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B Cell Apoptosis Triggered by Antigen Receptor Ligation Proceeds Via a Novel Caspase-Dependent Pathway

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In contrast to positive signaling leading to proliferation, the mechanisms involved in negative signaling culminating in apoptosis after B cell Ag receptor (BCR) ligation have received little study. We find that apoptosis induced by BCR cross-linking on EBV-negative mature and immature human B cell lines involves the following sequential, required events: a cyclosporin A-inhibitable, likely calcineurin-mediated step; and activation of caspase-2, -3, and -9. Caspase-2 is activated early and plays a major role in the apoptotic pathway, while caspase-9 is activated later in the apoptotic pathway and most likely functions to amplify the apoptotic signal. Caspase-8 and -1, which are activated by ligation of the CD95 and TNF-R1 death receptors, are not involved. Apoptosis induced by BCR ligation thus proceeds via a previously unreported intracellular signaling pathway. The Journal of Immunology, 1999, 163: 2483–2491.

Apoptosis, programmed cell death, or the cellular suicide program is a fundamental biological process that plays requisite roles in the development, differentiation, and maintenance of the cells of multicellular organisms. Inappropriate or dysregulated apoptosis, or failure to undergo programmed cell death has been implicated in a number of diseases and pathologic conditions (1). In the immune system, ligation of the Ag receptors on T and B lymphocytes (TCR (1), BCR3) may trigger survival signals leading to proliferation and differentiation or, alternatively, negative signals leading to apoptosis (2). Cellular selection between positive and negative signaling responses is determined by many factors, including cellular maturation state and tissue location, the nature and intensity of TCR or BCR ligation, and coligation of additional cell surface receptors in the appropriate temporal order (3, 4). The importance of apoptosis triggered by TCR ligation in shaping the T cell repertoire in the thymus and in homeostasis of mature peripheral T cells has been well documented (2, 3).

Considerable progress has been made in identifying the kinases, phosphatases, and signaling events that mediate proliferation after TCR or BCR ligation (15, 16). The same receptor-proximal signaling pathways are triggered in cells that die, rather than proliferate in response to Ag receptor cross-linking (3, 4). Apoptosis induced by TCR or BCR ligation is also undoubtedly additionally dependent on activation of caspase-type proteases, which form a death pathway able to mediate cell death by interfering with critical cellular functions and by disrupting cellular and genomic integrity (17). Although relatively little is known of caspase involvement in TCR- and BCR-induced apoptosis, activation of caspase-3 (Yama, apopain) has been found to precede apoptosis triggered by TCR ligation on murine thymocytes in vitro and in vivo in correlation with negative selection; and caspase-3 inhibitors have been reported to block such apoptosis (18, 19). Evidence for caspase-3 activation after BCR ligation has also been reported (20–22). The present studies, obtained with human B cell lines with mature and immature phenotypes, indicate that apoptosis induced by BCR ligation proceeds via a previously unreported caspase-2-, -3-, and -9-dependent pathway.
Materials and Methods

Cells and plasmids

Rat-1 fibroblasts were obtained from Dr. J. Jackson (The Scripps Research Institute, La Jolla, CA). The B104 human B lymphoma cell line was kindly provided by Dr. M. Mayumi (Fukui Medical University, Fukui, Japan) (23). This EBV-negative cell line, derived from a child with malignant lymphoma, expresses CD10, CD19, CD20, CD21, CD35, and CD40, as well as IgA and surface IgM and IgD with κ light chains. The B104 cell line does not express CD23 or surface IgG, IgA, or IgE. The DND Burkitt lymphoma B cell line was also obtained from Dr. Mayumi (24). This EBV-negative cell line expresses CD11a, CD18, CD40, and CD54, as well as surface IgM and IgD; other characteristics have not been reported. The ST486 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cell line is EBV negative and expresses surface IgM and IgA with κ light chains, but lacks surface IgG and IgD (25); other characteristics have not been reported. A clone ST486 cell line (ST486-M) was obtained by limiting dilution. The lymphoma cell lines were cultured in RPMI 1640 containing 10% FCS.

