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Mitochondria-Dependent Caspase-9 Activation Is Necessary for Antigen Receptor-Mediated Effector Caspase Activation and Apoptosis in WEHI 231 Lymphoma Cells

Marco J. Herold, Andreas W. Kuss, Christa Kraus, and Ingolf Berberich

Engagement of the B cell Ag receptor (BCR) on immature B cells leads to growth arrest followed by apoptosis. Concomitant signaling through CD40 sustains proliferation and rescues the cells from apoptosis. Previously, we have shown that cross-linking CD40 on B cells stimulates the expression of A1, an antiapoptotic member of the Bcl-2 family, and that transduction of the murine B lymphoma line WEHI 231, a model for immature B cells, with A1 protected the cells against BCR-induced apoptosis. Here we demonstrate that A1 strongly interferes with activation of caspase-7, the major effector caspase activated after BCR cross-linking on WEHI 231 lymphoma cells. The pathway leading to activation of the effector caspase cascade including caspase-7 is unclear. Using retrovirally transduced WEHI 231 cell populations, we show that a catalytically inactive mutant of caspase-7 is cleaved almost as efficiently as the wild-type form, arguing against autocatalysis as the sole activating process. In contrast, overexpression of cata
calytically inactive caspase-9 strongly interferes with caspase-7 processing, poly(ADP-ribose) polymerase cleavage, and DNA
laddering, suggesting a role for caspase-9 and hence for the mitochondrial pathway. The importance of the mitochondrial/caspase-9 pathway for BCR-triggered apoptosis is highlighted by our finding that both A1 and the mutant caspase-9 attenuate
BCR-induced apoptosis. Thus, our data suggest that the BCR-mediated apoptotic signal in immature B cells spreads via a

B cells develop in the bone marrow from committed pro
genitors to immature B cell Ag receptor (BCR)-bearing B cells. Inherent to Ig gene rearrangements is the gener
ation of BCRs recognizing self Ag. Therefore, mechanisms have
evolved to control or delete autoreactive B cell clones. Receptor editing, induction of energy, and induction of apoptosis are used to achieve self-tolerance of the emerging B cell repertoire. A major
time point for eliminating self-reactive B cells is at the transition
from the immature to the mature stage. At this developmental
phase, engagement of the BCR in the absence of costimulatory
signals leads to induction of apoptosis (reviewed in Ref. 1).

Apoptosis is characterized by morphological changes in the cell,
including shrinkage, chromatin condensation, membrane blebbing,
and formation of apoptotic bodies (reviewed in Ref. 2). Central
to the controlled disassembly of the cell is the activation of caspases,
a family of cysteinyl aspartate-specific proteases (reviewed in Ref.
3). Caspases are constitutively expressed and are present in cells as
inactive precursors (procaspases or zymogens) that consist of three
domains: a prodomain and a large and small subunit. Based on the
length of their prodomain, caspases are subdivided into initiator
(i.e., caspase-8 and -9) and effector caspases (i.e., caspase-3, -6,
and -7). To obtain full proteolytic activity, a precursor has to be
cleaved between the domains. The large and the small fragment
then combine to form an active enzyme. Triggering of the caspase
cascade takes place in multicomponent complexes either at the
plasma membrane (death-inducing signaling complexes at death
receptors, e.g., CD95) or in the cytoplasm (apoptosomes), where
the initiator caspase-8 and caspase-9 are activated, respectively
(reviewed in Refs. 2 and 3). The active initiator caspases subse
cquently cleave and thereby activate the downstream effector
caspases. Consecutive proteolytic degradation of a broad spectrum
of cellular targets including poly(ADP-ribose) polymerase (PARP)
or inhibitor of caspase-activated DNase ultimately leads to cell
death.

