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Cloning and Functional Analysis of BAG-1: A Novel Bcl-2-Binding Protein with Anti-Cell Death Activity

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Summary

Using a protein interaction cloning technique, we identified cDNAs that encode a novel Bcl-2-binding protein, termed BAG-1. The BAG-1 protein shares no significant homology with Bcl-2 or other Bcl-2 family proteins, which can form homo- and heterodimers. In gene transfer experiments using a human lymphoid cell line, Jurkat, coexpression of BAG-1 and Bcl-2 provided markedly increased protection from cell death induced by several stimuli, including staurosporine, anti-Fas antibody, and cytolytic T cells, relative to cells that contained gene transfer–mediated elevations in either BAG-1 or Bcl-2 protein alone. BAG-transfected 3T3 fibroblasts also exhibited prolonged cell survival in response to an apoptotic stimulus. The findings indicate that *bag-1* represents a new type of anti-cell death gene and suggest that some routes of apoptosis induction previously ascribed to Bcl-2-independent pathways may instead reflect a need for the combination of Bcl-2 and BAG-1.

Introduction

Cell death plays an important role in a wide variety of physiological circumstances in essentially all complex multicellular organisms (reviewed by Ellis et al., 1991). Several genes have been identified that participate as either inducers or repressors of programmed cell death. Among these is *bcl-2* (for B cell lymphoma 2), a blocker of cell death that was first discovered by virtue of its involvement in the t(14;18) chromosomal translocations found in the majority of non-Hodgkin's B cell lymphomas (Tsujimoto and Croce, 1986). Gene transfer–mediated elevations in Bcl-2 protein levels have been shown to render cells relatively more resistant to induction of apoptosis by an impressive variety of stimuli and insults, suggesting that this protein regulates a distal step in a final common pathway for apoptotic cell death (reviewed by Reed, 1994). Furthermore, elements of this pathway appear to be well conserved throughout evolution (Vaux et al., 1992b; Hengartner and Horvitz, 1994).

The biochemical mechanism of action of the Bcl-2 protein remains enigmatic, principally because its predicted amino acid sequence shares no significant homology with other proteins whose functions are known. For this reason, we have attempted to identify proteins that can bind to Bcl-2. Here we describe the molecular cloning and functional characterization of cDNAs encoding a novel protein that interacts physically and functionally with the Bcl-2 protein. Gene transfer–mediated elevations in the levels of this Bcl-2-binding protein can prolong cell survival in some circumstances and also can cooperate with Bcl-2 in the suppression of apoptosis. These properties suggest that the gene encoding this protein represents a novel type of anti–cell death gene, which we have termed BAG-1, for Bcl-2-associated athanogene 1 (from the Greek word athanos, which refers to anti-death).

Results

Molecular Cloning of cDNAs for BAG-1

To identify cDNAs encoding proteins that can bind to Bcl-2, a mouse embryo cDNA library in a λ phage expression vector was screened with recombinant human Bcl-2 protein. Phage clones that produced proteins capable of binding to recombinant Bcl-2 protein were then detected by incubation of filters with a human-specific anti-Bcl-2 monoclonal antibody, resulting in a single positive clone from a screen of ~10^6 phage clones. DNA sequencing revealed an 830 bp insert, containing a 630 bp coding region in-frame with the upstream T7/10 protein sequences derived from the λEX-lox cloning vector.

