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B Cell Receptor-Mediated Apoptosis of Human Lymphocytes Is Associated with a New Regulatory Pathway of Bim Isoform Expression

Shahul Mouhamad,* Laurence Besnault,* Marie Thérèse Auffredou,* Corinne Leprince,† Marie Françoise Bourgeade,* Gérald Leca,*† and Aimé Vazquez2*

Studies in Bim-deficient mice have shown that the proapoptotic molecule Bim plays a key role in the control of B cell homeostasis and activation. However, the role of Bim in human B lymphocyte apoptosis is unknown. We show in this study that, depending on the degree of cross-linking, B cell receptors can mediate both Bim-dependent and apparent Bim-independent apoptotic pathways. Cross-linked anti-μ Ab-mediated activation induces an original pathway governing the expression of the various Bim isoforms. This new pathway involves the following three sequential steps: 1) extracellular signal-regulated kinase-dependent phosphorylation of the BimEL isoform, which is produced in large amounts in healthy B cells; 2) proteasome-mediated degradation of phosphorylated BimEL; and 3) increased expression of the shorter apoptotic isoforms BimL and BimS. The Journal of Immunology, 2004, 172: 2084–2091.

APOPTOSIS plays a key role in the establishment and maintenance of the immune system (1). It is one of the major physiological mechanisms regulating selection and regulation of the B lymphocyte pool. B cell apoptosis is mediated principally by the B cell receptor for Ag (BCR) and cell interactions, including signals involving soluble factors or cell-cell interactions (2–7). The molecular machinery of cell death has been studied extensively, and many of the components involved have been identified. The members of the Bcl-2 family of proteins are among the best-characterized regulators of apoptosis (8). These proteins contain Bcl-2 homology (BH) domains and can be classified into three subfamilies on the basis of structural and biological properties (9). Members of the first subgroup, which includes Bcl-2 and Bcl-xL, contain four different BH domains (BH1, BH2, BH3, and BH4) and inhibit some apoptotic pathways (10). The members of the second subgroup, such as Bak and Bak, contain three BH domains (BH1, BH2, and BH3) and are directly involved in the induction of apoptosis, by promoting the release of apoptotic factors from the mitochondria (11). Members of the third subfamily possess only the BH3 domain and are therefore called BH3-only proteins. This subgroup is composed of proapoptotic proteins that may interact with and inhibit the antiapoptotic activity of Bcl-2 or Bcl-xL or directly activate the proapoptotic Bax or Bak proteins. At least nine mammalian BH3-only proteins have been identified to date (Bad, Bik, Hrk, Bid, Noxa, PUMA, Bmf) (12, 13).

Bim is a BH3-only protein that is particularly important in the control of lymphocyte apoptosis (14–17). At least 12 isoforms of Bim are generated by alternative splicing (18–20). However, three isoforms (BimEL, BimL, and BimS) are predominant in various cell types, including lymphocytes (19). All these isoforms induce apoptosis, with the smallest form, BimS, having a stronger proapoptotic effect than the larger BimL and BimEL forms (18). These isoforms also differ in their sequestration to the microtubule-associated dynein motor complex, through binding to dynein light chain LC8 via a short peptide motif present in BimEL and BimL, but absent from BimS. In response to apoptotic stimuli, BimEL and BimL are released from microtubules and transported to the mitochondria, where they associate and inhibit antiapoptotic proteins, such as Bcl-2 (21). Shorter forms, such as BimS, have been shown to bind directly to the proapoptotic Bax protein (13, 20). Bim production may also be regulated transcriptionally in response to signaling by growth factors or cytokines in various cell types (22–27). Phosphorylated Bim has also been detected in hematopoietic and neuron cells, suggesting that, as for other BH3-only proteins, Bim activation may also be regulated by phosphorylation (28–31).

Studies with knockout mice have demonstrated that Bim plays a key role in the control of lymphocyte development. Indeed, large numbers of lymphoid cell accumulate in Bim−/− animals, and this accumulation is associated with changes in immune functions. For instance, thymocytes lacking Bim are refractory to the induction of apoptosis by TCR stimulation. In the absence of Bim, B lymphocyte numbers increase dramatically, and this expansion of the B cell population is associated with the accumulation of serum Igs and the development of autoimmune diseases. In addition, the sensitivity of pre-B cells and autoreactive B cells to apoptotic stimuli was also found to be low in Bim−/− mice (14, 15, 32). These data from animal models show that Bim plays a crucial role in the establishment and control of homeostasis in the B lymphocyte...
pools. However, the precise role of Bim in the control of human B lymphocyte apoptosis is not documented.

We previously reported that, depending on the degree of cross-linking, the BCR can induce two different pathways leading to mitochondrial activation and the death of human B cells. One of these pathways, induced by cross-linked anti-μ Abs, involves mitochondrial activation via the caspase-8-dependent cleavage of BID. The second pathway, which occurs in the presence of soluble anti-μ Abs, results in the caspase-independent activation of mitochondria (5). We investigated the role of Bim in human B lymphocyte apoptosis by studying the involvement of Bim in these two different BCR-induced apoptotic pathways.

