. Similarly, Bcl-xL expression could only be detected by Western blotting with anti-Bcl-xL mAb in WEHI-231 cells transduced with retrovirus encoding Bcl-xL (data not shown).

The extent of apoptosis initiated by antigen receptor cross-linking can be quantitated based on the analysis of DNA content. The induction of apoptosis results in activation of nucleases that cleave the genome of apoptotic cells into nucleotide fragments. Cells undergoing apoptosis will therefore have less total DNA than nonapoptotic cells. Using a dye that quantitatively binds DNA, such as PI, these populations can be discriminated. To determine whether HSH2 expression alters the apoptotic response of WEHI-231 cells to stimulation through the BCR, cells expressing empty vector, HSH2, or Bcl-xL were stimulated with polyclonal anti-IgM F(ab′)2 Ab and then assayed for apoptosis initiated in response to stimulation of cells through the BCR. To determine whether HSH2 is capable of inhibiting apoptosis initiated by antigen receptor cross-linking, WEHI-231 cells transduced with empty vector, HSH2, or Bcl-xL were stimulated with polyclonal anti-IgM F(ab′)2 Ab and then assayed for apoptosis by ANV and PI staining. HSH2 expression enhanced the survival of WEHI-231 cells following antigen receptor stimulation relative to empty vector controls. Forty-eight hours after stimulation, ~60% of the HSH2-expressing WEHI-231 cells contained sub-G0/G1 levels of DNA. In contrast to control WEHI-231 cells, only 37% of HSH2-transducing WEHI-231 cells contained sub-G0/G1 levels of DNA at the 48-h time point. Indeed, at both the 24- and 48-h time points, approximately half of the number of HSH2-expressing cells had undergone apoptosis when compared with control cells transduced with empty vector. The results of these initial experiments suggest that HSH2 expression is capable of inhibiting apoptosis initiated in response to stimulation of cells through the BCR.

To confirm that HSH2 is capable of inhibiting apoptosis initiated by antigen receptor cross-linking, WEHI-231 cells transduced with empty vector, HSH2, or Bcl-xL were stimulated with polyclonal anti-IgM F(ab′)2 Ab and then assayed for apoptosis by ANV and PI staining. HSH2 expression enhanced the survival of WEHI-231 cells following antigen receptor stimulation relative to empty vector controls. Forty-eight hours after stimulation, ~60% of the HSH2-expressing WEHI-231 cells contained sub-G0/G1 levels of DNA (data not shown). HSH2 expression decreased the percentage of cells undergoing apoptosis relative to empty vector controls. Consistent with the data for sub-G0/G1 DNA content shown in Fig. 1A, there was an ~50% decrease in the number of HSH2-expressing cells undergoing apoptosis at both the early and the late stages relative to empty vector control cells at the 48-h time point. Taken together, the DNA content analysis and ANV staining clearly suggest that HSH2 is capable of regulating apoptosis initiated by BCR cross-linking in WEHI-231 cells.

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RESULTS

HSH2 Protects WEHI-231 Cells from Undergoing BCR-induced Apoptosis—Oda et al. (28) first identified human HSH2 after searching existing databases for genes that encode proteins with regions homologous to SH2 domains. The hsh2 gene encodes a 352-amino-acid protein with expression of transcripts restricted to cells of the hematopoietic lineage, including B and T lymphocytes. Based on a search of expressed sequence tag databases using the BLAST algorithm, we identified the mouse and rat homologues of human HSH2 consisting of 334 and 335 amino acids, respectively. Recently, another group has cloned the mouse homologue of HSH2 referred to as ALX (29). Primary amino acid sequence analysis of mouse HSH2 revealed an N-terminal SH2 domain and three conserved PXPF motifs that are likely to facilitate protein-protein interactions; however, no domains associated with catalytic activity were detected. Based on this sequence analysis, it was logical to hypothesize that HSH2 functions as an adaptor protein. Moreover, the expression of HSH2 in lymphocytes raised the possibility that it might function as a regulator of antigen receptor-mediated signal transduction.

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an SH2 domain and PXXP motifs suggests that it functions as an adaptor protein capable of facilitating the formation of protein complexes, thereby regulating signal transduction. Therefore, the possibility existed that HSH2-dependent inhibition of apoptosis may be due to its ability to directly regulate BCR-mediated signal transduction. In this regard, HSH2-dependent inhibition of apoptosis could be due to the fact that HSH2 functions as a negative regulator of antigen receptor signaling either by effectively decreasing the overall strength of the signal initiated in response to BCR cross-linking or by modulating one or more specific signal transduction pathways. Alternatively, HSH2 could be involved in inhibition of pro-apoptotic events or potentiation of anti-apoptotic processes that are downstream of BCR-mediated signaling pathways.