BAG-1 Protein Specifically Interacts with Bcl-2 In Vitro

An immobilized protein interaction (Far Western blot) assay was performed to confirm the ability of recombinant Bcl-2 protein to bind to BAG-1 protein. The *bag-1* partial cDNA described above was subcloned into the plasmid pGEX-3X and expressed in Escherichia coli as a GST–BAG-1 fusion protein. GST–BAG-1 and GST nonfusion protein were compared with regards to binding of baculovirus-produced Bcl-2, using the same filter binding assay employed for library screening. As shown in Figure 1A, Bcl-2 bound to GST–BAG-1 but not to GST protein. In this experiment, cell lysates from a murine lymphoid cell, S49.1, that had been stably infected with a recombinant retrovirus encoding human Bcl-2 protein (S49.1-BCL-2) or a control retrovirus (S49.1-NEO) (Miyashita and Reed, 1992) were also included. The prominent ~26 kDa band seen in S49-BCL-2 cell lysates represents direct binding of the anti-human Bcl-2 monoclonal antibody 4D7 to human Bcl-2 protein in the S49.1-BCL-2 cells, thus serving as a control for the antibody detection system. In addition, however, a faint band at ~28–30 kDa was also seen in both S49.1-BCL-2 and S49.1-NEO cells that may represent binding of recombinant Bcl-2 protein to endogenous BAG-1 protein (see below). As a control, Far Western blot analysis was performed using Sf9 lysates derived from cells infected with a β-galactosidase (β-gal)-expressing baculovirus (Figure 1A, right).

To determine whether the interaction of BAG-1 with Bcl-2 can also take place in solution, soluble GST–BAG-1 and GST control proteins were immobilized on glutathione–Sepharose and then incubated with lysates prepared from Sf9 cells that had been infected with either Bcl-2-

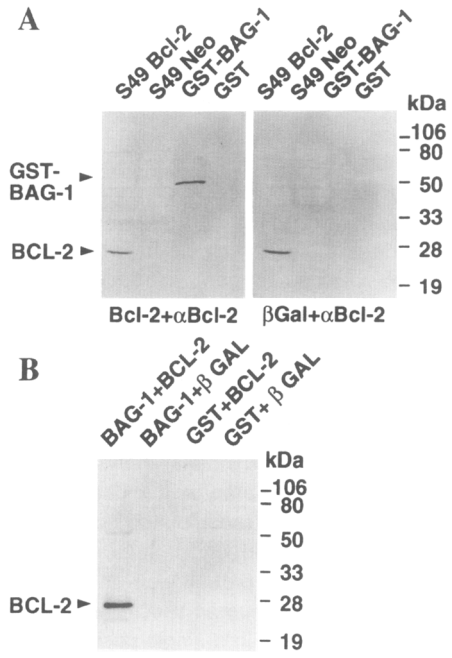

Figure 1. Interaction of Bcl-2 with BAG-1 Fusion Proteins

(A) Purified GST–BAG-1 (amino acids 8–219) fusion and GST nonfusion proteins (1 μg) and detergent lysates prepared from 10^6 S49-NEO and S49-BCL-2 cells were subjected to SDS–PAGE and transferred to nitrocellulose. The resulting filters were incubated with 1% (v/v) Sf9 lysates derived from cells infected with either Bcl-2 (left) or β-gal (right) baculoviruses, followed by anti-Bcl-2 antibody, which was detected by using an alkaline phosphatase–conjugated secondary antibody.

(B) GST–BAG-1 fusion and GST nonfusion proteins were immobilized on glutathione–Sepharose (~2.5 μg) and mixed with 500 μg of detergent lysates prepared from Sf9 cells infected with either Bcl-2 or β-gal-producing baculoviruses. The Sepharose beads were then washed extensively and associated proteins analyzed by SDS–PAGE/immunoblot assay using a Bcl-2-specific antiserum.

producing or control β-gal baculovirus. After the beads were washed, associated proteins were analyzed by immunoblotting using a Bcl-2-specific antibody (Reed et al., 1991). As shown, Bcl-2 protein was specifically recovered from Sf9 cell lysates on GST–BAG-1 Sepharose but not on beads that contained GST control protein (Figure 1B). Though the data are not presented here, Bcl-2 also did not associate with several other control GST fusion proteins. The interaction of BAG-1 with Bcl-2 was also confirmed by a yeast two-hybrid method (data not shown).