We show in this study that Bim expression is required for cross-linked anti-μ Ab-mediated apoptosis in various Burkitt cell lines (Ramos and BL41), whereas it appears not to be required for apoptosis triggered by soluble anti-μ Ab. Moreover, this involvement of Bim is associated with a previously unknown pathway that regulates the expression of Bim isoforms. This novel pathway involves three sequential steps: 1) phosphorylation of BimEL, the predominant isoform in healthy B cells, by extracellular signal-regulated kinase (ERK); 2) proteasome-dependent degradation of phosphorylated BimEL; and 3) de novo expression of Bim, mainly in the form of the more apoptotic isoforms, BimL and BimS.

Materials and Methods

Reagents

The murine DA44 Ab (anti-human IgM, IgG1) was obtained from hybridoma cell lines (American Type Culture Collection (ATCC), Rockville, MD) and was purified from ascitic fluids on protein A-Sepharose columns (Pharmacia, Uppsala, Sweden). The Fab(1')2 of goat anti-mouse IgG (cross-linker Ab (CL)) was obtained from Immunotech (Marseille, France). Human t(μ) was from R&D Systems (Wiesbaden, Germany). Z-Val-Ala-Arg-FLK-fluoromethyl ketone (Z-Val-Ala-Arg-FLK-fmk) was obtained from Bachem Biochemie SARL (Voisin le Bretonneux, France). PMA, MG 262, U0126, PD98059, Go 6983, and Ly-294002 were all obtained from Calbiochem (Strasbourg, France).

Cell lines

The Burkitt’s lymphoma BL41 cell line, kindly provided by A. Calender and G. Lenoir (Lyon, France), does not contain the EBV genome. The Ramos cell line was obtained from ATCC. For Ramos-bcl-2 isolation, the pSSVF-bcl2-neo vector bearing the human bcl-2 CDNA was used to transfect Ramos cells by electroporation, and stable transfectant was isolated. All cell lines were cultured in RPMI 1640 medium with glutamax supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, sodium pyruvate, and nonessential amino acids (from Life Technologies, Strasbourg, France). Human breast carcinoma MCF-7 cells were kindly provided by M. Chelbi-Alix (Institut Andre Lwoff, Villejuif, France).

Detection of apoptotic cells

Cells were washed in PBS, pelleted, and resuspended in PBS. Dot-blot light scatter profiles were analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Shrunken cells with relatively high levels of side light scattering and low levels of forward light scattering were considered to be apoptotic and were counted. The number of apoptotic cells is expressed as a percentage of the total population.

Western blot analysis and immunoprecipitation

Cells were lysed by incubation in 20 mM Tris, pH 7.4, 4 mM MgCl2, 0.5% SDS, and 25 U/ml benzamidine (Merck Eurolab, Strasbourg, France). The samples were centrifuged for 5 min at 4°C at 15,000 × g. The resulting supernatants were boiled for 3 min and frozen at −80°C or used immediately. Aliquots of the supernatants were used for protein determination (micro-bicinchoninic acid protein assay; Pierce, Rockford, IL). The 3× Laemmli buffer with 2-ME and bromophenol blue was added, and cell lysate proteins (30 μg/lane) were resolved by SDS-PAGE in 15% (37.5% acrylamide/1% bis-acrylamide) polyacrylamide gels. Proteins were then electrophoresed onto nitrocellulose filters with 0.45-μm pores, and the filters were blocked by incubation for 1 h with 5% nonfat milk powder in 1× TBS, 0.1% Tween 20. The filters were incubated for 1 h at room temperature or overnight at 4°C with rabbit anti-Bim Ab (Stressgen, Victoria, British Columbia, Canada), anti-Bcl-2 (C-2), anti-Bcl-xL (S-18), anti-phospho-ERK, anti-ERK2, α-tubulin Ab (TU-02) (Santa Cruz Biotechnologie, Santa Cruz, CA), anti-Bax mAb 6A7 (Treigen, Gaithersburg, MD), anti-CoxII Ab (Molecular Probes, Eugene, OR), polyclonal rabbit anti-LC8 antiserum (kindly provided by S. King, University of Connecticut, Farmington, CT) (33), or anti-actin Ab (Sigma-Aldrich, St. Louis, MO). Blots were washed three times for 10 min each, in 1× TBS, 0.1% Tween 20, and were then incubated for 1 h with peroxidase-labeled anti-mouse or anti-rabbit Ig. Blots were developed using the ECL detection system (Pierce). For immunoprecipitation, cells were lysed for 30 min on ice in Nonidet P-40 lysis buffer (50 mM of Tris, pH 7.5, 140 mM of NaCl, 0.1% Nonidet P-40, 5 mM of EDTA, 50 mM of NaF, 2 mM of sodium vanadate, 1 μg/ml aprotinin, 2 mM of PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin A; all from Sigma-Aldrich). The resulting cell lysate was centrifuged for 30 min at 13,000 × g, and incubated for 3 h at 4°C with 5 μg of Ab coated on 25 μl of protein G-Sepharose beads (Pharmacia). Immune complexes were resolved by SDS-PAGE. The gel was then electroblotted onto nitrocellulose membranes that were incubated with various Ab.

