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Involvement of Bik, a Proapoptotic Member of the Bcl-2 Family, in Surface IgM-Mediated B Cell Apoptosis

Aimin Jiang and Edward A. Clark

Apoptosis plays a central role in shaping the repertoire of circulating mature B lymphocytes, but the underlying molecular mechanisms regulating B cell fate are not well understood. Human B104 B lymphoma cells undergo apoptosis after surface Ig (sIg)M, but not sIgD, ligation; sIgM-mediated apoptosis of B104 cells apparently requires new gene transcription because actinomycin D can inhibit the apoptotic response. Here we report that expression of Bik, a proapoptotic member of the Bcl-2 family, is greatly increased after sIgM ligation. Bik expression was tightly controlled at both transcriptional and post-transcriptional levels. Whereas a calcineurin-dependent pathway was essential for Bik mRNA induction, both the phosphatidylinositol 3-kinase (PI3K)- and the calcineurin-dependent pathways were required for the sustained production of Bik protein. Consistent with these findings, sIgD ligation, which leads to the similar calcium mobilization and increases in Bik mRNA, induced only a transient activation of PI3K and did not lead to sustained Bik protein expression. Furthermore, sustained Bik protein expression correlated with B cell apoptosis, as treatment with either a calcineurin inhibitor or PI3K inhibitors blocked both sIgM-mediated sustained Bik protein induction and apoptosis. In addition, sIgM ligation strongly increased the amount of Bik associated with endogenous Bcl-x, but sIgD ligation did not. Studies with caspase inhibitors also revealed that Bik and Bcl-x interacted upstream of caspases in the B cell apoptosis cascade. Thus, Bik protein induction and, subsequently, sequestering of antiapoptotic Bcl-x by Bik may play an important role in regulating B cell apoptosis. The Journal of Immunology, 2001, 166: 6025–6033.

A hallmark of the immune system is the ability to recognize and eliminate foreign Ags while remaining nonresponsive to self Ags. B lymphocytes recognize and respond to foreign Ags through their B cell Ag receptor (BCR) complexes (1). The BCR complex is composed of surface Ig (sIg), which binds to Ag, and a disulfide-linked heterodimer Igα/Igβ, which is the signaling subunit of the BCR that is essential for the development of B lineage cells (2, 3). Because the assembly of the BCR involves somatic V gene rearrangement, self-reactive BCRs are also produced during B cell maturation. Consequently, tightly regulated programs for cell death are essential for proper selection of B lymphocytes. Signaling through the BCR has different consequences, depending on the developmental stage of the B cell and factors such as strength and duration of the signal (4, 5). Self-reactive immature or transitional immature B lymphocytes (6, 7) are thought to be the targets for negative selection, ultimately eliminated by programmed cell death or apoptosis (8, 9). In vivo analysis of autoantibody-transgenic mice demonstrated that encounter with Ag at an early stage of B cell development ultimately leads to elimination of Ag-reactive clones (10, 11). In vitro studies using purified immature B cells have established that immature B cells can be induced to die, whereas mature B cells proliferate (12–14). Although little is known about the underlying molecular mechanisms, Ag-driven apoptosis of immature B cells seems to be dependent on new gene expression and protein synthesis in contrast to CD95-mediated apoptosis (12, 15, 16).

Apoptosis is essential for normal development, tissue homeostasis, and immune function; its altered regulation can trigger cancer, autoimmunity, and degenerative disorders (17, 18). Genetic studies of the nematode Caenorhabditis elegans have identified three genes, ced-3, ced-4, and ced-9, which are essential for a basic apoptotic pathway and are also conserved in mammals (19–22). Dismantling of the cells is conducted by Ced-3 and its mammalian counterpart caspases, but initiation of many apoptotic responses is regulated by CED-9 and homologous Bcl-2 family members. At least 15 Bcl-2 family members have been identified in mammalian cells and several others in viruses (21, 22). Some members, such as Bcl-2, Bcl-x, A1, Mcl-1 and Bcl-w, promote cell survival, which is opposed by members in two other subfamilies. Close relatives such as Bax exhibit considerable sequence homology with Bcl-2, possessing three of the four Bcl-2 homology (BH) domains, whereas distant homologues such as nematode EGL-1 (23) and mammalian Bad, Bik, Hrk, Bim and Bid share only the short BH3 domain with the Bcl-2 family (21, 22). This domain allows them to bind to the prosurvival Bcl-2 like molecules and neutralize their functions. Different mechanisms including expression levels, dimerization patterns, and post-translational modification are used to regulate different BH3-only proteins in different types of apoptosis (21, 22). Removal of IL-3, an apoptotic signal, results in dephosphorylation of Bad and subsequent interaction between Bad and Bcl-x, triggering apoptosis (24, 25). A more apoptotic form of Bid is generated by caspase 8 cleavage after CD95 engagement (26, 27).