Predicted Amino Acid Sequence of BAG-1 Reveals a Novel Protein

Using the partial *bag-1* cDNA as a hybridization probe for cDNA library screening, we obtained additional *bag-1* cDNAs, thus deducing the complete open reading frame for the 219 amino acid mouse BAG-1 protein (Figure 2). BAG-1 is novel and shares no similarity with Bcl-2 or its homologous proteins. The BAG-1 protein is acidic (predicted pI, 4.81) and contains multiple glutamic acid residues (31 of 219). Although this raised the possibility of Ca^{2+} binding, no clear homology was observed with other

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MAKTEEMVQT EEMETPRLSV IVTHSNERYD    30
LLVTPQQGNS EPVVQDLAQL VEEATGVPLP    60
FQKLIFKGKS LKEMETPLSA LGMQNGCRVM    90
LIGEKSNPEE EVELKKLKDL EVSAEKIANH   120
LQELNKELSG IQQGFLAKEL QAEALCKLDR   150
KVKATIEQFM KILEEIDTMV LPEQFKDSRL   180
KRKNLVKKVQ VFLAECDTVE QYICQETERL   210
QSTNLALAE                          219
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Figure 2. Predicted Amino Acid Sequence of BAG-1 Protein

The predicted amino acid sequence of the encoded BAG-1 protein is presented.

Ca^{2+}-binding proteins of either the EF-hand or non-EF-hand type. A region in the mouse BAG-1 protein (residues 37–73) shows as much as 50% amino acid sequence identity (66% similarity) with several ubiquitin and ubiquitin-like proteins. The region from amino acid ~100 to amino acid ~212 is predicted to assume a largely α-helical conformation and contains segments that on Edmundson wheel plots are predicted to be amphipathic in nature and thus good candidates for participation in coiled-coil interactions with other proteins. Kyte–Doolittle plots failed to reveal any hydrophobic domains in BAG-1 that would suggest the presence of a transmembrane domain or hydrophobic leader sequence.

Gene Transfer Analysis of BAG-1 Function Reveals Anti-Cell Death Activity

A cDNA encoding full-length BAG-1 protein was subcloned into a mammalian expression plasmid, pCEP-4, that contains a hygromycin phosphotransferase gene and was introduced into Jurkat T cell lines that had previously been stably transfected with either a *bcl-2* (pZip–Bcl-2) or a control (pZip–NEO) expression plasmid (Torigoe et al., 1994a, 1994b). After selection in hygromycin, the BAG-1 transfectant cells were analyzed by immunoblotting using BAG-1- and Bcl-2-specific anti-peptide antisera. Elevated levels of BAG-1 protein were detected in the Jurkat–Bcl-2 and Jurkat–NEO transfectants that received the pCEP–4–BAG-1 plasmid but not those that were transfected with pCEP-4 parental control plasmid (Figure 3, left). Despite a predicted molecular mass of ~24.5 kDa, note that the BAG-1 protein migrates as a ~29–30 kDa protein, presumably owing to its acidic nature. As expected, Bcl-2 protein levels were markedly elevated in the Jurkat–Bcl-2 cells as compared with Jurkat–NEO cells (Figure 3, right).

We next explored the relative sensitivity of these doubly transfected T cells to induction of apoptosis by several stimuli. As shown in Figure 4, Jurkat T cells with gene transfer–mediated elevations in Bcl-2 protein were partially resistant to cytotoxicity induced by 1 μg/ml anti-Fas antibody. In contrast with Bcl-2, gene transfer–mediated elevations in BAG-1 by themselves had essentially no effect on the relative sensitivity of Jurkat T cells to anti-Fas-induced cytotoxicity. In combination with Bcl-2, however, elevations in BAG-1 protein levels resulted in markedly enhanced resistance to anti-Fas-induced apoptosis. Similar conclusions were reached on the basis of experiments where the protein kinase inhibitor staurosporine was employed to induce apoptosis in Jurkat T cells. As shown in