RNA interference (RNAi)

BL41 cells (107) were transfected twice at 3 days of interval by electroporation (500 μF, 300 V) with 10 μg of a mixture of three dsRNA DNA hybrids. Twenty-four hours after the second transfection, cells were either stimulated with cross-linked DA44 Ab or t(μ) or left untreated. Oligonucleotides were obtained from Operon Technologies (Palo Alto, CA). Control sense 5'-AGCCACCCCGCAUGUGUAUdTt(TT)-3'; Bim-1, sense 5'-CGCUUUAUGCCAAAGGGGd(TT)-3'; Bim-2, sense 5'-GACCGAGAAGUGAAGGAUGdT(TT)-3'; Bim-3, sense 5'-UUACCAAGCGGCGGAGCt(TT)-3'.

Subcellular fractionation and taxol treatment

BL41 cells (107) were washed twice in PBS and harvested in isotonic mitochondrial buffer (210 mM of mannitol, 70 mM of sucrose, 1 mM of EDTA, and 10 mM of HEPES, pH 7.5) supplemented with complete protease inhibitor cocktail (Sigma-Aldrich). They were homogenized by 30–40 strokes of a Dounce homogenizer. Unbroken cells and nuclei were pelleted by centrifugation at 760 × g for 10 min at 4°C, and are hereafter referred to as the enriched membrane/nuclear fraction. The resulting supernatants were centrifuged at 10,000 × g for 15 min to yield supernatants (cytosolic fraction) and pellets enriched in mitochondria. Taxol treatment was performed according to Puthalakath et al. (21). Briefly, cells were lysed in Triton X-100 lysis buffer (20 mM of Tris-HCl, pH 7.4, 135 mM of NaCl, 1.5 mM of MgCl2, 1% Triton X-100, 10% glycerol) supplemented with 0.5 mg/ml Pefabloc and 1 mg/ml of each for leupeptin, aprotinin, soy bean trypsin inhibitor, and pepstatin (Sigma-Aldrich). Lysates were treated with taxol (5 μg/106 cells in 0.5 ml) (Sigma-Aldrich) and aprotase (2 μ/ml) (Sigma-Aldrich) for 15 min at 30°C. The lysate was then loaded on a 1–0.2% sucrose cushion and centrifuged at 100,000 × g.

Confocal laser-scanning microscopy analysis

Cells were fixed by incubation with methanol for 5 min at −20°C and washed with PBS. They were then permeabilized by incubation with 0.05% Triton X-100 for 5 min at room temperature. After incubation and washings with 10% FCS PBS, the cells were incubated for 1 h at 4°C with rabbit anti-Bim and mouse anti-α-tubulin, each diluted at 20 μg/ml in 2% FCS PBS. The cells were stained with FITC-conjugated AffiniPure goat anti-rabbit IgG and tetrathoradamine isothiocyanate-conjugated AffiniPure donkey anti-mouse IgG from Jackson ImmunoResearch Laboratories (Mar- seille, France). For mitochondrial localization, cells were loaded with Mitotracker CMXRos (red dye; Molecular Probes) before fixation, were then permeabilized and labeled with the desired FITC-conjugated Affi- niPure Abs. Cell images were acquired by confocal microscopy with a CLSM confocal microscope (Leica, Deerfield, IL).

Results

BCR stimulation is associated with the expression of different Bim isoforms, depending on degree of cross-linking

BCR-mediated apoptosis is observed in various types of human B cells depending on the degree of BCR cross-linking. For instance, soluble anti-μ Ab promote apoptosis in Ramos Burkitt lymphoma cells, whereas further cross-linking of BCR is required to induce apoptosis in BL41 Burkitt lymphoma cells (5, 7) (Fig. 1A). We
investigated the role of Bim in these two apoptotic pathways by carrying out Western blotting to analyze Bim expression in non-activated control cells and cells activated by incubation for 48 h with various stimuli. Both Ramos and BL41 cells spontaneously produce large amounts of Bim as the extralarge isoform (BimEL) (Fig. 1A). After 48 h of activation with the soluble murine anti-μ mAb (DA44), BimEL levels remained unchanged, regardless of whether cells did (Ramos) or did not (BL41) undergo apoptosis. In contrast, if apoptosis was triggered in Ramos and BL41 cells by anti-μ Ab cross-linked with F(ab')2 goat anti-murine Ig Ab (DA44 + CL), BimEL levels decreased considerably, whereas levels of large (BimL) and short (BimS) isoforms of Bim increased significantly. In contrast, the induction of BL41 apoptosis by the death receptor TRAIL (as well as TGF-β; data not shown) was not associated with the differential expression of Bim isoforms. We therefore investigated the kinetics of Bim isoform expression upon stimulation with soluble or cross-linked DA44 mAb (Fig. 1B). In activated BL41, the expression of Bim displayed a three-step pattern of regulation. After a short period of stimulation (1 h in Fig. 1), BimEL disappeared and a form with lower levels of electrophoretic mobility was expressed. After 4 h of stimulation, this form of Bim with a higher apparent m.w. disappeared. The third step in the regulation of Bim depended on whether DA44 Ab was cross-linked or not. Upon activation with soluble anti-μ Ab (DA44), BimEL was expressed again, at levels similar to those observed in nonactivated cells (Fig. 1B). This pattern was observed in both Ramos cells (in which DA44 induces apoptosis) and BL41 cells, in which soluble DA44 does not promote apoptosis (Fig. 1, A and B, and data not shown). In contrast, if BL41 cells (Fig. 1B) or Ramos cells (data not shown) were stimulated with cross-linked anti-μ Ab (DA44 + CL), the levels of BimEL produced were lower than those in control cells, whereas the levels of BimL, and to a lesser extent BimS, were higher than those in control cells (Fig. 1B). This regulation of Bim expression was not affected by the broad caspase inhibitor zVAD-fmk (100 μM), showing that the three steps regulating Bim isoform expression induced by cross-linked anti-μ Ab were caspase independent (Fig. 1C).