Flag-tagged crmA cDNA was created by PCR amplification from plasmid pBSR3 (kindly provided by Dr. D. Pickup, Duke University Medical Center, Durham, NC) by using the following primers: 5′-CCG GAA TTC ACC ATG GAC TAC AAA GAC GAT GAC AAG ATG ATT ACC ACC ACC ACC ATC TGG -3′ and 5′-GGG GCC GGC GGT ACC GCT TAT TTC TTT AGT TGT TGG AGA GCA-3′. The amplified flag-tagged crmA fragment was digested with EcoRI and inserted into the EcoRI site of pcDNA3 (Invitrogen, San Diego, CA). The pHook-2 vector was also from Invitrogen. The pRSC-lacZ vector and a dominant-negative (DN) caspase-9 mutant (C287A) in pRSC-lacZ have been described (26). The wt (1) caspase-2 was directed mutagenesis and inserted into pRSV-lacZ and pcDNA3 (Invitrogen, Cambridge, MA) (27). To generate DN caspase-2, the active site cysteine was mutated to alanine (C303A, TGC GCC) by site-directed mutagenesis and inserted into pRSV-lacZ and pcDNA3 (Invitrogen, Carlsbad, CA).

DNA transfection

Transient expression of crmA in B104 cells was induced by electroporation (250 V, 960 μF). B104 cells (1 × 10^7 cells) were mixed with 1 μg of vector only, or 10 μg of pcDNA3/flag-crmA together with 1 μg of pHook-2 vector (10:1). Transfected cells expressing a single chain Ab on their membranes were isolated 72 h after transfection by their reactivity with hapten-coated magnetic beads (pHook2; Invitrogen). CrmA expression was determined by the Western blotting procedure using anti-flag M2 mAb (Eastman Kodak, New Haven, CT). Rat-1 fibroblasts were transiently transfected with wt caspase-2 in the pBact H372 vector, DN caspase-2 in the pRSV-lacZ vector, wt caspase-2 in the pBact H372 vector plus DN caspase-2 in either the pRSV-lacZ or pcDNA3 vectors, or DN caspase-9 in the pRSV-lacZ vector using lipofectamine (Life Technologies, Gaithersburg, MD). Double-sized samples (10^6 cells) and double-thickness (1.5-mm) gels were used. The western blots were reprobed with mouse anti-actin mAb clone C4 (ICN Biochemicals, Aurora, OH) to verify equal loading.

Peptide inhibition studies were of two types. In the first, B cells (2 × 10^7 cells) were incubated for 10 min with 100 μg/ml aprotinin (Sigma, St. Louis, MO). After 10 min at 37°C, the lysates were centrifuged at 13,000 × g for 20 min, and aliquots (0.5 ml) of the clear cytosolic supernatants were incubated with 2 μM zVAD-fmk and zYVAD-fmk (Enzyme Systems Products), or zVAD-fmk to test for anti-IGM for the times specified in the text were subjected to SDS-PAGE analysis, and Western blotting. Western blotting with anti-caspase-3 or -8 (Phoenix Pharmaceuticals, Mountain View, CA) was also evaluated in Western blotting studies using mAb C2-10 (from Dr. G. Poirier, Quebec, Canada). Blots were routinely stripped (100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 70°C for 30 min, and reprobed with mouse anti-actin mAb clone C4 (ICN Biochemicals, Aurora, OH) to verify equal loading.

Apoptosis induction and detection

B104, ST486-M, and DND-39 cells were treated with 1 μg/ml purified DA4.4 mAb IgG to human IgM (ATCC) for the times specified in the various experiments. Cell viability was determined by the MTT assay system (Chemicon International, Temecula, CA) and occasionally by trypan blue exclusion. B104 cells transiently transfected with wt caspase-2 in the pRSV-lacZ vector and a dominant-negative (DN) caspase-9 mutant (C287A) in pRSC-lacZ have been described (26). The wt (1) caspase-2 was also evaluated in Western blotting studies using mAb C-2-10 (from Dr. J. Yuan (Harvard Medical School, Cambridge, MA) (27). To generate DN caspase-2, the active site cysteine was mutated to alanine (C303A, TGC GCC) by site-directed mutagenesis and inserted into pRSV-lacZ and pcDNA3 (Invitrogen, Carlsbad, CA).