Initiation and propagation of BCR-mediated signaling has been shown to be highly dependent on phosphotyrosine-based signal transduction cascades initiated seconds after antigen receptor engagement (7). To determine whether HSH2 expression decreases the magnitude of inducible protein tyrosine phosphorylation initiated by cross-linking of the BCR, WEHI-231 cells transduced with empty vector or HSH2 were stimulated with polyclonal anti-IgM F(ab’2)Ab for 24–48 h, after which cells were harvested and stained with PI to measure DNA content. Cells were analyzed using flow cytometry to quantitate the percentage of the total population that contained sub-G0/G1 levels of DNA at 24 and 48 h. B, quantitation of the percentage of viable cells (ANV-PI-) at 0, 24, or 48 h after stimulation of empty vector control (●), HSH2 (■), or Bcl-xL (▲) WEHI-231 cells with anti-IgM F(ab’2)Ab. C, quantitation of the percentage of control (open bar), HSH2- (gray bar), and Bcl-xL- (black bar) expressing WEHI-231 cells that are ANV-PI+, characteristic of early apoptotic cells. The data are representative of four independent experiments.

FIG. 1. HSH2 blocks BCR-induced apoptosis in WEHI-231 cells. A, WEHI-231 cells were transduced with retrovirus encoding the empty p-MSCV-puro vector (empty vector) or with virus containing either HSH2:p-MSCV-puro or Bcl-xL:p-MSCV-puro, after which the cells were cultured in the presence of puromycin to select for homogeneous expression of HSH2 or Bcl-xL. Empty vector control (open bar), HSH2- (gray bar), and Bcl-xL- (black bar) expressing WEHI-231 cells (1 × 10^5 cells/ml) were stimulated with polyclonal anti-IgM F(ab’2)Ab for 24–48 h, after which cells were harvested and stained with PI to measure DNA content. Cells were analyzed using flow cytometry to quantitate the percentage of the total population that contained sub-G0/G1 levels of DNA at 24 and 48 h.
Ab. HSH2 was not observed to significantly follow stimulation of Indo-1-AM-loaded cells with polyclonal anti-IgM F(ab')2 Ab or in the presence of polyclonal anti-IgM F(ab')2 Ab for varied periods of time. Western blotting to detect HSH2 with polyclonal anti-HSH2 Ab to rule out cross-contamination of the mitochondrial and cytosolic subcellular fractions, membranes were stripped and reprobed with polyclonal Ab specific for the cytosolic protein Apaf-1 or monoclonal Ab against the mitochondrial protein Hsp60, respectively.

Activation of PLCγ promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to yield inositol 1,4,5-trisphosphate, which triggers the release of Ca2+ from the endoplasmic reticulum, promoting capacitative entry of Ca2+ through plasma membrane channels. Therefore, Ca2+ mobilization was measured following stimulation of Indo-1-AM-loaded cells with polyclonal anti-IgM F(ab')2 Ab. HSH2 was not observed to significantly affect Ca2+ mobilization (data not shown), suggesting that upstream activation of PLCγ is likely to be normal and that NFAT function downstream is unaffected.

Activation of the serine/threonine kinase AKT is regulated by PI3K and correlates with enhanced cell proliferation and survival (37, 38). Therefore, it was of interest to determine whether HSH2 expression enhances AKT activation, leading to increased survival and decreased apoptosis of WEHI-231 cells. Western blot analysis with phospho-AKT-specific Ab revealed that HSH2 expression does not potentiate, and actually appears to diminish, phosphorylation of AKT in response to BCR cross-linking (Fig. 2B). Therefore, HSH2 does not inhibit apoptosis via an AKT-dependent mechanism and must enhance survival and prevent apoptosis via an alternate mechanism.