**FIGURE 1.** BCR stimulation can trigger differential expression of various Bim isoforms. A, Ramos and BL41 cells were cultured for 48 h without (control) or with mouse DA44 mAb (2.5 μg/ml) in the absence (DA44) or presence of goat anti-mouse Ig (20 μg/ml) (DA44 + CL) or TRAIL (200 ng/ml). Whole cell extracts were subjected to SDS-PAGE, and Bim isoforms were detected by immunoblotting with anti-Bim Ab. Apoptotic cells were identified as shrunken cells with high side scatter and low forward scatter properties on flow cytometry. Apoptotic cells were counted, and their numbers were expressed as a percentage of the total number of cells. BL41 cells were activated for various periods of time with either soluble (DA44) or cross-linked DA44 Ab (DA44 + CL) in the absence (B) or presence of zVAD-fmk (100 μM) (C). Bim isoforms were detected by immunoblotting with anti-Bim Ab. Data are representative of at least three different experiments.

We next investigated the mechanisms by which BimEL expression was controlled. BimEL was rapidly converted to a molecular form with a higher apparent m.w. within 5 min of stimulation (Fig. 2A). This conversion involved the phosphorylation of BimEL because after in vitro treatment with acid phosphatase, the Bim present in these lysates displayed an electrophoretic mobility similar to that of BimEL in control cells (Fig. 2B). To determine the nature of the kinases responsible for this phosphorylation of BimEL, we investigated, in preliminary experiments, the effect of a large panel of inhibitors specific for the various kinases activated after BCR stimulation (protein kinase C (PKC), protein kinase A, Btk, Src, Syk, phosphatidylinositol-3 kinase, mitogen-activated protein kinase, ERK, p38, and c-Jun N-terminal kinase (JNK); data not shown). From these experiments, we observed that the phosphorylation of BimEL was prevented by two different inhibitors (U0126 and PD98059) of mitogen-activated protein/ERK kinase-dependent ERK activation and by the PKC inhibitor Go 6983, whereas the phosphatidylinositol-3 kinase inhibitor Ly-294002 had no effect (Fig. 2C). In addition, we also observed BCR-mediated ERK phosphorylation was decreased by U0126 and the PKC inhibitor Go 6983, suggesting that under these conditions, BCR-triggered ERK activation is also partly regulated by PKC (4). Because putative sites of ERK phosphorylation were present in the region of BimEL interacting with the LC8 dynein L chain (21, 29, 34), we investigated whether this phosphorylation of BimEL modified Bim behavior by interfering with the binding of this protein to LC8. Similar amounts of LC8 were associated with Bim (Fig. 2D), regardless of whether the immunoprecipitated BimEL was in nonphosphorylated (nonactivated cells) or phosphorylated (BL41 cells stimulated for 30 min) form. Thus, BimEL is associated with LC8 in Burkitt cells, and BCR-mediated phosphorylation of BimEL does not disrupt this association. As the phosphorylation of BimEL was followed by its disappearance and as proteasome-dependent protein degradation is often associated with phosphorylation, we then investigated whether the down-regulation of BimEL expression resulted from proteasome-dependent degradation. Indeed, the