Results

Ligation of the BCR on mature and immature human lymphoma cell lines leads to apoptotic cell death

Cross-linking of mlgM on B104 cells, a human B lymphoma cell line with a mature phenotype, induced rapid cell death, as previously reported (23). Cell death (MTT assay) was detectable at 4 h, and 50% cell loss occurred ~10 h after BCR ligation (Fig. 1A). BCR cross-linking on ST486-M cells, a clonal human B lymphoma cell line with an immature phenotype, induced cell death with slower kinetics (Fig. 1A), as did BCR ligation on DND-39 cells, a human B lymphoma cell line with a mature phenotype (data not shown). BCR-ligated B104 cells were enlarged with electrolute cytoplasm and eccentric nuclei containing condensed chromatin; the cells did not show DNA fragmentation as earlier
reported (29). Dying ST486-M and DND-39 cells, in contrast, exhibited the classic morphologic features of apoptosis, as well as DNA fragmentation.

Cell death triggered by BCR ligation on these three human B lymphoma cells is clearly apoptotic because it is not passive but, rather, is triggered by ligation of a cell-activating membrane receptor and involves requisite caspase activation, because it was largely inhibited by preincubating the cells with certain irreversible active site caspase inhibitors (Fig. 1B). Furthermore, BCR ligation led to time-dependent exposure of PS on the membrane of intact cells (Fig. 1C), an accepted measure of early apoptotic cellular changes (28). The evolution from annexin V- and PI-negative cells, to annexin V-positive and PI-negative cells and, finally, to annexin V- and PI-positive cells is evident.

**Activation of caspase-3 induced by BCR ligation on B104 cells is required for apoptosis**

Programmed cell death induced by many agents is associated with caspase-3 activation, and subsequent cleavage of various substrates by the activated enzyme (30, 31). Evidence for caspase-3 cleavage or activation 4–24 h after BCR ligation on various human B cell lines has been obtained (20–22). In the present study, initial cleavage of caspase-3 into 20- and 17-kDa fragments was evident 2 h after BCR cross-linking on B104 cells, and maximal cleavage occurred 4–8 h after BCR ligation (Fig. 2A). The observed cleavage represents activation, because B104 cell extracts acquired the ability to cleave zDEVD-AFC, a synthetic substrate of caspase-3-like enzymes (data not shown), as well as a natural protein substrate, PARP, a DNA repair enzyme (Fig. 2B), with the same kinetics. Caspase-3 was also cleaved after BCR ligation on ST486-M cells; significant cleavage was apparent 8 h after BCR cross-linking, and maximal cleavage occurred 12–24 h after BCR ligation (data not shown). Caspase-3 activation was blocked by preincubating B104 cells with zDEVD-fmk, an irreversible caspase-3 inhibitor, before BCR ligation (Figs. 2C, as was PARP cleavage (data not shown).

Because the caspase-3 inhibitor, zDEVD-fmk, also potently inhibited cell death induced by BCR ligation on B104 and ST486-M cells (Fig. 1B), these findings strongly suggest that caspase-3, or a caspase-3-like enzyme, is a required participant in the BCR signaling pathway leading to the death of mature and immature human B cell lines. Of note, these results contrast with two recent reports in which Ac-DEVD-CHO failed to inhibit BCR-induced apoptosis of cloned Ramos B cells (21) and WEHI-231 immature murine B cells (32). The reasons for the different results are not known.

**Caspase-3 activation and apoptosis after BCR ligation are dependent on activation of one or more upstream caspases**

BCR-induced activation of caspase-3 and apoptosis was inhibited by preincubating B104 and ST486-M cells with zVAD-fmk, an irreversible inhibitor of numerous caspases, before the addition of anti-IgM (Figs. 1B and 2C). These findings provided suggestive evidence for the involvement of another caspase in caspase-3 activation after BCR ligation, because zVAD-fmk is a relatively weak caspase-3 inhibitor (33). Definitive evidence for involvement of an additional caspase upstream of caspase-3 came from the demonstration that the cowpox-encoded caspase inhibitor, crmA, blocked BCR-induced apoptosis after expression in B104 cells. CrmA, although a good caspase-8 (K_i = 950 pM) and caspase-1 (K_i = 10 pM) inhibitor, is not an effective caspase-3 inhibitor (K_i = 500 nM) (34). In these studies, B104 cells were transiently cotransfected with crmA and pHook-2 expression plasmids. In three independent experiments in which high levels of crmA expression were obtained, caspase-3 cleavage (Fig. 2D) and PARP cleavage and apoptosis (data not shown) were all markedly inhibited.