Only a few Bcl-2 family members, such as Bcl-2, Bcl-x, and Bax, have been studied in regard to their roles in immature B cell apoptosis (9). Examination of expression patterns of Bcl-2, Bcl-x, and A1 suggest that different Bcl-2 family members affect B cell...
fate at different stages of development (9). Genetic studies using transgenic mice expressing Bcl-2 and Bcl-x as well as knockout mice suggest that Bcl-x may play a more important role than Bcl-2 in regulation of immature (transitional) B cell apoptosis (28–31). CD40-induced rescue of BCR-mediated apoptosis involves increases in Bcl-x and A1 (32–34), suggesting that the overall balance of pro- and antiapoptotic Bcl-2 family members may affect BCR-regulated cell fate. However, the relative contributions of other Bcl-2 family members in B cell fate—especially BH3-only molecules—have not been addressed.

B104 B lymphoma cells express 20 times more sIgM than sIgD and undergo apoptosis after sIgM ligation but not after sIgD ligation (15, 35, 36). However, ligating either sIgM or sIgD on B104 cells induces similar increases in intracellular cytoplasmic free calcium concentration [Ca2+]i, or extracellular signal-regulated kinase (Erk) activity (35, 36). In this report, we found that Bik (37, 38), a proapoptotic member of the Bcl-2 family, is differentially induced after sIgM vs sIgD ligation. Engagement of sIgM but not sIgD results in sustained Bik protein induction, although both receptors induce similar levels of Bik mRNA. Both the phosphatidylinositol 3-kinase (PI3K) and the calcineurin-dependent pathways are required for sIgM-induced sustained Bik protein expression and apoptosis. Furthermore, sIgM, but not sIgD, ligation induces endogenous Bik to interact with endogenous Bcl-x in vivo. Our results suggest that sIgM-induced Bik may signal apoptosis by sequestering the Bcl-x protein.

Materials and Methods

Reagents and cells

F(ab')2 of goat anti-human IgM and IgD sera were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and Southern Biotechnology Associates (Birmingham, AL). Rabbit anti-human Akt phospho-serine 473-specific sera and rabbit polyclonal Abs to mouse Bik were purchased from Upstate Biotechnology (Lake Placid, NY). Goat anti-Bik, rabbit anti-p38 mitogen-activated protein kinase (MAPK), and rabbit anti-Erk sera were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-Bcl-x serum was purchased from Transduction Laboratories (Lexington, KY). Protein A-Sepharose and protein G-Sepharose were obtained from Amersham Pharma Biotech (Piscataway, NJ). LY 294002, wortmannin, and actinomycin D were purchased from Calbiochem (La Jolla, CA). Cyclosporin A (CsA) was obtained through Novartis Pharmaceuticals (East Hanover, NJ). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD) was purchased from Kamiya Biomedical (Seattle, WA). The B104 human B lymphoma line, originally kindly provided by Dr. M. Mayumi (Kyoto University Hospital, Kyoto, Japan), was grown in culture as described (35).

RNA isolation and RNase protection assays (RPAs)

Total RNAs were extracted using TRIzol reagent (Life Technologies, Grand Island, NY), following the protocol recommended by the manufacturer. RPAs were then performed according to the manufacturer’s instructions, using a RiboQuant RNase protection kit and human APO-2 or Bcl-x as a template. Using a RiboQuant RNase protection kit and human APO-2 or Bcl-x as a template, the RNA samples were then quickly denatured at 90°C and allowed to anneal at 56°C for 12–16 h. The samples were then treated with a RNase A and RNase T1 mixture following the protocol described in the kit. The samples were resolved by a 5% acrylamide sequencing gel that was prepared by adding 2 M urea to the sample RNAs. The gels were then dried and analyzed by autoradiography.

Cell death and flow cytometric assays

Cells (10 × 106 cells/ml) were incubated with either anti-IgM (1 µg/ml) or anti-IgD serum (1 µg/ml) at 37°C for 10–12 h. A total of 1–2 × 106 cells was collected and washed twice with sterile PBS. The pellets were then incubated with FITC-conjugated annexin V (Clontech Laboratories, Palo Alto, CA) for 10 min in the dark. The cells were subsequently analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA). Trypan blue dye exclusion assay was also used to count the percentage of dead cells after 18–24 h. This procedure yielded results similar to those obtained with annexin V assays (data not shown).