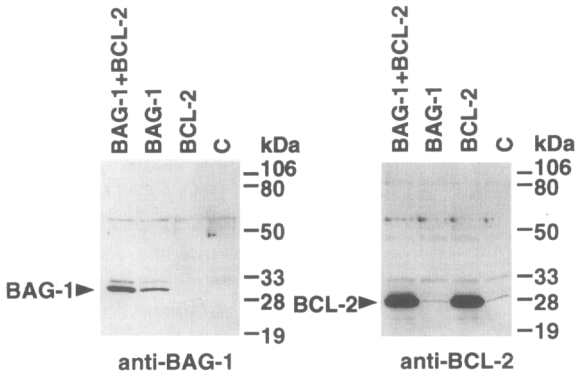

Figure 3. Immunoblot Analysis of BAG–1-Transfected Jurkat T Cells

Protein from cell lysates (50 μg) was subjected to SDS–PAGE/immunoblot analysis using either anti-BAG-1 (left) or anti-Bcl-2 antiserum (right). The arrows indicate the positions of the BAG-1 and Bcl-2 proteins. Control-transfected Jurkat cells (C) contained the pZip–NEO and pCEP-4 plasmids without inserted *bcl-2* or *bag-1* cDNAs.

Figure 4, BAG-1 alone provided only slightly enhanced protection from staurosporine-induced cell death. Bcl-2 was partially protective, with less than half of the cells still alive after 2 days. In contrast, the combination of BAG-1 and Bcl-2 afforded marked resistance to staurosporine-induced cell death, with ~80% of the cells surviving a

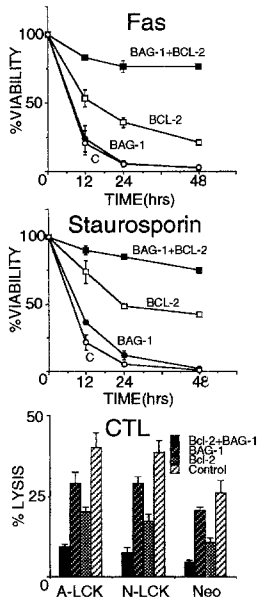

Figure 4. BAG-1 Cooperates with Bcl-2 to Increase Resistance to Cell Death in Jurkat T Cells

Transfected Jurkat cells were cultured at 5×10^5 cells/ml in complete medium containing either 1 μg/ml of anti-Fas monoclonal antibody 2D1 (top) or 10 μM staurosporine (middle). The percentage viable cells were then determined at various times thereafter by trypan blue dye exclusion. Equivalent data were obtained using MTT assays (data not shown). Jurkat cells were labeled with ^{51}Cr (bottom) and mixed with CTLL-2 cytolytic T cells that had been transfected with expression plasmids, producing constitutively activated Lck kinase (Tyr→Phe505), normal Lck kinase, or neo control plasmid alone. Specific Cr release was measured 4 hr later. All data represent mean ± standard deviation (n = 3).

2 day treatment with 10 μM staurosporine. The differential survival of these Jurkat T cell transfectants was not attributable to variations in rates of spontaneous cell death, since cell viability was >95% for all four cell lines when cultured for up to 3 or 4 days without anti-Fas antibody or other stimuli. Comparable results were obtained when several independent clones of tranfected Jurkat cells were evaluated (data not shown).