**Caspase-8 and -1 are not activated by ligation of the BCR on B104 cells**

Caspase-8 and -1 are known activators of caspase-3 (30). Although we considered it unlikely that caspase-8 was involved in BCR-triggered apoptosis of B104 cells because of the absence of death domains (1) in either membrane IgM or the associated CD79a and
CD79b proteins, the observed inhibition of caspase-3 activation and apoptosis by crmA, a good caspase-8 and caspase-1 inhibitor (34), suggested the possibility that BCR components interacted directly, or via an intermediate protein(s) with a death effector domain (1)-containing adaptor protein(s), and thereby recruited caspase-8 or caspase-1. However, Western blotting analyses provided no evidence for cleavage of caspase-8 or caspase-1 during the first 8 h after BCR ligation on B104 cells (data not shown). Thus, neither caspase-8 nor caspase-1 was directly activated after BCR ligation. A minor amount of caspase-8 cleavage was detected 12 h after BCR ligation, but this most likely represents secondary or feedback activation, rather than direct activation. Studies using other approaches have also shown that upstream events in the BCR and death receptor apoptotic pathways differ (21, 22).

Caspase-9 plays a minor role in apoptosis after BCR ligation

Because caspase-3 is activated in vitro in the presence of caspase-9, cytochrome c, Apaf-1, and dATP (26, 35), we evaluated the possibility that BCR ligation on B104 cells led to caspase-9 activation. Although caspase-9 is present in very low concentrations in B104 cells, it was not cleaved during the first 8 h after BCR ligation on B104 cells, although modest cleavage became apparent 12 h after BCR ligation (Fig. 3A). This late cleavage was most likely mediated by caspase-3, or by a caspase-3-like enzyme via a feedback mechanism, because it was blocked by low concentrations of the irreversible caspase-3 inhibitor, zDEVD-fmk (Fig. 3B). In this regard, caspase-3 has been shown to directly activate caspase-9 in vitro (36).

Transfection studies with DN caspase-9 (26) were conducted to definitively assess the role of caspase-9 in apoptosis of B104 cells triggered by BCR ligation. Apoptosis induced by BCR ligation was evaluated in B104 cells transiently transfected with either DN caspase-9 or lacZ containing vector. DN caspase-9 only modestly inhibited apoptosis of blue cells induced by BCR cross-linking in this experiment (27 ± 6% viability for DN transfected vs 6 ± 5% viability for vector-transfected cells); these data are representative of five identical studies.

Caspase-2 is activated early and plays a major, essential role in BCR-induced apoptosis

Because of the lack of a major role for caspase-9 in caspase-3 activation after BCR ligation, we hypothesized the involvement of another CARD (1)-containing caspase; such caspasas include human caspases-1, -2, -4, -5, -8, -9, -10, and -13 (37, 38). Of these, significant roles for caspase-1 and -8 were eliminated by the experiments presented above; and caspase-9 was found to play a minor role. Caspase-10 was considered an unlikely candidate, because it is a receptor-type caspase with death effector domains (39). We focused on caspase-2, because it is closely related to caspase-9 (30) and contains a functional CARD domain (40, 41). In addition, caspase-2 is highly expressed in lymphocytes and participates in certain B and T cell apoptotic reactions (42, 43).
irreversible caspase-3 inhibitor, to the cytosols, whereas the addition of 1 mM zVDVAD-fmk blocked cleavage (data not shown). Because the caspase specificity of VDAD has not been analyzed, it is possible that other caspases cleave and are inhibited by this peptide. However, the similar kinetics of caspase-2 cleavage after BCR ligation with those obtained for the ability of cytosols of BCR-ligated cells to cleave VDVAD, strongly suggest that caspase-2 is activated early after BCR ligation.

To assess the importance of a caspase-2-like enzyme for BCR-induced apoptosis, B104 and ST486-M cells were preincubated with various concentrations of zVDVAD-fmk for 2 h before BCR ligation, and cell death was assessed 18 h (B104 cells) or 36 h (ST486-M) later. Apoptosis of both cell types was inhibited by zVDVAD-fmk in a dose-dependent manner (Fig. 4D). These findings document a major essential role for caspase-2 in BCR-induced apoptosis. In the same experiments, B104 cells were also transfected with DN caspase-9 alone and together with DN caspase-2. DN caspase-9 only modestly inhibited apoptosis induced by BCR ligation (22 ± 6% viability), similar to the values reported above. However, BCR-induced apoptosis was almost completely blocked (80 ± 5% viability) in B104 cells cotransfected with DN caspase-2 and -9 together (Fig. 4D). These findings, which represent the combined results of three identical experiments, indicate that caspase-2 and -9 are both important in BCR-induced apoptosis, with caspase-2 playing the major role. Blocking both caspases essentially abrogated apoptosis triggered by BCR ligation.