Cell lysates, immunoprecipitation, and Western blot analysis

B104 cells were resuspended at 1–1.5 × 106 cells/ml in complete RPMI 1640 medium for 30 min at 37°C before stimulation for the indicated times. Final concentrations of F(ab')2 of either goat anti-human IgM or goat anti-human IgD serum were 1 µg/ml. Wortmannin (1 nM), LY 294002 (5 µM), or CsA (150 ng/ml) was added 30 min before anti-IgM stimulation. Incubations were quickly quenched on ice by addition of 10 vol ice-cold PBS and centrifuged at 500 × g for 8 min at 4°C. Supernatants were aspirated, and cell pellets were washed once with 1 ml PBS before lysis with 500 µl of 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, and 1 mM EDTA (pH 7.5)) with protease inhibitors (1 µM PMSF, 2 µg/ml aprotinin, and 2 µg/ml leupeptin) and phosphatase inhibitors (50 µM NaF and 1 µM sodium orthovanadate). Following incubation on ice for 15 min, the lysed cells were centrifuged at 16,000 × g for 15 min at 4°C. Cell lysates were denatured by boiling with one-third volume of 4× SDS sample buffer, resolved by 12% SDS-PAGE, and transferred to nitrocellulose in non-SDS-containing transfer buffer (25 mM Tris, 0.2 M glycine, and 20% methanol (pH 8.5)). For immunoprecipitations of the p85 subunit of PI3K, the cell lysates were incubated with 1 µg anti-p85 PI3K serum. After mixing samples overnight at 4°C by constant rocking, immune complexes were captured by mixing the samples for 2 h at 4°C with 30 µl packed protein A-Sepharose beads. For either Bik or Bcl-x immunoprecipitation, cell lysates were preincubated with 15 µl packed protein G-Sepharose beads and 1 µg goat IgG or rabbit IgG, respectively. After 3 h of constant rocking at 4°C, the mixtures were centrifuged at 16,000 × g for 1 min at 4°C. The supernatants were added to 20 µl packed protein G-Sepharose beads and 1.5 µg of either goat anti-Bik serum or rabbit anti-Bcl-x serum, then mixtures were incubated with constant rocking at 4°C for another 3 h. The immune complexes were then washed, denatured, resolved by 10% (for PI3K) and 15% SDS-PAGE (for Bik and Bcl-x), transferred to nitrocellulose, and subjected to immunoblotting according to the manufacturer’s instructions.

Data presentation

All experiments shown are representative of between three and five similar experiments.

Results

Apoptosis of B104 cells mediated by sIgM requires new early gene transcription

Early studies have suggested that new gene transcription and new protein synthesis are necessary for sIgM-induced apoptosis (15, 36). To further study the requirements for new gene transcription in B cell apoptosis, we tested the effects of actinomycin D, a transcription inhibitor, on sIgM-induced apoptosis of B104 cells. Actinomycin D has been shown to effectively inhibit new gene transcription in B lymphoma cell lines (39). Anti-IgM stimulation resulted in a strong apoptotic response, leading to a 40% increase in apoptotic cells after 10 h, as detected by annexin V binding (Fig. 1). However, addition of actinomycin D at a concentration (12.5 ng/ml) that has been found not to induce any toxic effects in B104 cells (data not shown) blocked the sIgM-mediated apoptosis (Fig. 1), suggesting that new gene transcription is required for sIgM-mediated apoptosis. The blockage of sIgM-mediated apoptosis by actinomycin D was only observed when actinomycin D was added within 3 h after anti-IgM stimulation (Fig. 1), suggesting that gene transcription essential for apoptosis probably occurred within a few hours.
Bik protein was also affected after sIgM and sIgD ligation. To our surprise, only sIgM stimulation resulted in substantial and sustained Bik protein induction with a maximal 5-fold increase at 6 h (Fig. 2C). Stimulation with sIgD induced only a weak and transient increase in Bik protein expression with a maximal 1.9-fold increase at 30 min. Thus, Bik expression appears to be regulated at a post-transcriptional level, and sustained protein increases of Bik correlate with sIgM-mediated cell death.

Both calcineurin- and PI3K-dependent pathways are required for sIgM-mediated sustained Bik protein expression and apoptosis

Ligation of sIgM induces activation of two major signaling pathways: phospholipase C-γ-mediated calcium signals and PI3K (5, 45–47), so we next examined the relative roles of these pathways in sIgM-induced Bik mRNA expression, Bik protein expression, and apoptosis.