*BiM isoforms and human BCR-mediated apoptosis*

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prior treatment of BL41 cells with the proteasome inhibitor MG 262 (100 nM) prevented the disappearance of the phosphorylated form of BimEL observed after stimulation with soluble or cross-linked DA44 Ab (DA44 + CL) in the absence (−) or presence (+) of the proteasome inhibitor MG 262 (100 nM). F. BimEL was detectable by immunoblotting for 1 or 24 h without (O) or with cross-linked DA44 mAb (DA44 + CL) in the absence (DA44 + CL) or presence of U0126 (40 μM) (DA44 + CL/U0126). BimEL was present in the soluble fraction, together with Bcl-2, and only a small proportion of BimEL was found in the insoluble fraction containing the microtubules. Thus, only a small proportion of BimEL is associated with microtubules in healthy B cells. Following DA44 + CL activation, Bim showed a less diffuse distribution in BL41 cells, giving a punctuate pattern of staining (Fig. 4Ac) consistent with mitochondrial localization. Indeed, co-staining with Mito Tracker red showed that DA44 + CL stimulation led to the translocation of Bim to mitochondria (Fig. 4Ad). This translocation was confirmed by subcellular fractionation, which showed that following activation with DA44 + CL for 48 h, Bim (particularly the BimL and BimS isoforms) was concentrated in the mitochondrial-enriched fractions (Fig. 4C). Thus, BimEL displayed a diffuse cellular distribution without preferential location in the microtubules in nonapoptotic human B cells, whereas Bim, and particularly the more proapoptotic BimL and BimS isoforms, was detected in the mitochondria following apoptotic stimulation by cross-linked anti-μ Ab (DA44 + CL).

**Subcellular distribution of Bim in B cells**

Bim has been reported to be sequestered in microtubules in nonapoptotic cells (21). We therefore investigated the cellular distribution of BimEL in nonactivated BL41 cells. Confocal microscopy showed that BimEL was not exclusively associated with microtubules in healthy BL41 cells (Fig. 4Aa). Bim was found to be more diffusely distributed in B cells, including the nucleus, and displayed a granular pattern of staining in the cytoplasm consistent with microtubule-independent association with intracellular membranes. As previously reported (21), Bim was more closely associated with microtubules in human breast carcinoma MCF-7 cells (Fig. 4Ab). To confirm these microscopy observations, we next separated soluble and insoluble tubulin by treating of Triton X-100 BL41 cell lysates with taxol to stabilize the microtubules, as previously described (21). We then used Western blotting to investigate BimEL levels in the soluble (Fig. 4B, lane S) and insoluble (lane P) fractions, and to compare these fractions with the unfractionated sample (lane T). In nonactivated BL41 cells, most of the BimEL was present in the soluble fraction, together with Bcl-2, and only a small proportion of BimEL was found in the insoluble fraction containing the microtubules. Thus, only a small proportion of BimEL is associated with microtubules in healthy B cells. Following DA44 + CL activation, Bim showed a less diffuse distribution in BL41 cells, giving a punctuate pattern of staining (Fig. 4Ac) consistent with mitochondrial localization. Indeed, co-staining with Mito Tracker red showed that DA44 + CL stimulation led to the translocation of Bim to mitochondria (Fig. 4Ad). This translocation was confirmed by subcellular fractionation, which showed that following activation with DA44 + CL for 48 h, Bim (particularly the BimL and BimS isoforms) was concentrated in the mitochondrial-enriched fractions (Fig. 4C). Thus, BimEL displayed a diffuse cellular distribution without preferential location in the microtubules in nonapoptotic human B cells, whereas Bim, and particularly the more proapoptotic BimL and BimS isoforms, was detected in the mitochondria following apoptotic stimulation by cross-linked anti-μ Ab (DA44 + CL).
Bcl-2 in nonactivated and apoptotic BL41 cells. Bcl-2 was present We investigated the binding of the various isoforms of Bim to Involvement of Bim in DA44/H11001 Down-regulation of Bim expression inhibits DA44 FIGURE 3. 2088 BIM ISOFORMS AND HUMAN BCR-MEDIATED APOPTOSIS fi ed as shrunken cells with high side scatter and low forward were identi c for human actin. Apoptotic cells fi with anti-Bim Ab. The amount of protein loaded in each lane was deter- of PMA (6 ng/ml), and Bim isoforms were detected by immunoblotting B, or rTRAIL (200 nM)) in the absence or the presence combination of three different RNAi speci fi s, BL41 cells were transfected twice with either control RNAi ( ), or Ramos cells ( ) or normal Ramos and BL41 cells (Fig. 5D). We found that the overexpression of Bcl-2 inhibited the apoptosis of Ramos cells mediated by soluble anti-μm Ab (DA44). In contrast, DA44 + CL treatment resulted in similar levels of apoptosis in all three cell lines, regardless of their level of Bcl-2 expression. Furthermore, the pattern of Bim isoform expression promoted by DA44 + CL activation was similar (decrease in BimEL levels and increase in BimL and BimS levels), regardless of the amount of Bcl-2 present in these cells. Altogether, these data indicate that the role of Bim during DA44 + CL-mediated apoptosis seems to be independent of the binding of this molecule to Bcl-2. BimL, and principally BimS, isoforms were detected in Bax immune complexes isolated from DA44 + CL-activated BL41 cells (Fig. 5E), suggesting that interactions between Bax and the BimL and BimS isoforms may be important for the apoptosis of human B cells induced by cross- linked anti-μ Ab.