Caspase-2 activation occurs upstream of caspase-3 activation in the BCR signaling pathway leading to apoptosis

To determine the sequence of involvement of caspase-2 and -3 in BCR-mediated apoptosis, B104 cells were preincubated with several concentrations of zDEVD-fmk or zDEVD-fmk before BCR cross-linking. The caspase-2-like inhibitor, zVDVAD-fmk, but not the caspase-3 inhibitor, zDEVD-fmk, inhibited caspase-2 cleavage in BCR-ligated B104 cells in a dose-dependent manner (Fig. 4E). The zVDVAD-fmk peptide also inhibited the generation of the 17- and 20-kDa fragments characteristic of activated caspase-3 (Fig. 4F), as did zDEVD-fmk (Fig. 2C) in BCR cross-linked B104 cells. Although a 22–23-kDa caspase-3 cleavage product was formed in the presence of low concentrations of zVDVAD-fmk (Fig. 4F), this cleavage product lacked caspase-3 activity, as lysates of cells pretreated with zVDVAD-fmk before BCR ligation were unable to cleave either zDEVD-pNA or PARP (data not shown). Presumably, the inactive 22–23-kDa cleavage product is generated by a non-zVDVAD-fmk-inhibitable caspase. These studies indicate that caspase-2 activation precedes and is required for caspase-3 activation after BCR cross-linking.

Caspase-2 activation occurs upstream of caspase-9 involvement in the BCR signaling pathway leading to apoptosis

In the transient transfection studies described earlier, Rat-1 cells were also transfected with wt caspase-2 plus DN caspase-9. Caspase-2-induced apoptosis (90 ± 4% cell death) was blocked in the presence of DN caspase-9 (18 ± 5% death) (Fig. 4C), suggesting that caspase-9 acts downstream of caspase-2.

Caspase-2 and -3 activation and apoptosis induced by BCR ligation are dependent on a CsA-inhibitable step

CsA (100 ng/ml) completely blocked apoptosis of B104 and ST486-M cells induced by BCR ligation (data not shown), findings that are consistent with previous findings (29, 45). In dose-response studies, very low concentrations of CsA inhibited caspase-2 cleavage (Fig. 5) as well as caspase-3 cleavage (data not shown) after BCR ligation on B104 cells. PARP cleavage was also inhibited (data not shown). Thus, caspase-2 and -3 activation and apoptosis induced by BCR ligation are dependent on an upstream CsA-inhibitable step.

Discussion

These studies show that BCR ligation triggers a previously undescribed intracellular signaling cascade involving a CsA-inhibitable step and sequential activation of caspase-2, -3, and -9 (Fig. 6). Caspase-8 and -1, upstream caspases activated by ligation of the

FIGURE 3. Caspase-9 plays a minor role in BCR-induced apoptosis of B104 cells. A, Evaluation of caspase-9 cleavage after BCR ligation. Samples were taken at intervals after BCR ligation and evaluated for caspase-9 cleavage by immunoblotting with a polyclonal Ab to caspase-9. Double-sized samples and double-thickness gels were used. B, The caspase-3 inhibitor, zDEVD-fmk, blocks late activation of caspase-9 after BCR ligation. Cells were incubated with various concentrations of zDEVD-fmk for 2 h before BCR-induced apoptosis, B104 and ST486-M cells were preincubated with several concentrations of zVDVAD-fmk for 2 h before BCR ligation, and evaluated for caspase-9 cleavage 16 h later.
CD95 and the TNF-R1 death receptors, were not activated by BCR ligation. Although most of the present studies were performed with B104 cells, the results of key experiments conducted with ST486-M cells indicate that the signaling pathway mediating apoptosis after BCR ligation is not unique to the B104 cell line. Because B104 cells exhibit a mature phenotype and ST486-M cells an immature phenotype, the apoptotic pathway is not dependent on the maturation state of the B cell. The signaling pathway also does not correlate with the presence of apoptotic phenotypic characteristics, because ST486-M cells exhibit such features, whereas B104 cells do not.