Apoptosomes are high-m.w. complexes containing at least
apoptotic protease-activating factor 1 (Apaf-1), cytochrome c (Cyt
c), dATP, and caspase-9 (Ref. 4 and reviewed in Ref. 3). Apaf-1,
procaspase-9, and dATP are located in the cytoplasm, whereas Cyt
c is normally sequestered in the mitochondria. When released, Cyt
c in concert with dATP triggers a conformational change in
Apaf-1, leading to its oligomerization. Procaspase-9 is simulta
neously recruited into this nascent complex and is activated by
cleavage. This pathway will be referred to as the mitochondrial/caspase-9 pathway. Release of Cyt c from mitochondria follows a
wide variety of stress signals, including UV, oxidative stress, an
ticancer drugs, and growth factor withdrawal (reviewed in Ref. 5).
Oligomerization of the proapoptotic “multidomain” Bcl-2 family
members Bak or Bax in the outer mitochondrial membrane seems
to be obligatory to initiate mitochondrial dysfunction by most if
not all of these stimuli (6–9). In contrast, antiapoptotic Bcl-2
family members such as Bcl-2, Bcl-xL, and A1 appear to sta
bilize mitochondria by inhibiting the formation of such olig
gomers (10, 11).

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Abbreviations used in this paper: BCR, B cell Ag receptor; PARP, poly(ADP-
ribose) polymerase; Apaf-1, apoptotic protease-activating factor 1; Cyt c, cytochrome
c; dnC9, dominant negative caspase-9.

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Engaging the BCR induces apoptosis in a variety of B cells, including primary human activated memory cells and various B cell lines. Mitochondria seem to link the BCR signals to the downstream apoptosis-inducing machinery, i.e., the caspases (12–14). In keeping with this, primary mature B cells lacking A1 are highly sensitive to BCR-mediated apoptosis (15).

For WEHI 231, a murine B lymphoma serving as a model for BCR-induced apoptosis at the immature stage of B cell development, we and others have shown that ectopic expression of anti-apoptotic Bcl-2 proteins protects against deleterious effects of BCR cross-linking, thereby arguing for a mitochondrial gateway to apoptosis in tolerance induction (14, 16–21). Again, the physiological importance is highlighted in transgenic mice overexpressing Bcl-xL in the context of a transgenic self-reactive BCR. B cells in these animals efficiently escaped developmental arrest and deletion (22). Recently, however, Ruiz-Vela et al. (20) presented data suggesting that the activation of the effector caspase-7 is independent of Cyt c release and processing of caspase-9.

In this report, we used retrovirally transduced WEHI 231 cells to assess the importance of the mitochondrial/caspase-9 pathway for BCR-induced apoptosis. We show that A1 inhibits processing of caspase-9 and caspase-7. In addition, a mutant of caspase-9 with an inactivated enzymatic center, dominant negative caspase-9 (dnC9), strongly interferes with processing of caspase-7, degradation of PARP, and oligosomal fragmentation of DNA. Both A1 and dnC9 alleviate apoptosis induced by cross-linking the BCR. These results suggest that mitochondria and caspase-9 are critically involved in BCR-triggered caspase activation and apoptosis in immature B cells.

Materials and Methods

Cell culture
WEHI 231 cells (murine B cell lymphoma line) and 293T cells (human embryonic kidney cell line 293 expressing SV40 T Ag) were obtained from American Type Culture Collection (Manassas, VA). The B cell line was maintained in RPMI 1640 medium supplemented with 4.8 mM L-glutamine, 1 mM sodium pyruvate, 1 × nonessential amino acids (Life Technologies, Rockville, MD), 5% (v/v) FCS, streptomycin (0.2 mg/ml), and penicillin (500 U/ml). The 293T cells were propagated in DMEM supplemented with 10% (v/v) FCS and streptomycin.

Reagents
Anti-IgM (AffiniPure F(ab′)2 goat anti-mouse IgM) was obtained from ICN Pharmaceuticals (Costa Mesa, CA) and used at 7.5 μg/ml. Biotinylated anti-IgM (AffiniPure F(ab′)2 goat anti-mouse IgM; working concentration, 2 μg/ml) used for survival experiments was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Etoposide was purified from Calbiochem (La Jolla, CA). ImmunoResearch Laboratories (West Grove, PA). Etoposide was purified from Calbiochem (La Jolla, CA).