Finally, phospho-specific antibodies against p38, JNK, and ERK were used to assess the extent of MAPK activation. As before, either empty vector or HSH2-transduced WEHI-231 cells were stimulated with polyclonal anti-IgM F(ab')2 Ab, and lysates were prepared for analysis by Western blotting. In Fig. 3C, lysates were probed using phospho-ERK-specific Ab to measure ERK activation. Comparison of empty vector with HSH2-expressing WEHI-231 cells indicated that ERK activation is not significantly affected by expression of HSH2. Likewise, activation of p38 as determined by Western blotting with phospho-p38-specific Ab is equivalent in empty vector and HSH2-expressing WEHI-231 cells (Fig. 3C). In contrast, HSH2 expression significantly enhanced JNK activation relative to empty vector control cells based on Western blotting with phospho-JNK Ab (Fig. 3C). Analysis of MAPK activation once again neither supports a generalized attenuation of BCR-mediated signaling in HSH2-expressing cells nor supports the conclusion that there is a global effect on signal transduction. Rather, based on the findings, it appears that HSH2 promotes a selective increase in JNK activation, which would not be predicted to account for the observed inhibition of apoptosis as JNK activation has generally been demonstrated to be pro-apoptotic in WEHI-231 cells (39, 40).

**Attenuation of BCR-mediated Apoptosis by HSH2**

Biochemical analysis of early signal transduction events following antigen receptor stimulation did not reveal a clear mechanism by which HSH2 is able to inhibit apoptosis. Therefore, experiments were conducted to analyze the subcellular localization of HSH2 and early events associated with the apoptotic process itself that occur downstream of proximal BCR-mediated signal transduction to further elucidate the potential mechanism by which HSH2 attenuates apoptosis. Subcellular fractionation of WEHI-231 cells incubated in medium alone or in the presence of anti-IgM F(ab')2 Ab for varied periods of time was performed to determine the subcellular localization of HSH2. Data depicted in Fig. 3 reveal that HSH2 is localized to the mitochondrial fraction of WEHI-231 cells as well as the cytoplasmic fraction. HSH2 was not detected in the nuclear fraction isolated from WEHI-231 cells, regardless of whether they had been stimulated with anti-IgM F(ab')2 Ab (data not shown). Although the amount of HSH2 contained in the mitochondrial fraction appears to be significantly higher than that contained in the cytoplasm, there is a 10-fold greater amount of total protein in the cytoplasm versus the mitochondrial fraction. When this difference in total protein is taken into account, ~85% of HSH2 is cytoplasmic, whereas 15% is contained in the mitochondrial fraction. The level of HSH2 in the cytoplasm and mitochondrial fractions was not observed to change in response to anti-IgM F(ab')2 Ab within the 4-h time period examined. Moreover, confocal microscopy did not demonstrate significant recruitment of HSH2 to the BCR activation complex within 1–60 min after BCR ligation (data not shown). Thus, it is apparent that HSH2 is constitutively localized to the mitochondrial fraction, where it may play a role in regulating mitochondrial stability.

The reduction in mitochondrial membrane potential (ΔΨm) has previously been shown to play a critical role in the induction of apoptosis in WEHI-231 cells. Indeed, prevention of mitochondrial membrane depolarization is thought to be the primary mechanism by which Bcl-xL blocks apoptosis (41, 42). Therefore, DiOC6 staining was used to assay changes in ΔΨm of WEHI-231 cells stimulated with polyclonal anti-IgM F(ab')2 Ab. HSH2 expression resulted in an ~50% decrease in the number of cells with reduced ΔΨm after antigen receptor stimulation relative to control cells transduced with empty vector (Fig. 4, A and B). Although HSH2 was not as effective at blocking mitochondrial membrane depolarization as Bcl-xL, the effect was clearly significant. The percentage of decrease in HSH2-expressing cells undergoing apoptosis relative to control cells was also consistent with the decreased percentage of sub-G1/G0 and ANV-positive cells observed earlier. The data support the conclusion that HSH2 inhibits apoptosis at the level of mitochondrial membrane depolarization (or earlier) and that maintenance of mitochondrial stability is likely to be responsible for inhibition of downstream events such as genomic DNA cleavage and phosphatidylserine translocation.