BCR ligation via either sIgM or sIgD leads to a rapid increase of [Ca²⁺], in B104 cells (35, 36), and elevation of [Ca²⁺], in turn activates calcineurin, a phosphatase essential for sIgM-mediated apoptosis of B104 cells (15, 36). Therefore, we tested the involvement of the calcineurin-dependent pathway in Bik mRNA induction. Treatment with CsA completely blocked sIgM-induced Bik mRNA increases (Fig. 3A). Initial sIgM-induced expression of Bik protein during the first 4 h of activation was not blocked by CsA, but Bik protein levels were not sustained in the presence of CsA (Fig. 3B). After 6 h in the presence of CsA, Bik protein level was only slightly above levels in unstimulated cells. One possibility is that the amount of Bik mRNA before activation is sufficient for short-term Bik protein production, because some Bik mRNA was already expressed in unstimulated B104 cells (Fig. 3A). In confirmation of earlier studies (15, 36), CsA also completely blocked sIgM-mediated apoptosis (Fig. 3C). Thus, a calcineurin-dependent pathway is essential for sIgM-mediated Bik mRNA induction, sustained Bik protein expression, and apoptosis.

Upon BCR ligation, the PI3K pathway is activated (47–50), which has also been implicated in regulation of B cell apoptosis (5). Therefore, we next tested the effect of wortmannin, a PI3K inhibitor, on the induction of Bik. Wortmannin only delayed Bik mRNA increases; after sIgM stimulation, Bik mRNA levels still increased 4-fold by 4 h (Fig. 3A), which was similar to the increase after anti-IgM stimulation alone. The delayed induction of Bik mRNA increases in the presence of wortmannin may be due to the fact that wortmannin can inhibit calcium influxes (51). Wortmannin treatment also did not affect rapid induction of Bik protein, but it did reduce sustained Bik protein production to basal levels by 6–10 h (Fig. 3B). Similarly, sustained Bik protein expression was also inhibited by another specific PI3K inhibitor, LY 294002, under conditions where PI3K activity was completely blocked (data not shown). These results suggest that the PI3K-dependent pathway is required for sIgM-mediated sustained Bik protein expression. Both wortmannin and LY 294002 inhibited sIgM-mediated apoptosis (Fig. 3C). Moreover, the efficiency of protection by LY 294002 correlated with its ability to block Akt phosphorylation (Fig. 3C and data not shown). Thus, the PI3K pathway is required for both sIgM-mediated sustained Bik protein expression and apoptosis. Consistent with other studies on IgM vs IgD signaling (52), pretreatment of anti-IgD did not interfere with the apoptotic response caused by sIgM ligation (Fig. 3C), suggesting that additional signals that are essential for apoptosis are generated by sIgM ligation. Together with the fact that sIgM signaling also dominated sIgD signaling for Bik protein expression (data not shown), our
data strongly suggest the involvement of PI3K-dependent Bik protein expression in sIgM-mediated apoptosis of B104 cells. In summary, the PI3K inhibitor wortmannin and calcineurin inhibitor CsA had different effects on Bik mRNA induction and did not affect early increases in Bik protein. However, both agents blocked sIgM-mediated sustained Bik protein expression and apoptosis. Thus, again there was a clear correlation between sIgM-mediated sustained Bik protein expression and apoptosis.

Ligation of sIgM but not sIgD results in a strong and sustained activation of the PI3K pathway

Because wortmannin blocked sustained Bik protein expression (Fig. 3B), we next compared the activation of PI3K after sIgM vs sIgD ligation. Inducible tyrosine phosphorylation of the p110 catalytic subunit and p85 regulatory subunits correlates with sIgM-mediated activation of PI3K in various B cell lines (53); therefore, we measured the phosphorylation of PI3K as an indicator of PI3K activity. Anti-IgM stimulation resulted in a strong and sustained tyrosine phosphorylation of the p110 catalytic subunit with a peak of 3-fold induction at 2 min (Fig. 4A). In contrast, sIgD ligation induced a weaker and transient phosphorylation of p110 (Fig. 4A), suggesting that sIgM and sIgD ligation differ in activating PI3K. Together with the fact that wortmannin blocks sustained Bik protein expression, these results suggest that sIgD ligation may not lead to sustained Bik protein expression in part because of insufficient PI3K activation. It would not be surprising to find that activation of PI3K is required for expression of apoptotic Bik protein and probably apoptosis, because inhibition of PI3K blocks sIgM-induced growth inhibition (54).