Plasmids
The bicistronic retroviral vector pEYZ/MCS containing a chimeric selection marker consisting of the enhanced yellow fluorescent (Y) and the zeocin-resistant (Z) proteins was previously described (19). pEYZ/MCS and pEZN/MCS are analogous to pEYZ/MCS but contain the green fluorescent marker (G) fused to either the neomycin (N)- or the zeocin (Z)-resistant protein. Oligonucleotides encoding a flag-peptide were inserted into the MCS of pEZN/MCS (→pEZN/MCS-N) and pEYZ/MCS (→pEYZ/MCS-F) to routinely flag-tag encoded proteins at the C terminus. Caspase-7 and caspase-9 DNAs were obtained by conventional RT-PCR technique from RNA isolated from WEHI 231 cells. The mutant forms of caspase-7 and caspase-9 were generated by PCR techniques. Both mutants encode a protein with a cysteine to serine mutation at their active center.

All DNAs were inserted into the EcoRI and BamHI of pEZN/MCS-Flag or pEYZ/MCS-Flag using EcoRI/BamHI restriction sites for caspase-7 and EcoRIBamHI restriction sites for caspase-9 DNAs. pEYZFm1 was described previously (19). The pEGN/MCS-Flag expression vector encoding human Bcl-2 was generated by releasing a full-length cDNA of Bcl-2 from pBluescript KS-Bcl2 (a gift from J. Tropmpair, Institut fůr Medizinische Strahlenkunde und Zellforschung, University of Wûrzburg, Wûrzburg, Germany) by EcoRI digestion and inserting it into the EcoRI restriction site of pEZN/MCS.

Transfection/infection assays and cell sorting
Recombinant retroviral particles were generated by using the pHIT packaging system essentially as described by Soneoka et al. (23). Transfection of 293T cells and infection of WEHI 231 cells have been described previously (19). Transduced cells were highly enriched (>80%) by treatment with the adequate antibiotics (250 μg/ml zeocin or 1 mg/ml G418) and/or by FACS (FACS Vantage; BD Biosciences, Mountain View, CA).

Determination of live cells
The percentage of live cells was determined as the number of events falling into the region of the live gate in a forward light scatter/side light scatter analysis on a FACS. To determine a stringent live gate, cell cultures were supplemented with 4 μg/ml propidium iodide and were incubated for 15 min at 37°C. Cells excluding propidium iodide were considered as live cells (19).

Western blot analysis
Whole-cell lysates were prepared by lysing 3 × 10^5 cells in 25 μl of 6× Laemmli buffer. Mitochondria containing cellular fractions and cytoplasmic fractions were prepared as described by Heiberg et al. (24). Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and analyzed by Western blotting.

Analysis of DNA fragmentation
Extraction of cellular DNA for the DNA fragmentation assay was conducted by lysing 2 × 10^6 cells as described by Doi et al. (14). For analysis, DNA equivalent to 5 × 10^6 cells was electrophoresed in a 2% agarose gel.

Analysis of caspase-3-like activity
Caspase-3-like (caspase-3, -6, and -7) activity in lysates of various WEHI 231 populations was determined by means of a colorimetric substrate (catalog no. 235400; Calbiochem) following the manufacturer’s instructions.

Results

Ligation of the BCR on WEHI 231 B lymphoma cells leads to processing and activation of effector caspases

Cross-linking of the Ag receptors on WEHI 231 cells induces growth arrest and apoptosis. We monitored cell death after BCR cross-linking as an increase in the number of propidium iodide-positive cells (Fig. 1A). In WEHI 231 cells, induction of apoptosis particularly correlates with processing, i.e., activation of caspase-7 (18). To confirm this observation by Brás et al. (18) for the WEHI 231 cell line used in our laboratory, we analyzed cleavage of endogenous procaspase-7 (p35) after BCR cross-linking. One day after stimulation, we observed significant amounts of the large fragment of caspase-7 (p20), which is indicative of active caspase-7 (Fig. 1B). Degradation of intact PARP (116 kDa) to the 85-kDa apoptotic fragment further supports the notion that caspases are indeed activated in WEHI 231 cells after BCR ligation (Fig. 1B, lower panel). In addition, we observed DNA ladder-like characteristic of apoptotic cells (Fig. 1C) and indicative for caspase-3 activation (see Discussion). Also, we directly measured a rise in caspase-3-like activity in cell extracts of Ag receptor-stimulated WEHI 231 cells (Fig. 4D and data not shown).