Next, Jurkat cells were labeled with ^{51}Cr and then co-cultured with cloned cytolytic T cells. Specific cytotoxic T lymphocyte (CTL)-induced ^{51}Cr release was then monitored in standard 4 hr cytotoxicity assays. These cloned T cells were previously shown to exhibit lymphokine-activated killer (LAK)-like cytolytic activity against Jurkat and certain other tumor cell lines through a CD3- and major histocompatibility complex–independent mechanism (Torigoe et al., 1994a, 1994b). Some of these cytolytic T cells had been stably transfected with expression plasmids encoding normal Lck kinase (N-Lck) or a constitutively active mutant version of Lck (A-Lck), since our previous investigations demonstrated that elevated levels of Lck kinase activity can enhance cytolytic T cell killing by increasing secretion of cytotoxic granules and expression of cell adhesion proteins (Torigoe et al., 1994a). Jurkat cells with elevations in BAG-1 protein exhibited some slight but reproducible (n = 3) resistance to lysis induced by CTLs. Jurkat cells with elevated levels of Bcl-2 protein were relatively more resistant than the BAG-1 transfectants, but not as resistant as Jurkat cells that contained gene transfer–mediated elevations in both Bcl-2 and BAG-1 proteins. Depending on the particular CTL clone examined, Jurkat cells overexpressing the combination of Bcl-2 and BAG-1 experienced only 16%–23% as much lysis as control-transfected Jurkat cells, compared with 76%–79% for the BAG-1 single transfectants and 40%–50% for the Bcl-2 single transfectants. Taken together, these data indicate that BAG-1 can augment the anti–cell death function of Bcl-2.

Analysis of BAG-1 Function in 3T3 Fibroblasts

In contrast with Jurkat T cells where BAG-1 alone only marginally enhanced cell survival, gene transfer studies using BALB/c 3T3 fibroblasts suggested that BAG-1 can have significant anti–cell death activity in some cells even when transfected without Bcl-2 expression plasmids. In these experiments, 3T3 cells were stably transfected either with the BAG-1 expression plasmid pRc/CMV–BAG-1 or with the pRc–CMV plasmid lacking an inserted *bag-1* cDNA. For comparison, 3T3 cells were also transfected with a Bcl-2 expression plasmid. Initially, a polyclonal line of G418-resistant BAG-1 transfectants was obtained but found to contain BAG-1 protein at levels not detectably above those seen in control-transfected 3T3 cells (lanes 1 and 2 of Figure 5). Individual clones from this bulk-transfected line were therefore obtained and some identified that had elevations in BAG-1 protein, presumably reflecting integration of the plasmid DNA into sites favorable for expression. The clone shown in Figure 5 (lane 3; clone number 19), for instance, contained ~3-fold higher levels of BAG-1 than the NEO control transfectants (lane 2). In

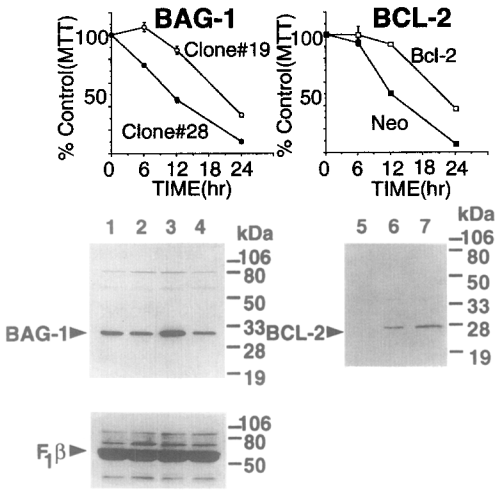

Figure 5. Expression and Function of BAG-1 in BALB/c 3T3 Cells

BALB/c 3T3 cells were stably transfected with pRc/CMV–BAG-1 (lane 1), pRc/CMV (lane 2), pZip–NEO (lane 5), or pZip–Bcl-2 (lane 6) plasmid DNAs. In some cases, independent clones were isolated from the pRc/CMV–BAG-1-transfected line: clones number 19 (lane 3) and number 28 (lane 4). Immunoblot analysis was performed (30 μg of cellular proteins) using antibodies specific for either the BAG-1 (lanes 1–4) or human Bcl-2 (lanes 5–7) proteins and using an ECL method. The t(14;18)-containing line RS11846 was included as a positive control (lane 7) for Bcl-2. The blot (lanes 1–4) was subsequently incubated with an antiserum specific for F_1–β-ATPase (detected by a colorimetric assay) to verify loading of equal amounts of protein.