We also examined phosphorylation of Akt after sIgM or sIgD ligation, a direct target of PI3K. Phosphorylated Akt was detected within 15 min after sIgM ligation and sustained for at least 3 h (Fig. 4B). In contrast, a much weaker and more transient phosphorylation of Akt was consistently observed after sIgD ligation (Fig. 4B and data not shown). Phosphorylation of Akt in B104 cells is dependent on PI3K because wortmannin and LY 294002 completely blocked sIgM-mediated Akt phosphorylation (data not shown). These data suggest that activation of PI3K-dependent kinases such as Akt may determine whether Bik protein expression is sustained after sIgM vs sIgD stimulation. Further studies are needed to elucidate the role of Akt in Bik expression.

**FIGURE 2.** Stimulation of sIgM but not sIgD stimulation induces both mRNA and protein expression of Bik. A, Stimulation of sIgM and sIgD resulted in changes in the mRNA levels of Bcl-2 family members. RPAs were performed using total RNAs and a human APO-2 template set as described in Materials and Methods. One of five similar experiments is shown. B, Quantification of Bik mRNA levels after sIgM and sIgD stimulation. The resulting gel from RPA was dried, and the amounts of Bik mRNA relative to L32 and GAPDH were quantified by phosphoimaging using a Storm model 840 Phospholmager (Molecular Dynamics). C, Stimulation of sIgM but not sIgD stimulation induced strong and sustained Bik protein expression. B104 cells were stimulated for the indicated times with either anti-IgM or anti-IgD. Cell lysates (100 μg/sample) were analyzed by immunoblotting with either anti-Bik (top) or anti-p38 MAPK (bottom). The relative amounts of the Bik protein were quantified by using densitometry.
necessary to elucidate the connection between PI3K-dependent kinases such as Akt and Bik protein expression.

**slgM stimulation results in increased and sustained interaction between Bik and Bcl-x**

Bik can interact with Bcl-x when the proteins are overexpressed together (37). Bcl-x is required for immature B cell survival (30, 31), and after CD40 ligation, it is up-regulated and can help prevent slgM-mediated apoptosis (32–34). Therefore, we asked whether Bik could interact with Bcl-x under physiological conditions, in this case, after slgM vs slgD engagement. Lysates of B104 cells were immunoprecipitated with anti-Bcl-x serum and then blotted with either a Bik or a Bcl-x antiserum. Bcl-x protein levels were not significantly changed after either slgM or slgD ligation (Fig. 5A). Bik protein was detectable in Bcl-x immunoprecipitates but not in IgG control precipitates from stimulated cells (Fig. 5A), suggesting that Bik-Bcl-x interactions are specific. The amounts of Bik associated with Bcl-x significantly increased after slgM stimulation by 3- to 4-fold at 6 h (Fig. 5A). In contrast, upon slgD stimulation, the amount of Bik decreased after 2 h to 0.6-fold at 4–8 h, despite there being slightly more Bcl-x available (Fig. 5A). Thus, whereas slgM stimulation produced more Bik-Bcl-x interactions, slgD stimulation did not.

Conversely, Bcl-x was also detectable in Bik precipitates (Fig. 5B), confirming that the interaction between Bik and Bcl-x is specific. The amounts of Bcl-x interacting with Bik were again higher after slgM stimulation (Fig. 5B). In contrast, upon slgD stimulation, the total amount of Bik protein expression and Bik protein associated with Bcl-x quickly diminished over time (Fig. 5B). Taken together, our data support a model claiming that sustained Bik protein expression and Bik-Bcl-x interactions play an important role in slgM-mediated apoptosis of B104 cells.

**Bik acts upstream of caspases in B cell apoptosis cascades**

Caspases are also activated during slgM-mediated apoptosis of B104 cells (41, 55). Because the relationship between caspase activation and Bik-Bcl-x regulation was not clear, we tested the effects of caspase inhibitors on Bik-Bcl-x interactions. The relative amounts of Bik mRNA were quantified by phosphoimaging using a Storm model 840 PhosphoImager (Molecular Dynamics). Both calcineurin- and PI3K-dependent pathways are required for slgM-mediated sustained Bik protein expression. Cell lysates (equivalent to 1 × 10^6 cells) were analyzed by immunoblotting with either anti-Bik (top) or anti-p38 MAPK (bottom). The relative amounts of the Bik protein were quantified by using densitometry.