As a pilot experiment for studies described below, we also monitored the processing of flag-tagged procaspase-7 after BCR ligation in retrovirally transduced populations. If necessary, infected cells were further enriched on the basis of the chimeric selection marker (green fluorescent protein conferring resistance to zeocin), the expression of which was coupled to the expression of procaspase-7 by an internal ribosomal entry site (see Materials and Methods). Routinely, populations >90% positive for green fluorescent protein were used for experiments. Typically, there was a...
15- to 30-fold overexpression of exogenous procaspase-7 compared with the endogenous enzyme (Fig. 1D). Increased expression of procaspase-7 did not influence proliferation of the cells or their response to apoptosis-inducing agents (Abs to the BCR or etoposide; data not shown). As observed with endogenous caspase-7, we found strong cleavage of ectopically expressed caspase-7 and degradation of PARP ~24 h after BCR ligation (Fig. 1E). Absence or presence of a flag tag at the C terminus of caspase-7 did not influence processing (data not shown). In most of our experiments, significant amounts of processed caspase-7 (p20) were detected, starting at 20–24 h poststimulation (Fig. 1F and data not shown).

Experiments by Ruiz-Vela et al. (20) suggest that cleavage of caspase-7 is initiated by an autoproteolytic process. If so, mutation of the active center of procaspase-7 should inhibit or at least slow down the cleavage of the proform of caspase-7. Therefore, a mutant of caspase-7 was constructed, in which the cysteine of the active center was changed to a serine, which renders the protein enzymatically inactive. When we compared the processing of ectopically expressed wild-type procaspase-7 with the mutant, no strong difference was found after BCR ligation (Fig. 2). Taking into account that overexpression of enzymatically inactive caspase-7 was 15- to 30-fold compared with the endogenous enzyme, we conclude that strong auto and/or transprocessing of caspase-7 is rather unlikely.

**Caspase-9 is processed after BCR stimulation in WEHI 231 cells**

Cleavage of effector caspases such as caspase-7 is normally dependent on the preceding activation of an initiator caspase. Because we and others have described the protective role of Bcl-2 family members, which are known to stabilize mitochondria, on Ag receptor-stimulated WEHI 231 cells (14, 16–21), activation of the mitochondrial-dependent initiator caspase-9 was examined. To date, only very low BCR-induced caspase-9 activity, but no processing, has been observed for this initiator caspase (18, 20). This does not necessarily argue against a role for caspase-9 in this pathway, because caspase-9 can be activated without prior proteolytic processing (25). Accordingly, we reevaluated this issue by analyzing WEHI 231 cell populations retrovirally transduced with procaspase-9. As shown in Fig. 3, triggering this population of WEHI

![FIGURE 1. BCR ligation activates caspases and induces cell death in WEHI 231 cells. A, WEHI 231 wild-type cells were stimulated as indicated or were left untreated (medium). Cells were incubated with propidium iodide (5 μg/ml) and analyzed by FACS. B, WEHI 231 wild-type cells were stimulated as indicated or were left untreated (medium). Cells were lysed in Laemmli buffer. The extracts were analyzed by Western blotting for caspase-7 (pro-caspase-7 (35 kDa) and large fragment (20 kDa); upper panel) and PARP (intact protein (116 kDa) and cleaved protein (85 kDa); lower panel). C, Analysis of chromosomal DNA. After stimulating the cells as indicated, cells were lysed in SDS buffer. Phenol/chloroform-extracted cellular DNAs were electrophoresed in a 2% agarose gel. D, WEHI 231 cells overexpressing procaspase-7 and control cells were lysed in Laemmli buffer. Three-fold dilutions of the procaspase-7 lysate and undiluted control lysate were analyzed with a caspase-7-specific Ab. The slower migration of transduced caspase-7 is due to the N-terminal flag tag. Mitogen-activated protein kinase (ERK) served as loading control. E and F, WEHI 231 cells transduced with procaspase-7 (35 kDa) were stimulated by cross-linking the BCR with specific Abs. Untreated cells served as control (medium). Laemmli lysates of the cells were analyzed by Western blotting with caspase-7- and PARP-specific Abs.