Discussion
Observations in Bim−/− mice have shown that Bim plays a critical role in controlling the homeostasis and activation of murine B lymphocytes (15). However, the precise role of Bim in the BCR-mediated activation leading to apoptosis in B cells is unclear. In Burkitt’s lymphoma cells, which can be considered to be a tumoral equivalent of normal germinal center B lymphocytes (37), we previously observed that, depending on the degree to which it was cross-linked with anti-μ Ab, human BCR activates two different apoptotic pathways that merge at the mitochondrial level. One of these pathways, activated in the Ramos Burkitt’s lymphoma cell line, when stimulated with soluble anti-μ Ab, leads to caspase-independent mitochondrial activation. In contrast, the second pathway, observed in the BL41 Burkitt’s lymphoma cell line stimulated with cross-linked anti-μ Ab, is caspase-8 dependent and associated with the cleavage and activation of the BH3-only protein Bid (5, 38). In this study, we found that these two pathways also differ in terms of the role played by Bim. Indeed, the down-regulation of Bim expression did not affect soluble anti-μ Ab-mediated apoptosis in Ramos cells (Fig. 3B). In contrast, cross-linked anti-μ Ab-mediated apoptosis was highly sensitive to Bim expression (Fig. 3, A and C). Consistent with data obtained in Bim−/− mice, the apoptotic signal provided by the death receptor TRAIL, in these cells was not affected by the absence of Bim (15). However, the biological relevance of this observation remains to be precise. These two pathways may reflect the diversity of apoptotic signals provided by the BCR throughout the emergence of

Involvement of Bim in DA44 + CL-mediated B cell apoptosis does not depend on binding to Bcl-2
We investigated the binding of the various isoforms of Bim to Bcl-2 in nonactivated and apoptotic BL41 cells. Bcl-2 was present in the Bim immune complexes prepared from control BL41 cells (Fig. 5A). After 48 h of activation with DA44 + CL, the amount of Bcl-2 present in Bim immune complexes had increased only slightly. Similar results were obtained when Bcl-xL levels were determined in these various Bim immune complexes (Fig. 5A). Furthermore, the immunoprecipitation of Bcl-2 from lysates of DA44 + CL-activated BL41 cells showed that only BimEL bound to Bcl-2 in these cells, with no BimL or BimS detected in Bcl-2 immune complexes (Fig. 5B, lane labeled Bcl-2) in conditions in which BimL and BimS were present in the crude supernatants (Fig. 5B, lane labeled pre-IP lysate) and Bcl-2 levels remained constant following DA44 + CL activation (Fig. 5C). Thus, the BimEL present in healthy cells can associate with Bcl-2 without inducing apoptosis, and the production of BimL and BimS in apoptotic cells is not associated with a major increase in Bim/Bcl-2 complexes, suggesting that Bim activity may be independent of Bcl-2 in this context. To verify this point, we therefore investigated whether the overproduction of Bcl-2 affected BCR-mediated apoptosis and Bim isoform expression. For this, we compared the response to DA44 or DA44 + CL activation of a Ramos cell clone stably transfected with the human bcl-2 gene (Ramos-Bcl-2) with that of normal Ramos and BL41 cells (Fig. 5D). We found that the overexpression of Bcl-2 inhibited the apoptosis of Ramos cells mediated by soluble anti-μm Ab (DA44). In contrast, DA44 + CL treatment resulted in similar levels of apoptosis in all three cell lines, regardless of their level of Bcl-2 expression. Furthermore, the pattern of Bim isoform expression promoted by DA44 + CL activation was similar (decrease in BimEL levels and increase in BimL and BimS levels), regardless of the amount of Bcl-2 present in these cells. Altogether, these data indicate that the role of Bim during DA44 + CL-mediated apoptosis seems to be independent of the binding of this molecule to Bcl-2. BimL, and principally BimS, isoforms were detected in Bax immune complexes isolated from DA44 + CL-activated BL41 cells (Fig. 5E), suggesting that interactions between Bax and the BimL and BimS isoforms may be important for the apoptosis of human B cells induced by cross-linked anti-μ Ab.
mature B cells and during germinal center activation, resulting in terminal B cell differentiation and the selection of memory B cells. Indeed, we previously reported that these two BCR-mediated apoptotic pathways are affected differently by costimulatory signals, such as CD40, which are important coregulators of B cell activation (2, 5, 39).

**FIGURE 4.** Subcellular distribution of Bim in B cells. A, BL41 cells (a), MCF-7 cells (b), or BL41 cells activated by incubation for 48 h with cross-linked DA44 Ab in the presence of zVAD-fmk (100 μM) (c and d) were fixed and stained with rabbit anti-Bim Ab, followed by FITC-labeled donkey anti-rabbit and murine anti-α-tubulin Ab, followed by goat red-labeled anti-murine Ab (a, b, and c) or incubated for 30 min with Mito Tracker red dye (d) and analyzed by confocal microscopy. The expression of Bim isoforms was detected by immunoblotting with anti-Bim Ab. B, Soluble (S) and insoluble (P) fractions were prepared by treating Triton X-100 BL41 cell lysates with taxol to fix cytoskeleton proteins and centrifugation. Total cell extracts (T) served as controls. Proteins were separated by gel electrophoresis, and membranes were probed with Abs against Bim, Bcl-2, LC8, or α-tubulin. C, Lysates from BL41 cells activated by incubation with cross-linked DA44 Ab for 48 h were separated into cytosolic (cyt.), enriched membrane/nuclei (m/n), and enriched mitochondrial (mito.) fractions. A total of 30 μg of each fraction was separated by gel electrophoresis and analyzed by immunoblotting with Abs against Bim, CoxII, LC8, α-tubulin, Bcl-2, or actin. Data are representative of at least two different experiments.