**FIGURE 3.** The involvement of calcineurin- and PI3K-dependent pathways in slgM-mediated Bik mRNA expression, sustained Bik protein expression, and apoptosis. A, Inhibition of calcineurin with CsA and inhibition of PI3K by wortmannin (Wm) affected slgM-mediated Bik mRNA expression differently. B104 cells were stimulated with anti-IgM, CsA plus anti-IgM, and wortmannin plus anti-IgM or anti-IgD for the indicated times. Total RNAs were extracted, and RPA was performed as described in Materials and Methods. The relative amounts of Bik mRNA were quantified by phosphoimaging using a Storm model 840 PhosphoImager (Molecular Dynamics). B, Both calcineurin- and PI3K-dependent pathways are required for slgM-mediated sustained Bik protein expression. Cell lysates (equivalent to 1 × 10^6 cells) were analyzed by immunoblotting with either anti-Bik (top) or anti-p38 MAPK (bottom). The relative amounts of the Bik protein were quantified by using densitometry. C, Treatment of B104 cells with either wortmannin, LY 294002, or CsA inhibits slgM-mediated apoptosis. B104 cells were treated with wortmannin (1 nM), LY 294002 (5 μM), CsA (150 ng/ml), or anti-IgD (1 μg/ml) for 30 min before the addition of anti-IgM (1 μg/ml). Cells were collected 10 h after stimulation, and annexin V assays were performed as described in Materials and Methods. Annexin V binding-positive cells were scored as apoptotic cells.
Bik IS INVOLVED IN B CELL APOPTOSIS

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FIGURE 4. Stimulation of sIgM but not sIgD results in a strong and sustained activation of the PI3K pathway. A, Ligation of sIgM led to stronger and more sustained tyrosine phosphorylation of the p110 catalytic subunit of PI3K than did sIgD stimulation. B104 cells were stimulated with either anti-IgM or anti-IgD serum for the indicated times, then lysed and immunoprecipitated with anti-p85 PI3K mAb; precipitates (equivalent to 6 \times 10^6 cells) were subjected to Western blotting with anti-phosphotyrosine mAb (PY) (top) or anti-p85 PI3K mAb (bottom). The p85 mAb coprecipitates the p110 catalytic subunit of PI3K, the dominant tyrosine phosphorylation bands detected by anti-phosphotyrosine blotting. The relative amounts of the phosphorylated p110 subunit were quantified by using densitometry. B, Stimulation of sIgM results in stronger and more sustained phosphorylation of Akt, a direct downstream target of PI3K, than does sIgD stimulation. Whole-cell lysates (100 \mu g/sample) were analyzed by immunoblotting with either anti-Phospho-S473-Akt (top) or anti-p38 MAPK (bottom). The relative phosphorylation of Akt was quantified by using densitometry.

“apoptosis (41, 55) had no effect on Bcl-x protein expression (Fig. 6A). The sIgM-mediated Bik protein expression was also not significantly changed after the treatment of Z-VAD (Fig. 6A). Z-VAD treatment also did not change the amount of Bcl-x in Bik precipitates or the amount of Bik that coprecipitated with Bcl-x before or after sIgM ligation (Fig. 6B). Thus, inhibition of caspases does not reduce Bik-Bcl-x interactions, suggesting that caspases are activated after Bik and Bcl-x interact during the apoptotic process.

Discussion

In this report, we showed that Bik, a pro-apoptotic Bcl-2 family member, was induced differentially after apoptotic sIgM ligation vs nonapoptotic sIgD ligation. Sustained Bik protein induction after sIgM engagement correlated strongly with B cell apoptosis. Indeed, sIgM stimulation dramatically increased the amount of Bik that associates with Bcl-x, whereas nonapoptotic sIgD stimulation did not. These data support a model claiming that sIgM engagement signals B cells to apoptosis by up-regulating Bik to antagonize the survival function of Bcl-x. Our studies with caspase inhibitors also suggest that Bik and Bcl-x play a role upstream of caspases in B cell apoptosis.