![FIGURE 2. BCR ligation leads to processing of wild-type and enzymatically inactive caspase-7 in WEHI 231 cells. WEHI 231 were retrovirally transduced with wild-type procaspase-7 (C7) or a mutant form in which the cysteine of the active center was changed to a serine (C7 m). After stimulating the cells via the BCR as indicated, Laemmli lysates of the cells were analyzed by Western blotting with caspase-7-specific Abs.
231 cells via the BCR induces low but unambiguous cleavage of caspase-9 from its proform (p47) to the large fragment of the active caspase (p35). This shows for the first time that caspase-9 is not only activated but is also processed after BCR ligation in WEHI 231 and opens the possibility that caspase-9 might initiate BCR-induced activation of downstream caspases.

dnC9 inhibits caspase-7 processing and interferes with PARP degradation and chromosomal DNA fragmentation

To investigate whether caspase-9 processing is only secondary to an ongoing apoptotic process or is actually required for caspase-7 processing, we generated populations of WEHI 231 cells overexpressing dnC9 (C→S exchange in the active center, also referred to as kinase-dead caspase-9) in cells previously transduced with caspase-7. Compared with endogenous caspase-9, transduced dnC9 was 25- to 50-fold overexpressed in different populations (data not shown). As a control, only the relevant selection marker was coexpressed together with caspase-7. Double-positive cell populations were enriched on the basis of green (coexpressed with caspase-7) and yellow (coexpressed with dnC9) fluorescent marker proteins by FACS. An example of the purity of the sorted cells is shown in Fig. 4A. When the BCR was ligated on cells expressing the enzymatically inactive mutant of caspase-9, processing of caspase-7 was indeed strongly reduced (Fig. 4B, upper panel).
To functionally test for caspase activation in these cells, we again looked for degradation of PARP and the appearance of DNA laddering after BCR ligation. The dnC9 clearly protected WEHI 231 cells against both destructive processes, i.e., it inhibited PARP cleavage (Fig. 4B, lower panel) and fragmentation of the chromosomal DNA (Fig. 4C). Furthermore, we compared cell extracts of dnC9 transduced and control cells for effector caspase activity. We used a colorimetric substrate, which is cleaved by caspase-3-like (caspase-3, -6, and -7) activity. Consistent with the finding that dnC9 blocks BCR-mediated PARP and DNA degradation, we found that the presence of overexpressed dnC9 reduced BCR-inducible caspase-3-like activity to almost undetectable levels over the whole period of observation (Fig. 4D and data not shown).

Thus, our results suggest that activation of caspase-9 is central and obligatory for the activation of the whole cascade of downstream effector caspases.

**Antiapoptotic members of the Bcl-2 family prevent BCR-inducible caspase activation**

We previously showed that CD40 signaling, which alleviates BCR-mediated apoptosis in WEHI 231 cells (26), boosts the expression of the antiapoptotic Bcl-2 family member A1 (19). Furthermore, ectopic expression of A1 protects these cells against the deleterious consequence of BCR-induced signals (19). To address whether damage of mitochondria is upstream of caspase-9 activation, we first tested whether A1 can stabilize mitochondrial function in WEHI 231. As expected, A1 was able to reduce the production of reactive oxygen species after engaging the BCR on WEHI 231 cells (data not shown). This result is in keeping with the observation that A1 localizes to mitochondria and stabilizes their function in epithelial cells (27). Thus, if disruption of mitochondrial function or integrity is upstream of BCR-mediated caspase-9 processing in WEHI 231, sufficient levels of A1 should interfere with the cleavage of procaspase-9. To test this, WEHI 231 cells expressing procaspase-9 in the absence or presence of A1 were analyzed. Indeed, WEHI 231 cells overexpressing A1 no longer exhibited caspase-9 processing after BCR ligation, whereas the respective control populations expressing caspase-9 only in combination with the relevant selection marker activated this initiator caspase (Fig. 5A). As a further control, we used etoposide known to activate caspases via the mitochondrial pathway. Again, A1 strongly inhibited caspase-9 activation (Fig. 5A), thus demonstrating the stabilizing potential of A1 on mitochondria. In addition, A1 also strongly interfered with caspase-7 activation after BCR ligation, further supporting a central role of caspase-9 (Fig. 5B). Bcl-2 itself (normally not highly expressed in WEHI 231), when ectopically expressed had the same effect as A1 and protected against BCR-stimulated caspase-7 processing (Fig. 5C). Thus, these data indicate that both the initiator caspase-9 and the effector caspases are downstream of mitochondria in BCR signal-induced apoptosis.