Clear-cut activation of caspase-2 and -3 was evident 2 h after BCR cross-linking on B104 cells, and peak activation occurred 4–8 h after BCR ligation. This time frame correlated with the onset of rapid cell death. Qualitatively identical results were obtained with ST486-M cells, except that caspase-2 and -3 activation and cell death triggered by BCR ligation occurred several hours later than in B104 cells. Experiments with cell-permeable fmk derivatives of caspase peptide substrates, which are irreversible active site inhibitors, showed that caspase-2 and -3 were required for apoptosis. Studies with B104 cells expressing DN caspase-2 provided definitive evidence for the requirement of caspase-2 for BCR-triggered apoptosis.

Studies with fmk derivatives of caspase substrates also strongly suggested that caspase-2 activation occurs upstream of, and is required for caspase-3 activation after BCR ligation, because a caspase-2 inhibitor blocked caspase-2 as well as caspase-3 activation, whereas a caspase-3 inhibitor prevented caspase-3, but not caspase-2 activation. In other systems, caspase-2 activation has also been reported to occur upstream of caspase-3 activation in

![Figure 4](http://www.jimmunol.org/)
apoptosis induced by etoposide, γ-irradiation, serum withdrawal, and treatment with atractyloside, which opens the permeability transition pore (PTP) (1, 46, 47). A caspase-2 to caspase-3 sequence is in agreement with the general conception that caspases with large CARD domains form complexes with specific proteins and are activated within such apoptotic signaling complexes, while caspases with short prodomains are directly activated and act as downstream effector caspases (48). However, a caspase-3 to caspase-2 hierarchy has been reported after CD95 ligation (43), and for caspase-2 cleavage induced in cytosolic extracts by addition of cytochrome c (49); in another study, caspase-2 was shown to be activated by a caspase-3-like activity (43). Whether a caspase-3 to -2 sequence is necessary for apoptosis remains to be determined.

It is likely that there is an intervening caspase(s) between caspase-2 and -3 in the BCR-triggered apoptotic pathway (Fig. 6), because caspase-2 is unable to activate caspase-3 in vitro (41, 50). This possibility is also supported by the finding that caspase-3 cleavage and activation after BCR ligation were blocked by crmA, which is a poor inhibitor of caspase-2 and -3 (27).

The mechanism of caspase-2 activation after BCR ligation was not addressed in these studies. There are, however, several possibilities. First, the presence of similar CARD domains in caspase-2 and -9 suggests the possibility that caspase-2 is activated by a caspase-9-like mechanism involving formation of a ternary complex with cytochrome c and Apaf-1 (26, 35). If so, however, Apaf-1 is not likely to be involved, because the CARD domains of caspase-2 and Apaf-1 do not interact (51). It is possible, however, that caspase-2 interacts with an unidentified Apaf-1-like molecule. A second possibility is that caspase-2 activation is mediated by homotypic interactions between the CARD domains of caspase-2 and an adaptor molecule, in analogy to the activation of caspase-2 by binding to the death domain-containing adaptor molecule, RAIDD/CRADD (40, 41). A third possibility is that caspase-2 is directly activated by proteolytic cleavage by another caspase. Finally, the recent demonstration that procaspase-2 is present in the intermembrane space of liver mitochondria and T cell hybridoma mitochondria, but is released in activated form after PTP opening, provides support for an autocatalytic mechanism of activation (47). Our current studies are addressing the mechanism of caspase-2 activation.

**FIGURE 5.** Caspase-2 activation and apoptosis after BCR ligation are dependent on a CsA-inhibitable step. Effect of CsA on caspase-2 cleavage after BCR ligation. Caspase-2 cleavage was assessed 16 h after BCR ligation on B104 cells that had been preincubated with CsA.

**FIGURE 6.** Key events in the apoptotic pathway triggered by ligation of the BCR on B104 cells.
In addition to the demonstrated role of caspase-2, perhaps the most surprising finding in these studies is the lack of a major role for caspase-9 in BCR-triggered apoptosis. Caspase-9 cleavage was not detected until 12 h after BCR ligation on B104 cells, whereas caspase-2 and -3 were activated 2–4 h after BCR ligation on these cells. Thus, caspase-9 involvement is a later event in BCR-induced apoptosis than caspase-2 and -3 activation. Furthermore, DN caspase-9 only modestly inhibited BCR-induced apoptosis, indicating that it plays a relatively minor role in this apoptotic pathway. Although the mechanism of this late activation of caspase-9 was not addressed in this work, it is likely that caspase-3 is responsible, because caspase-9 cleavage after BCR ligation was blocked in cells preincubated with zDEVD-fmk, an active site caspase-3 inhibitor. Furthermore, caspase-3 has been previously reported to possess the ability to cleave caspase-9 in vitro (36). It is likely that caspase-9 functions in this system to enhance apoptosis via a feedback mechanism (Fig. 6).