**FIGURE 5.** Involvement of Bim in DA44 + CL-mediated B cell apoptosis is not dependent on its binding to Bcl-2. A, Lysates from nonactivated BL41 cells (0) or BL41 cells activated by incubation for 48 h with DA44 + CL (48) were incubated with rabbit Ig control (Ig) or Bim Ab (Bim), and immune complexes were analyzed by immunoblotting with Ab against Bcl-2, Bcl-xL, and Bim. B, BL41 cell lysates were cultured for 48 h with cross-linked DA44 mAb. Cell lysates were immunoprecipitated with Ab against Bcl-2. Crude lysates (Lysate) or Bcl-2 immune complexes were separated by gel electrophoresis and analyzed by immunoblotting with Abs against Bim and Bcl-2. C, BL41 cells were activated by incubation for various periods of time with cross-linked DA44 Ab, and Bcl-2 levels in crude supernatants were analyzed by immunoblotting with anti-Bcl-2 Ab. D, Bcl-2 levels were quantified by immunoblotting in lysates from nonactivated BL41, Ramos, and Bcl-2-transfected Ramos cells. BL41, Ramos, and Ramos-Bcl 2 cells were cultured for 48 h without (control) or with cross-linked (DA44 + CL) or soluble DA44 mAb (DA44). Apoptotic cells were assessed by flow cytometry, as in Fig. 1, and Bim isoforms were detected by immunoblotting. E, BL41 cells were cultured for 48 h with cross-linked DA44 mAb. Cell lysates were incubated with control murine Ig or anti-Bax Ab. Crude lysates (Lysate), control Ig, or Bax immune complexes were separated by gel electrophoresis and analyzed by immunoblotting with anti-Bim Ab. Data are representative of at least three different experiments.
Nonapoptotic human B lymphocytes, including human resting tonsilar B cells and the Burkitt’s lymphoma cell lines Ramos and BL41, produce large amounts of Bim, primarily in the extralarge isoform (BimEL). We found that human BCR triggered an original pathway leading to a regulated expression of Bim through its various isoforms. This pathway involved three different steps: 1) ERK-mediated phosphorylation of BimEL; 2) proteasome-dependent degradation of phosphorylated BimEL; and 3) a third step that differs according to the nature of BCR activation. Soluble anti-μ Ab reinitiated BimEL production at levels similar to those in non-activated cells. In contrast, cross-linked anti-μ Ab triggered only low levels of BimEL, and instead induced the expression of two smaller isoforms of Bim: BimL and BimS. These isoforms differ in apoptotic potential. Indeed, as already reported in other systems (18, 20), overexpression of the smaller form BimS had a greater apoptotic effect than did the overexpression of BimEL, whereas BimL exhibited an intermediate apoptotic power in BL41 cells (data not shown). The molecular basis of the differences in pro-apoptotic properties of the Bim isoforms is not yet fully understood, but various observations have suggested that the ability of the various isoforms to bind LC8 may play an important role. Indeed, rBimL, mutated within the LC8-binding domain, was found to induce more strongly apoptosis than wild-type rBimL in transfected cells (21). Differences in the ability of the various isoforms to bind with other molecules more directly involved in the apoptotic process, such as Bcl-2 and Bax, may also account for these differences (see below) (13, 20, 21, 40, 41). Nevertheless, the observation that cross-linked anti-μ Ab stimulation leads to the preferential up-regulation of expression of the more apoptotic isoforms, BimL and BimS, is consistent with the involvement of Bim in this BCR-mediated apoptotic pathway.

Two major pathways of Bim expression have been described in other cell types. A first pathway, observed in cells that do not spontaneously produce Bim, such as neurons, fibroblasts, and thymocytes, is dependent on the de novo synthesis of Bim in response to apoptotic stimuli or serum deprivation (22–27). The second pathway, observed in Bim-transfected cells and tumor cells such as MCF-7, is based on changes in the localization of Bim within cells. Thus, in healthy cells, Bim is sequestered to the microtubule-associated dynein motor through its binding to LC8. In response to apoptotic stimuli, Bim is released from microtubules and translocated to the mitochondria, where it binds Bcl-2 and neutralizes its antiapoptotic activity (21). Lei and Davis (30) recently reported that the release of Bim from dynein complexes could be controlled by the JNK-dependent phosphorylation of Bim in response to UV radiation and serum deprivation. Various other groups have also reported that the JNK pathway can control the de novo expression of Bim (22–24). In our experimental conditions, we found no evidence for the involvement of JNK in Bim regulation in B cells. Indeed, none of the three steps regulating the expression and subcellular distribution of Bim was sensitive to specific inhibitors of JNK. Furthermore, no JNK activity was detected in Burkitt cells activated with soluble or cross-linked anti-μ Ab (data not shown). These results suggest that the mechanism of Bim activation differs in B cells and other cell types. We found that Bim expression in B cells was regulated by another member of the mitogen-activated protein kinase family: ERK. In our experimental conditions, ERK plays a different role to JNK. Although the region identified as important for the binding of BimEL to LC8 has several ERK phosphorylation sites (such as Ser1109 and Thr1110, as reported by Biswas and Greene (29)), our data show that ERK-mediated BimEL phosphorylation did not dissociate LC8 from BimEL. Instead, this phosphorylation led to the degradation of BimEL by the proteasome pathway. The nature of the phosphorylated residues responsible for proteasome activity is still unclear, but these residues must be present in BimEL and BimL, but not in BimS. When very small amounts of BimL and BimS were detected in BL41 or Ramos cells, anti-μ Ab and PMA stimulation also promoted the phosphorylation of BimL, but not of BimS (Figs. 1 and 3). It also remains to verify whether PKC can directly phosphorylate specific residues in Bim different from those phosphorylated by ERK or whether it acts indirectly, mediating ERK activation, as previously reported (35, 36).