The large fragment of caspase-9 accumulates in the mitochondria containing cellular fraction

Caspase-9 activation depends on the formation of apoptosomes consisting of Apaf-1, Cyt c, dATP, and caspase-9 (4). Despite cleavage of caspase-9 in our experiments, we have not been able to detect significant release of Cyt c from the mitochondrial intermembrane space to the cytosol after BCR ligation (Fig. 6A). This was also true for experiments in which we already observed basal amounts of Cyt c in the cytoplasm of untreated cells (data not shown). When we compared the cytosolic and mitochondrial fractions for caspase-9, we found a possible explanation for this apparent discrepancy. Whereas only a minor amount of procaspase-9 is found in the mitochondria-containing fraction of unstimulated cells compared with the cytosol, it is exactly this fraction in which the large fragment of caspase-9 (p35) is strongly enriched after BCR stimulation (Fig. 6B, upper panel, C9 + eyz). In contrast, almost no processed form of caspase-9 (p47) is found in the cytoplasm. As expected, antiapoptotic A1 strongly interfered with the formation and the accumulation of cleaved caspase-9 in the mitochondrial fraction (Fig. 6C, lower panel, C9 + A1). Even though the current level of analysis does not allow unequivocal

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**FIGURE 5.** Bcl-2 family members inhibit processing and activation of caspases in BCR-stimulated WEHI 231 cells. A, Procaspase-9 (C9)-transduced WEHI 231 cells were “superinfected” with A1 encoding recombinant retroviruses (A1) or with the relevant control viruses (eyz) and were stimulated as indicated. Laemmli extracts of the cells were analyzed by Western blotting for caspase-9 (Fig. 4B, lower panel) and fragmentation of the chromosomal DNA (Fig. 4C). Furthermore, we compared cell extracts of dnC9 transduced and control cells for effector caspase activity. We used a colorimetric substrate, which is cleaved by caspase-3-like (caspase-3, -6, and -7) activity. Consistent with the finding that dnC9 blocks BCR-mediated PARP and DNA degradation, we found that the presence of overexpressed dnC9 reduced BCR-inducible caspase-3-like activity to almost undetectable levels over the whole period of observation (Fig. 4D and data not shown). Thus, our results suggest that activation of caspase-9 is central and obligatory for the activation of the whole cascade of downstream effector caspases.

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allocation of caspase-9, the association with the mitochondria-enriched fraction is consistent with the notion that minor amounts of an apoptosomelike structure are already deposited on the surface of mitochondria in unstimulated cells where it can quickly sense very low amounts of released Cyt c.

**dnC9 supports survival of BCR-induced WEHI 231 cells**

Our molecular analyses show that dnC9 has the same potential as A1 in inhibiting caspase-7 cleavage and PARP degradation. Because we have previously shown that A1 interferes with BCR-activated cell death, we next explored whether dnC9-transduced WEHI 231 cells are also more resistant to this type of cell death. We approached this question by determining the survival rate after BCR ligation in A1- and dnC9-transduced populations relative to a control population. Fig. 7 shows that dnC9 supports, over the period of 2 days, survival of Ag receptor-stimulated WEHI 231 cells to the same extent as A1. Again, the data indicate the importance of the mitochondrial/caspase-9 pathway for BCR-released apoptotic signals.

**Discussion**

In the present study, we used retrovirally transduced WEHI 231 B lymphoma populations to analyze BCR-triggered apoptosis. Overexpression of enzymatically inactive caspase-9 strongly interfered with caspase-7 processing, PARP degradation, DNA laddering, and induction of cell death. In addition, the antiapoptotic Bcl-2 family member A1 suppressed caspase-9 processing. These data indicate that the mitochondria in combination with caspase-9 are the gateway to caspase activation and apoptosis in immature B cells after engaging the Ag receptor.