Caspase activation has also been implicated as an early event in the induction of apoptosis in WEHI-231 cells (43–45). Therefore, experiments were conducted to determine whether HSH2 expression affects caspase activation. WEHI-231 cells were stimulated with polyclonal anti-IgM F(ab')2 Ab, and then cell lysates were assayed for caspase activation by Western blotting to detect caspase cleavage products. HSH2 expression was observed to cause a modest delay in the kinetics and a decrease in the magnitude of activation of caspases 9, 3, and 7 relative to empty vector-transduced cells (Fig. 5). Additionally, the kinetics and magnitude of PARP cleavage, a known substrate of caspases 3 and 7, were also modestly decreased in HSH2-expressing cells. In contrast, Bcl-xL expression almost completely blocked detectable cleavage of caspases 9, 3, and 7, as well as PARP (Fig. 5). Although there is a detectable decrease in caspase activation in WEHI-231 cells expressing...
HSH2, it is uncertain whether the modest block in caspase activation is responsible for the inhibition of apoptosis. In this regard, evidence suggests that WEHI-231 cells may use proteases other than caspases, such as calpain and cathepsin, to initiate the apoptotic response (44, 46, 47). This could explain why there are minimal detectable caspase cleavage products relative to uncleaved pro-caspases even in the empty vector control cells (Fig. 5).

Expression of Endogenous HSH2 Correlates with Survival of WEHI-231 Cells—Experiments were performed to monitor endogenous HSH2 expression in response to BCR-mediated signal transduction in WEHI-231 cells. WEHI-231 cells were stimulated with polyclonal anti-IgM F(\(\text{ab}')_2\) Ab for 12–48 h, after which cells were harvested and incubated with DiOC\(_6\) to monitor mitochondrial depolarization using flow cytometry. The percentage of cells in which mitochondrial depolarization occurred in response to BCR ligation was quantitated for each time point. This could explain why there are minimal detectable caspase cleavage products relative to uncleaved pro-caspases even in the empty vector control cells (Fig. 5).

Expression of Endogenous HSH2 Correlates with Survival of WEHI-231 Cells—Experiments were performed to monitor endogenous HSH2 expression in response to BCR-mediated signal transduction in WEHI-231 cells. WEHI-231 cells were stimulated with polyclonal anti-IgM F(\(\text{ab}')_2\) Ab for varied periods, cell lysates were prepared, and the protein content in cell lysates was analyzed. Equivalent amounts of protein from each time point were separated by SDS-PAGE, and the level of HSH2 was detected by Western blotting with anti-HSH2 polyclonal Ab. As can be seen in Fig. 6A, HSH2 expression decreases significantly between 12 and 24 h in response to BCR signaling. The observed decrease in HSH2 was not due to a loading artifact because the amount of actin present in all samples was comparable (Fig. 6A). Moreover, HSH2 does not appear to translocate to the detergent-insoluble fraction in response to BCR ligation (data not shown). HSH2 contains a PEST domain, and it has been shown that PEST domains are involved in rapid proteolytic degradation of proteins. Thus, it is possible that the induction of apoptotic signals in WEHI-231 cells leads to the proteolytic degradation of endogenous HSH2. In contrast, retroviral-mediated expression of HSH2 in WEHI-231 cells results in a severalfold increase in HSH2 expression that is resistant to anti-IgM induced degradation, presumably because the enhanced level of HSH2 expression overwhelms the proteases in the cell (Fig. 6B).

CD40-mediated signaling has been shown to confer protec-
harvested and processed as described in the methods. 

Western blotting with anti-caspase Abs was performed to detect caspase activation based on the appearance of cleaved subunits (indicated by arrows). Similarly, Western blotting with anti-PARP Ab was conducted to measure PARP cleavage. The percentage of cells in which mitochondrial depolarization occurred in response to BCR ligation was quantitated for each sample. B, WEHI-231 cells (2 x 10^5/sample) were incubated in medium alone or in the presence of polyclonal anti-IgM F(ab')_2 Ab with or without anti-CD40 mAb (1C10) for 24 h. Cells were harvested and lysed, and the protein content of detergent-soluble lysates was measured. Equivalent amounts of protein were separated by SDS-PAGE, and the separated proteins were transferred to nitrocellulose. HSH2 expression was monitored by Western blotting with anti-HSH2 polyclonal Ab. Protein loading was monitored by reprobing membranes with anti-actin Ab. 

**DISCUSSION**

These studies demonstrate that expression of the adaptor protein HSH2 in the B lymphoma cell line WEHI-231 results in significant attenuation of apoptosis in response to ligation of the BCR. HSH2 was observed to cause a 50% decrease in apoptosis based on flow cytometric analysis of PI and ANV staining. Although HSH2 exerted a significant effect on the apoptotic response, it was not as efficient as the known anti-apoptotic protein Bcl-xL. 