Although the molecular mechanisms are not well understood, agonist-immature B cell apoptosis appears to depend on new gene expression and protein expression (5, 12, 15). Actinomycin D inhibits apoptosis of B104 cells even when added 3 h after sIgM ligation, suggesting that there is a window of new gene transcription that is essential for B cell apoptosis (Fig. 1). Stimulation of sIgM results in a strong and rapid induction of Bik mRNA among other apoptosis-related genes (Fig. 2A and data not shown), making Bik an excellent candidate gene for regulating B cell apoptosis. B104 cells express both sIgM and sIgD, but only sIgM ligation results in apoptosis, and there are several differences in the signaling pathways induced by these receptors (15, 36, 56). However, some early results were puzzling: whereas both increase of [Ca^{2+}], and the downstream calciumurin-dependent pathway are critical for apoptosis, apoptotic sIgM and nonapoptotic sIgD stimulation induce similar increases in [Ca^{2+}] (35, 36). Similar observations were also made of CD38-positive B-chronic lymphocytic leukemia cells: sIgD ligation induces [Ca^{2+}], mobilization and tyrosine kinase activation as efficiently as sIgM ligation, although only sIgM ligation resulted in apoptosis (52). Our data reported here show that expression of Bik is tightly controlled at both the mRNA and protein level. Whereas the calcium/calciurein-dependent pathway is essential for Bik mRNA induction, both the PI3K-dependent and the calcium/calciurein-dependent pathways are required for sIgM-mediated sustained Bik protein expression and apoptosis (Fig. 3). Furthermore, sIgM, but not sIgD, ligation results in strong activation of PI3K and sustained Bik protein expression (Figs. 2, 3, and 4), suggesting that sIgD ligation may not sustain Bik protein expression in part because of insufficient activation of PI3K. Thus, although sIgD ligation results in similar calcium signals and Bik mRNA induction that are essential for B cell apoptosis, it fails to provide a strong enough PI3K-dependent signal for sustained Bik protein expression and apoptosis. Ligation of sIgD, like sIgM ligation, rapidly induces increases in [Ca^{2+}], Erk activity, protein kinase C activity, and Bik mRNA (Refs. 35, 36, 52, and 56 and Fig. 2). The weak and transient activation of PI3K and Akt phosphorylation via sIgD are two of the earliest differences we have detected between sIgM and sIgD signaling in these cells (Fig. 4). Activation of PI3K is required for both sIgM-mediated sustained

FIGURE 5. Ligation of sIgM but not sIgD results in increased and sustained Bik-Bcl-x interactions. A, The amounts of Bik that interact with endogenous Bcl-x were increased after sIgM stimulation but not sIgD stimulation. B104 cells were stimulated for either anti-IgM or anti-IgD serum for the indicated times, lysed, and immunoprecipitated with anti-Bcl-x or anti-Bik serum (bottom). The relative amounts of Bik or Bcl-x were quantified by densitometry. B, Ligation of sIgM but not sIgD increases Bik-Bcl-x association. Whole-cell lysates were immunoprecipitated with anti-Bik serum or a control goat IgG. The final precipitates (equivalent to 5 \times 10^6 cells/lane) were resolved by 15% SDS-PAGE and probed with either anti-phospho-S473-Akt (top) or anti-p38 MAPK (bottom). The relative phosphorylation of Akt was quantified by using densitometry.
Bik protein expression and apoptosis (Fig. 3). Consistent with the model claiming that PI3K-dependent sustained Bik protein expression is required for apoptosis, we and others (52) have also observed that sIgM ligation-induced phenotypes dominated sIgD-induced signals in respect to Bik protein expression as well as apoptosis (Fig. 3C and data not shown). Taken together with the fact that PI3K-dependent Akt is also activated during BCR-mediated apoptosis of several immature B cell lines (57), this suggests that regulation of Bcl-2 family members by the PI3K-Akt pathway may affect cell fate in general. Although the possible proapoptotic role of PI3K and Akt in B cell apoptosis is contrary to the survival role of Akt (24, 25), it has been previously reported that the PI3K pathway can be apoptotic (54). Further studies are necessary to elucidate the underlying mechanisms and context for pro- vs anti-apoptotic signaling. However, we showed that inhibition of Bik protein expression, either through the mRNA level by actinomycin D or CsA or through the protein level by PI3K inhibitors, reduced sIgM-mediated apoptosis (Fig. 3C), suggesting a strong correlation between sIgM-mediated sustained Bik protein expression and apoptosis. Supporting the involvement of Bik in sIgM-mediated apoptosis of B104 cells, previous studies showed that expression of Bik induced apoptosis in a variety of cells including the T lymphoma cell line H9 (37, 38). Tight control of Bik gene expression at multiple steps is probably essential, given the fact that Bik possesses an already expressed BH3 domain and is thus constitutively active (54).