Caspase-7 has been suggested to be activated by an autocatalytic process (20). Our experiments addressed this issue at two levels: 1) the initial activation of wild-type caspase-7 in the presence of dnC9 (Fig. 4B), and 2) the "amplification" step with a kinase-dead caspase-7 (Fig. 2). Our observation that a dnC9 totally blocks caspase-7 processing after BCR ligation indicates an absolute requirement for active caspase-9 in the initial phase of caspase-7 cleavage. Furthermore, the observation that kinase-dead caspase-7 is processed to a similar extent as wild-type caspase-7 seems to argue against strong autocatalytic activation or transprocessing of this effector kinase. Of course, overexpression of the mutant did not eliminate the expression of endogenous caspase-7, which would provide caspase-7 activity after BCR engagement. Even though exogeneous mutant caspase-7 was 15- to 30-fold more highly expressed than the endogenous enzyme, our experiments cannot rule out that processed endogenous caspase-7 contributes to the processing of the inactive mutant.

Besides caspase-7, activation of caspase-3 after BCR ligation seems very likely. Whereas PARP can apparently be cleaved by both caspase-3 and -7 (28, 29), chromosomal DNA degradation observed in our experiments rather specifically points to the activation of caspase-3 because various cell types deficient for this

**FIGURE 6.** The large fragment of caspase-9 mainly localizes to the mitochondria containing cellular fraction. A, WEHI 231 cells were stimulated as indicated. Thereafter, cells were lysed in 0.1% digitonin-containing buffer. The cytoplasm was separated from the digitonin-insoluble material including nuclei, mitochondria, and endoplasmatic reticulum by centrifugation. The pellet was dissolved in Laemmli buffer. Cytoplasmic (cyto) and mitochondria-containing (mito) fractions were analyzed by Western blotting for Cyt c. B, Procaspase-9-transduced WEHI 231 cells were stimulated as indicated. Thereafter, cells were lysed in 0.1% digitonin-containing buffer. Cytoplasmic (cyto) and mitochondria-containing (mito) fractions prepared as in A were analyzed by Western blotting for caspase-9 specific proteins.

**FIGURE 7.** A1 and dnC9 alleviate BCR-induced cell death. WEHI 231 transduced with A1, dnC9, or the relevant control (eyz) were stimulated as indicated. The percentage of live cells was determined on the basis of propidium iodide exclusion by FACS analysis (19).
effector caspase (including primary thymocytes, T cells, murine embryonic fibroblasts, and hepatocytes) exhibit strong impairment or even absence of DNA degradation (30, 31). Consistent with our conclusion, caspase-3 activation after BCR ligation has been observed in B cells, including primary human memory B cell blasts and various B cell lines (12, 13, 32). However, in agreement with the finding of Bras et al. (18), we were unable to directly demonstrate processing of procaspase-3 after BCR ligation by Western blotting, even though the caspase-3-specific Ab used in the experiments did detect the large fragment of caspase-3 in extracts of etoposide-treated WEHI 231 (data not shown). It is possible that levels of caspase-3 cleavage below the level of detection are still sufficient for DNA degradation.

Cleavage of effector caspases such as caspase-3 and -7 is normally dependent on the preceding activation of initiator caspases in a death-inducing signaling complex at the plasma membrane (caspase-8) or in an apoptosisome in the cytoplasm (caspase-9) (2). Experiments by Yoshida et al. (33) clearly argue against the involvement of caspase-8 in BCR-mediated apoptosis because over-expression of either CrmA (the cowpox virus caspase inhibitor protein known to preferentially block caspase-8) or a dominant negative form of the adapter Fas-associated death domain protein did not block BCR-mediated apoptosis in WEHI 231, whereas it inhibited Fas-dependent cell death. Even though Scott et al. (34) described a Fas-dependent pathway in which caspase-8 activation could not be observed, the inability of dominant negative Fas-associated death domain protein to interfere with BCR-dependent apoptosis makes it rather unlikely that Fas is involved in the initiation of the caspase cascade.

In contrast, caspase activation via the mitochondria should be involved because the protective role of Bcl-2 family members like A1 and Bcl-xL is well established for the immature B cell line WEHI 231 (14, 16–21). Consistent with this, we show for the first time processing of caspase-9 after BCR ligation. Furthermore, the absence of effector caspase activation in the presence of dnC9 demonstrates an obligatory role of this effector caspase for initiation of the whole caspase cascade. This is in line with the finding that mitochondria are central to BCR-triggered apoptosis in various B cells (12–14). Interestingly, there is one report on a mature and an immature human B cell line in which the authors place caspase-9 activation downstream of caspase-2 and -3 (32). This result is in seeming contrast to our findings. However, in the human B cell lines analyzed by Chen et al. (32), activation of caspase-2 and -3 occurred rather early, i.e., within 2–4 h. Thus, one explanation to reconcile the data lies in the possibility that the BCR first generates a low-level activation of caspase-2 and -3 via an unknown mechanism. This first wave of caspases would then trigger the strong effector caspase response via caspase-9. We are currently testing this possibility.