The observation that HSH2 did not completely protect against mitochondrial destabilization, nor did it significantly block caspase activation, both of which were completely blocked in

**FIG. 5.** HSH2 expression exerts only a slight effect on caspase (Casp) activation and PARP cleavage in WEHI-231 cells stimulated through the BCR. Empty vector control, HSH2-, and Bcl-xL- expressing WEHI-231 cells (1 x 10^5 cells/ml) were stimulated with polyclonal anti-IgM F(ab')_2 Ab for 24–48 h, after which cells were harvested and lysed and prepared. The protein concentration of the sample was determined, and an equivalent amount of protein from each sample was subjected to SDS-PAGE, after which the separated proteins were transferred to nitrocellulose. Western blotting with anti-caspase Abs was performed to detect caspase activation based on the appearance of cleaved subunits (indicated by arrows). Similarly, Western blotting with anti-PARP Ab was conducted to measure PARP cleavage as shown by the appearance of lower molecular mass cleavage products (indicated by the arrow). ECL and autoradiography were used to visualize bands of interest. The results presented are representative of three independent experiments.

**FIG. 6.** HSH2 expression decreases in response to BCR-mediated signal transduction in WEHI-231 cells. A, BCR-mediated loss of HSH2 expression. WEHI-231 cells (2 x 10^5/sample) incubated in medium alone (NT) or in the presence of polyclonal anti-IgM F(ab')_2 Ab for varied periods of time. Cells were harvested and lysed, and the protein concentration of the detergent-soluble lysates was determined. Equivalent amounts of protein for each sample were separated by SDS-PAGE, and the separated proteins were transferred to nitrocellulose. HSH2 expression was monitored by Western blotting with anti-HSH2 rabbit polyclonal Ab. To confirm equal protein loading, membranes were stripped and reprobed with anti-actin polyclonal Ab. HSH2 and actin bands were visualized using ECL and autoradiography. B, retroviral-mediated expression of HSH2 in WEHI-231 cells transduced with empty p-MSCV-puro alone or with retrovirus encoding HSH2 were incubated in medium alone or in the presence of polyclonal anti-IgM F(ab')_2 Ab for 12–24 h. Cells were harvested and processed as described in panel A.

**FIG. 7.** CD40-mediated signal transduction protects WEHI-231 cells from undergoing BCR-induced apoptosis and maintains expression of HSH2. A, CD40-mediated signaling and retroviral-mediated expression of HSH2 are equally effective in protecting WEHI-231 cells from undergoing BCR-induced apoptosis. Empty vector control, HSH2-, and Bcl-xL- expressing WEHI-231 cells (1 x 10^5 cells/ml) were stimulated with polyclonal anti-IgM F(ab')_2 Ab with or without anti-CD40 mAb (1C10, 1.5 µg/ml) for 24 h, after which cells were harvested and incubated with DiOC_6 to monitor mitochondrial depolarization using flow cytometry. The percentage of cells in which mitochondrial depolarization occurred in response to BCR ligation was quantitated for each sample. B, WEHI-231 cells (2 x 10^5/sample) were incubated in medium alone or in the presence of polyclonal anti-IgM F(ab')_2 Ab with or without anti-CD40 mAb (1C10) for 24 h. Cells were harvested and lysed, and the protein content of detergent-soluble lysates was measured. Equivalent amounts of protein were separated by SDS-PAGE, and the separated proteins were transferred to nitrocellulose. HSH2 expression was monitored by Western blotting with anti-HSH2 polyclonal Ab. Protein loading was monitored by reprobing membranes with anti-actin Ab.

HSH2 or anti-CD40 mAb alone (Fig. 7A). This finding suggests that HSH2 and CD40-mediated signaling might be involved in a common anti-apoptotic pathway. Therefore, experiments were performed to monitor HSH2 expression in WEHI-231 cells stimulated through the BCR in the presence or absence of anti-CD40 mAb. As can be seen, stimulation of WEHI-231 cells via the BCR leads to a significant decrease in HSH2 expression within 24 h (Fig. 7B). However, when WEHI-231 cells are stimulated through the BCR in the presence of anti-CD40 mAb (1C10), HSH2 expression does not decrease significantly (Fig. 7B). These findings demonstrate that CD40-dependent signaling stabilizes HSH2 expression and therefore support the hypothesis that CD40 and HSH2 may regulate survival via a common pathway.