Because caspase-9 is activated in the presence of cytochrome c and Apaf-1 in vitro (26, 35), the lack of early caspase-9 activation after BCR ligation suggests the possibility that cytochrome c is not released into the cytosol after BCR ligation. However, this would be surprising in view of the rapidly accumulating evidence of mitochondrial involvement and cytochrome c release as early requisite events in the response of intact cells to numerous apoptotic stimuli (38, 52, 53). Indeed, in preliminary studies, we find detectable release of cytochrome c into the cytosol 2 h after BCR ligation on B104 cells, and maximal release 4 h after ligation. The lack of early caspase-9 cleavage in B104 cells after BCR ligation is thus particularly striking, especially in view of the low concentrations of this caspase in B cells, and the recent demonstration that procaspase-9 is present in the intermembrane space in liver mitochondria together with cytochrome c (47). B cells most likely contain Apaf-1, although we have not verified this. A possible explanation for the lack of early caspase-9 cleavage after BCR ligation is that mitochondrial Bcl-X<sub>L</sub> prevents effective interaction of Apaf-1, or Apaf-1-cytochrome c complexes with the relatively small amounts of caspase-9 in B cells. Such an autoregulatory process would be analogous to the demonstrated binding of overexpressed Bcl-X<sub>L</sub> to Apaf-1, with resulting inhibition of caspase-9 activation (51, 54). Current studies are addressing this hypothesis.

In confirmation of earlier reports (29, 45), apoptosis triggered by BCR ligation was blocked by CsA. In the present studies, the CsA-dependent step was placed upstream of caspase-2 and -3 cleavage, because activation of these caspases was blocked by treatment of the cells with nanomolar CsA concentrations before BCR crosslinking. CsA binds to two intracellular proteins, cyclophilin A, a cytoplasmic protein, and cyclophilin P, an inner mitochondrial membrane protein. Cyclophilin P represents an attractive target for CsA because of its relationship to the PTP (55), which is associated with apoptotic events (56). Nevertheless, cyclophilin P is not likely to represent the target for the antiapoptotic actions of CsA, because FK506, another immunosuppressant, also inhibits BCR-induced apoptosis in several B cell types (57), but does not alter mitochondrial PTP function (55); FK506, like CsA, targets calcineurin, a calcium- and calmodulin-regulated phosphatase. Therefore, the most likely target for CsA is cyclophilin A. The CsA-cyclophilin A complex functions by binding to and inhibiting the enzymatic activity of calcineurin (58). Inhibition of calcineurin phosphatase activity by CsA and FK506 interferes dramatically with the transcription of IL-2, numerous other cytokines, and various genes involved in cellular activation (59, 60). It is possible that CsA blocks BCR-triggered caspase-2 activation and/or apoptosis by inhibiting the transcription of gene(s) required for these processes. Alternatively, CsA may function by blocking the calcinurin-mediated dephosphorylation of a regulatory protein involved in Bcl-2 or Bcl-X<sub>L</sub> interactions with death-promoting family members such as BAD (61), another type of caspase-regulating protein, or a critical upstream kinase, such as Akt or Raf-1 (62, 63). In this regard, recent evidence indicates that calcineurin promotes apoptosis by dephosphorylating BAD (64).

The findings presented in this work indicate that the BCR signaling pathway leading to cell death involves a novel calcineurin and caspase-2, -3-, and -9-dependent pathway (Fig. 6). Further studies are clearly needed to define intervening steps, and to characterize the intracellular mechanisms that regulate cell fate decisions leading to proliferation and cell growth, or, alternatively, to cell death after BCR ligation.

Acknowledgments

We gratefully acknowledge gifts of reagents from J. Yuan (Harvard Medical School, Cambridge, MA); M. Mayumi, Fukui Medical University (Fukui, Japan); J. Jackson, The Scripps Research Institute (La Jolla, CA); X. Wang, Southwestern Medical Center (Dallas, TX); and D. Pickup, Duke University Medical Center (Durham, NC). We also thank Emanuella Bonfoco for helpful comments and Catalina Howe and Joan Gausepohl for assistance with the manuscript.

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