Bcl-2 family members play a major role in regulating apoptosis. The involvement of Bcl-x in regulating immature B cell apoptosis is well established (30, 31). However, the biochemical process by which Bcl-x represses cell death and the mechanisms that regulate its functions are still not well understood. Death-promoting members such as Bad, Bax, Bak, and Bik can form heterodimers with Bcl-x, and their relative concentration may act as a rheostat for the apoptosis program (21). Although a Bik and Bcl-x interaction has not been detected under physiological conditions, several lines of evidence pointed toward that possibility. Exogenous Bik interacts with exogenous Bcl-x when overexpressed, and BH3-dependent heterodimerization of Bik and Bcl-x is required, albeit insufficient, for promoting cell death (37, 60). In addition, Bik is localized around the nuclear envelope and in cytoplasmic membranes (37, 38), whereas Bcl-x is in the cytosol and outer membranes of mitochondria (61, 62); thus, the location of Bik and Bcl-x also favors a possible Bik-Bcl-x interaction. Here, we showed for the first time that endogenous Bik does interact with endogenous Bcl-x and that an apoptotic signal (sIgM ligation) but not a similar nonapoptotic signal (sIgD ligation) increased Bik and Bcl-x association (Fig. 5). The sIgM-mediated sustained Bik protein expression may be necessary for apoptosis probably because high levels of Bik are required to block survival function of Bcl-x (Figs. 3 and 5). Our data support a model claiming that the balance between proapoptotic Bik and antiapoptotic Bcl-x may play an important role in determining B cell fate and that sIgM engagement signals apoptosis in part by up-regulating Bik to antagonize the function of Bcl-x (Fig. 6A). Consistent with a role of Bik-Bcl-x interactions in B cell apoptosis, overexpression of Bcl-x can rescue Bik-induced apoptosis (37); this suggests that the relative proportion of Bcl-x that is not associated with Bik may determine whether B cells survive.

Our studies do not rule out the possible involvement of other proapoptotic Bcl-2 members such as Bax or Bak in B cell apoptosis, and further studies are necessary. Our studies do suggest that it is the accumulation of Bik rather than the initial production of Bik that determines whether a cell undergoes apoptosis (Fig. 3). Possibly, the degradation of Bik protein plays some role in its regulation. Ubiquitin/proteasome-dependent degradation has been shown to play an important role in regulation of Bcl-2, Bax, and Bid and their function in the regulation of apoptosis (63–65). The sIgM-induced Bk-Bcl-x interactions coincide with sustained Bik protein production (Fig. 5), suggesting that the interaction between Bcl-x and Bik may somehow affect the degradation of Bik protein. However, further studies are required to investigate how the Bik-Bcl-x interaction affects the regulation of Bik during B cell apoptosis.

Caspases play a central role in apoptosis, including BCR-mediated apoptosis. Different sets of caspases seem to be used for B cell receptor-mediated apoptosis. In the WEHI 231 B cell line, caspase 7 is activated and responsible for BCR-mediated apoptosis (66). Caspase 2 is activated early and is an initial caspase in IgM-mediated apoptosis of B104 cells (55). Bcl-2 family members can act both upstream and downstream of caspases in regulation of apoptosis; however, the relationship between caspase activation and Bik in B cell apoptosis has not been established. Our studies show that inhibition of caspase activity has no effect on the expression of Bik and Bcl-x and their interaction (Fig. 6); this suggests that caspases act downstream of Bik and Bcl-x during B cell apoptosis. These results are consistent with previous studies showing that Bik-mediated apoptosis was blocked by caspase inhibitors (67). The identification of Bik and Bcl-x as regulators of B cell apoptosis also fits well with several observations of sIgM-mediated apoptosis of B104 cells. Caspase 8 is not activated during apoptosis of B104 cells (55). Consistent with this finding, we found that Bid, another BH3-only Bcl-2 family member that is cleaved and activated by caspase 8 in CD95-mediated apoptosis (26, 27), was not induced after sIgM stimulation (data not shown). Recent findings showed that the apoptotic BH3-only Bcl-2 members Bik and Bid induced cytochrome c release without mitochondria membrane-potential changes (68). This may explain why caspase 9 was not activated early but cytochrome c was still released in the cytosol.
within 2 h after sIgM stimulation (55). In addition, our data suggest that Bcl-x is normally dominant and promotes the survival of unstimulated cells, and only after an apoptotic stimulus does Bik gradually increase and shift the Bik/Bcl-x balance to trigger apoptosis (Fig. 5). It is possible that, under some conditions, very high Bcl-x protein levels, even after apoptotic stimuli (Figs. 5 and 6), prevent activation of caspase 9 (69). However, further studies are needed to elucidate the mechanisms by which the Bik-Bcl-x interaction regulates caspase activation during B cell apoptosis.

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