**FIG. 6.** HSH2 expression decreases in response to BCR-mediated signal transduction in WEHI-231 cells. A, BCR-mediated loss of HSH2 expression. WEHI-231 cells (2 x 10^5/sample) were incubated in medium alone (NT) or in the presence of polyclonal anti-IgM F(ab')_2 Ab for varied periods of time. Cells were harvested and lysed, and the protein concentration of the detergent-soluble lysates was determined. Equivalent amounts of protein for each sample were separated by SDS-PAGE, and the separated proteins were transferred to nitrocellulose. HSH2 expression was monitored by Western blotting with anti-HSH2 rabbit polyclonal Ab. To confirm equal protein loading, membranes were stripped and reprobed with anti-actin polyclonal Ab. HSH2 and actin bands were visualized using ECL and autoradiography. B, retroviral-mediated expression of HSH2 in WEHI-231 cells transduced with empty p-MSCV-puro alone or with retrovirus encoding HSH2 were incubated in medium alone or in the presence of polyclonal anti-IgM F(ab')_2 Ab for 12–24 h. Cells were harvested and processed as described in panel A.

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cells expressing Bcl-xL. Nevertheless, the results strongly suggest that HSH2 modulates the BCR-induced apoptotic response of WEHI-231 cells either by directly affecting signal transduction via the BCR or by regulating downstream events (e.g., mitochondrial destabilization) associated with the apoptotic process itself.

The fact that HSH2 is an adaptor protein and that it prevents apoptosis of WEHI-231 cells raised the possibility that this was due to the alteration of one or more signal transduction processes initiated by ligation of the BCR. Indeed, it was initially hypothesized that HSH2 might attenuate BCR-mediated signal transduction, thereby inhibiting the functional apoptotic response. However, the results from experiments to analyze basic components of the pathways that are activated by BCR ligation do not support this conclusion. Overall inducible tyrosine phosphorylation in control and HSH2-expressing cells was comparable, suggesting that protein tyrosine kinase activation is grossly normal. Analysis of Ca\(^{2+}\) mobilization revealed only a slight change in the overall Ca\(^{2+}\) flux response, once again indicating that activation of PLC\(\gamma\) upstream and NFAT activation downstream are likely to be comparable between HSH2-expressing and control cells. Of particular interest was whether HSH2 potentiated activation of AKT, which has been shown to play an important role in promoting survival and proliferation in the B cell (52, 53). Analysis of AKT phosphorylation did not reveal increased phosphorylation of either Thr-308 or Ser-473 on AKT, the phosphorylation of which is correlated with activation of this kinase (54). Thus, it does not appear that HSH2 blocks apoptosis through increased activation of AKT. Finally, activation-dependent phosphorylation of the MAPKs ERK, p38, and JNK was analyzed in control versus HSH2-expressing cells. Although ERK and p38 phosphorylation was comparable, the phosphorylation of JNK was significantly increased in cells that express HSH2. The potential involvement of specific MAPKs in regulation of B cell apoptosis remains to be completely elucidated and is controversial. Studies examining activation of JNK in the context of B cell apoptosis have presented evidence suggesting that JNK activation may actually promote apoptosis (39, 40). Expression of a dominant-negative form of JNK was shown to protect cells from undergoing growth arrest and apoptosis in response to BCR ligation (40). Moreover, pharmacological inhibitors of JNK have been shown to confer protection from apoptosis in a human B cell line (39). In contrast, studies have shown that CD40-mediated signaling and treatment of B cells with CpG DNA, both of which exert potent anti-apoptotic effects, potently induce activation of JNK (55–57). However, it should be noted that activation of JNK under these conditions does not appear to be required for the protective effect of these pro-survival agonists (55–57). Thus, it is not likely that the potentiation of JNK activation observed in these studies is responsible for the ability of HSH2 to attenuate apoptosis.

Because HSH2 does not cause global changes in BCR-mediated signal transduction, experiments were conducted to analyze the effect that HSH2 expression has on processes associated with apoptosis, including mitochondrial depolarization and caspase activation. Importantly, HSH2 was observed to protect against mitochondrial depolarization, which has been shown to play an important role in B cell apoptosis. The mechanism by which HSH2 blocks mitochondrial depolarization remains to be elucidated. However, results from subcellular fractionation studies clearly show that endogenous HSH2 is constitutively localized in the mitochondrial fraction, as well as the cytoplasmic fraction, isolated from WEHI-231 cells. Analysis of FLAG-tagged HSH2 expressed via retroviral transduction of WEHI-231 cells reflected the same pattern of subcellular localization as that observed for endogenous HSH2 (data not shown). Thus, it is possible that HSH2 physically interacts with one or more proteins in the outer membrane of mitochondria, thereby blocking depolarization in a direct manner, or that it recruits other proteins to mitochondria that play a role in stabilization of the membrane. In either case, prevention of mitochondrial destabilization is likely to play an important role in HSH2-dependent inhibition of the apoptotic response.