The biological relevance of this proteasome-dependent degradation of Bim is unclear, but should be considered in conjunction with the specific pattern of BimEL production and/or activity in healthy B cells. However, this activity, if any, is not prevented by sequestration in microtubules because we found only small amounts of BimEL associated with these structures and BCR-mediated activation did not lead to the dissociation of BimEL from LC8 or the microtubules. Instead, it led to the degradation of BimEL. Such regulation of BimEL expression is likely to be an important step facilitating complete redistribution of the various Bim isoforms produced in activated and/or apoptotic B cells. Activation by soluble anti-μ Ab induced the reinitiation of BimEL production, whereas activation with cross-linked anti-μ Ab completely changed the nature of the signal associated with Bim, with the more apoptotic isoforms BimL and BimS present and active. In addition, as previously described in autoreactive murine thymocytes (14), the differential production of Bim protein observed in our experimental condition was not associated with significant modification of the mRNA levels for the various Bim splicing variants (data not shown), indicating that posttranscriptional events were responsible for differences in Bim protein levels in both healthy and apoptotic B cells.

Various studies have revealed that Bim binds directly to the Bcl-2 and Bcl-xL molecules localized in the mitochondria, thereby inhibiting their antiapoptotic activities (14, 18, 20). Our data strongly suggested that Bim acts via a different mechanism in activation due to cross-linked anti-μ Ab. Bim was found to be present in mitochondria after activation with cross-linked anti-μ, but the level of binding to Bcl-2 or Bcl-xL molecules was similar in apoptotic and control cells (similar results were observed in healthy or soluble anti-μ Ab-activated cells; data not shown). Moreover, only the BimEL isoform of Bim was associated with Bcl-2, and we observed no binding of the BimL or BimS isoforms to Bcl-2. In addition, Bcl-2 overexpression in Ramos cells did not prevent the induction of apoptosis by cross-linked anti-μ Ab, suggesting that Bim activity during BCR-mediated cell apoptosis is probably independent of binding to Bcl-2. Interestingly, studies in Bim−/− mice also showed that lymphocytes from these mice and lymphocytes from transgenic Bcl-2 animals display different responses to various apoptotic stimuli. In particular, the absence of Bim inhibits the apoptosis of immature murine thymocytes more strongly than the overexpression of Bcl-2, which suggests that the mechanism by which Bim induces apoptosis may be less sensitive to Bcl-2 (14, 15). It has been shown that Bim, or at least some shorter isoforms of Bim such as BimS and BimAD, can bind to Bax, thereby directly activating this proapoptotic molecule (13, 20, 42). Such a mechanism may also be involved in our experimental conditions because, after activation with cross-linked anti-μ Ab, weak binding was observed between Bax and Bim, involving the BimS isoform preferentially. We have previously reported that cross-linked anti-μ Ab-mediated mitochondrial activation in BL41 cells depends on the caspase-8/Bid pathway (5). Thus, it is possible that Bim (through its shorter isoforms) may synergistically strengthen Bid-mediated Bax activation through a currently unknown mechanism rather than being directly responsible for Bax.
activation. The original pathway regulating Bim isoform expression in human B cells activated by cross-linked DA44Ab is thus relevant and pertinent to specific production of the Bim isoforms involved in the mitochondrial activation required for BCR-mediated apoptosis.

In conclusion, we show that depending on the extent to which it is cross-linked, human BCR may mediate Bim-dependent or apparent Bim-independent apoptosis. Moreover, the involvement of Bim in BCR-mediated apoptosis reveals a novel pathway for Bim regulation based on sequential phosphorylation by ERK and pro tease-dependent degradation of the BimEL isoform in healthy B cells, followed by an increased expression of the apoptotic isoforms BimL and BimS. Thus, the various isoforms of Bim play a critical role in B cell apoptosis, and our results provide new insight into an original mechanism controlling Bim expression and activity during BCR-mediated apoptosis, resulting in the control of human B lymphocyte homeostasis.

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References