Even though dnC9 seems to totally block certain indicators of apoptosis (cleavage of caspase-7, caspase-3-like activity, PARP and DNA degradation), the kinase-dead version of caspase-9 did not completely inhibit cell death. This could indicate: 1) because caspase-9 is downstream of the mitochondria, signals from the BCR can still damage the mitochondria, leading to the release of apoptotic effector molecules like apoptosis-inducing factor, which would kill the cells in a caspase-independent way (35), and 2) blockade of effector caspases is not complete and the undetectable activity of these enzymes is sufficient to eventually kill the cells.

It is intriguing that the large fragment of caspase-9 indicative of an active enzyme is preferentially associated with the mitochondrial preparation in WEHI 231 cells. However, because the mitochondria preparations used in our study contain other cellular compartments including nuclei, it is currently not possible to pinpoint the exact cellular structure to which the active caspase-9 fraction localizes. Several sites are possible.

First, caspase-9 might be found in the nucleus as observed for human epithelial cells (36). This localization pattern would be consistent with a need for active caspase-3 in the nucleus to degrade the inhibitor of caspase-activated DNase to allow DNA cleavage by caspase-activated DNase (37–39). However, in the precedent of epithelial cells, nuclear caspase-9 activation is downstream of a caspase-3-like activity (36). Because in WEHI 231 cells BCR-induced caspase-9 cleavage primarily depends on mitochondria, as indicated by the inhibition through A1 (Figs. 5A and 6C, lower panel), we do not favor this possibility.

Second, as described, for instance, for primary rat neurons and cardiomyocytes, caspase-9 could also be sequestered together with Cyt c within the mitochondrial intermembrane space (40–42). In these cells, >80% of all cellular caspase-9 localizes within mitochondria (42). In WEHI 231, however, only a small fraction of all caspase-9 would be locked up in the mitochondria to be released upon stimulation into the cytosol, where the majority of the protein is located anyway. We do not consider this a likely possibility.

Third, the minor fraction of caspase-9 might be localized not within mitochondria but onto the cytoplasmic surface of mitochondria. This localization could potentially sensitize the cells for mitochondrial-dependent caspase activation because even very low amounts of released Cyt c would readily “find” caspase-9. According to this scenario, Cyt c would be released from the mitochondria after BCR ligation and would be bound immediately. The resulting activation of this complex would then lead to activation of caspase-9. Subsequently, effector caspases-3 and -7 would be recruited, activated by cleavage, and then released, whereas active caspase-9 would remain in the complex. The model predicts that Apaf-1 or a homolog like the recently described proapoptotic caspase adaptor protein (43) will also be found in this compartment. Alternatively, caspase-9 could be activated, not in an apoptosisome-like complex, but via a yet unknown mechanism. Current experiments are addressing these issues.

Although damage to mitochondria have been emerging as central to BCR-induced apoptosis, it remains unclear how mitochondria are damaged and if there is one or more players in the game. The “multidomain” proapoptotic Bcl-2 proteins (i.e., Bak and Bax) should be most important because at least one of these proteins seems to be necessary for the induction of apoptosis by many stimuli (10, 44). Besides Bcl-2 proteins, lipids like ceramide or arachidonic acid are also known to impinge on mitochondria after BCR ligation (45–47). In keeping with this, an unusually low level of ceramide production correlated with resistance to BCR-induced apoptosis in a WEHI 231 variant (48). At the current stage, however, it is not clear whether physiological levels of these compounds are sufficient to induce apoptosis on their own or whether they only set the stage for Bak or Bax, for example, by lowering the threshold for Cyt c release. Clearly, additional experiments are needed to clarify the very important questions of how mitochondria are damaged and how apoptosis is induced after BCR ligation.

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