Analysis of caspase activation, which has been shown to occur in response to mitochondrial destabilization, revealed that HSH2 has only a modest effect on this process. Activation of caspases 9, 7, and 3, as well as PARP cleavage, were observed in control cells and cells transduced with HSH2 but not in those cells that were transduced with Bcl-xL. However, a slight delay in the kinetics of caspase activation was observed, and there did appear to be a slight decrease in the production of caspase and PARP cleavage products in HSH2-transduced cells. Whether the effects of HSH2 on caspase activation are responsible for the observed pro-survival activity of this protein is unclear. Indeed, the importance of caspase activation in apoptosis of WEHI-231 cells is itself controversial based on studies demonstrating a potential role for caspase-independent protease pathways during apoptosis (44, 46, 47). Thus, it is possible that the degree of caspase activation observed in WEHI-231 cells is not physiologically relevant and that other proteases are in fact responsible for mediating apoptosis. In this case, the modest effects of HSH2 on caspase activation may not be biologically significant if other proteases such as cathepsin B or calpain are activated and promote apoptosis (44, 46, 47). Whether HSH2 antagonizes the activation or function of these or other proteases has yet to be determined.

An important finding of these studies that supports the hypothesis that HSH2 functions as an anti-apoptotic regulatory adaptor protein is that its expression directly correlates with survival of WEHI-231 cells. Experiments demonstrated that BCR-induced apoptosis of WEHI-231 cells is accompanied by a significant decrease in HSH2 expression within 12 h. In contrast, simultaneous treatment of WEHI-231 cells with anti-CD40 mAb, which protects cells from undergoing BCR-induced apoptosis, was observed to maintain the level of HSH2 expression. Thus, there is a clear correlation between the expression of HSH2 and survival of WEHI-231 cells. Similarly, when HSH2 is expressed via retroviral transduction of WEHI-231 cells, it was shown that this results in a severalfold increase in the level of HSH2 in transduced cells, which remains constant regardless of whether cells have been stimulated through the BCR. These findings suggest that BCR-mediated signal transduction leads to the activation of one or more proteases that actively degrade HSH2. HSH2 does not contain a consensus caspase proteolytic cleavage site; therefore, it is unlikely that it is degraded via a caspase-dependent mechanism. However, HSH2 does contain a peptide region rich in proline, glutamate, serine, and threonine referred to as a PEST domain. PEST domains have been shown to play a role in promoting rapid proteolytic degradation of proteins mediated by calpains, a family of Ca\(^{2+}\)-dependent cysteine proteases (58–60). Indeed, studies have shown that BCR ligation promotes activation of calpains, leading to activation of caspase-7 and apoptosis in WEHI-231 cells (44). Moreover, it was demonstrated that CD40-dependent signaling promotes up-regulation of the endogenous calpain inhibitor calpastatin, which blocks the function of calpain and is associated with protection from undergoing apoptosis in response to BCR ligation (44, 49). Thus, it is possible that BCR-induced activation of calpains promotes proteolytic degradation of HSH2 and that this is blocked by CD40-mediated up-regulation of calpastatin in WEHI-231 cells. Although it
remains to be formally proven that calpains are responsible for
degradation of HSH2 in response to BCR-mediated signaling,
this represents an attractive hypothesis.

In conclusion, these studies demonstrate that the adaptor
protein HSH2 attenuates the apoptotic response of WEHI-231
cells to BCR ligation. Expression of HSH2 does not result in
global changes in BCR-mediated signal transduction. Thus, it
is unlikely that HSH2 prevents apoptosis through its ability to
quantitatively decrease the BCR signal or by specifically po-
tentiating known pro-survival pathways (e.g. the PI3K-AKT
pathway). However, HSH2 was observed to prevent mitochon-
drial stability remains to be confirmed. In either case,
the findings present a potentially novel paradigm by which an
adaptor protein modulates cell survival.

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The Adaptor Protein HSH2 Attenuates Apoptosis in Response to Ligation of the B Cell Antigen Receptor Complex on the B Lymphoma Cell Line, WEHI-231
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