At top, 3T3 transfectants were cultured at 10^4 cells/well (50%–70% confluent) for various times with 1 μM staurosporine, and the relative number of viable cells was determined by MTT assay and expressed as a control relative to untreated cells at the initiation of the experiment (mean ± standard deviation; n = 3).

many other G418-resistant clones, however, the levels of BAG-1 protein were essentially unchanged from baseline (clone number 28, for example; lane 4 of Figure 5).

Previously we showed that the protein kinase inhibitor staurosporine is a potent inducer of apoptosis in fibroblast cell lines (Jacobson et al., 1993), often producing more synchronous and reproducible cell death kinetics than serum withdrawal. We therefore challenged BAG-1 and control-transfected 3T3 cells with 1 μM staurosporine and monitored cell survival at various times thereafter. As shown, for example, by the results obtained with the BAG-1-overexpressing clone 19, gene transfer–mediated elevations in BAG-1 protein prolonged the survival of BALB/c 3T3 cells relative to other clones, such as clone 28, which had been transfected with the same *bag-1* cDNA–containing plasmid but failed to produce higher levels of BAG-1 protein (Figure 5). This prolongation of cell survival was typically similar to that seen for 3T3 cells that had been stably transfected with a Bcl-2-encoding expression plasmid.

Discussion

Using a protein interaction cloning technique, we have identified cDNAs encoding a novel Bcl-2-binding protein. With the exception of a ubiquitin-like domain, the predicted amino acid sequence of BAG-1 has no clear similarity to other known proteins and contains no motifs that would indicate a biochemical function for this protein. BAG-1 therefore may represent the prototype of a novel type of anti–cell death gene. Although Bcl-2 has been shown to bind to several homologous proteins, including Bax (for Bcl-2-associated x protein), Bcl-x_L, Bcl-x_S, and Mcl1 (Sato et al., 1994; Oltvai et al., 1993), BAG-1 lacks sequence homology with Bcl-2 and its related proteins. All Bcl-2 homologs identified thus far contain significant amino acid similarity in at least one of three well-conserved domains, which we have previously designated Bcl-2 domains A, B, and C (Sato et al., 1994). The BAG-1 protein lacks similarity to all three of these conserved domains, indicating that it is not a member of the Bcl-2 protein family.

BAG-1 contains a domain located at residues 37–73 that has sequence similarity with several ubiquitin and ubiquitin-like proteins, including the human and mouse Gdx-1 protein (50%, 43%), baculovirus ubiquitin-like protein (46%), and the mouse *Nedd8* gene (36%). Ubiquitin is a small 76 amino acid protein that is well conserved throughout evolution and that becomes covalently attached to proteins, typically as part of a nonlysosomal, ATP-dependent protein degradation pathway (reviewed by Hershko and Ciechanover, 1992; Rechsteiner, 1991). Ubiquitins contain a conserved lysine at position 48 that provides an ε amino acceptor group for covalent ligation to the carboxyl group of the C-terminal glycine of other ubiquitin molecules during the formation of branch chain ubiquitin–ubiquitin conjugates on target proteins. Interestingly, a lysine is found at position 67 within the ubiquitin-like domain of BAG-1 that corresponds to lysine 48 of ubiquitin. Internal lysines have also been shown to be the sites of ubiquitin conjugation in some other proteins that become polyubiquitinated and thereby targeted for degradation. Thus, one possible role of the ubiquitin-like domain in BAG-1 may be to serve as a site for attachment of ubiquitin and subsequent proteolytic degradation. In this regard, ubiquitin conjugation has been shown to regulate the turnover of other proteins that are either known or thought to play a role in cell death regulation in mammals, including the tumor suppressor p53 and some cyclins (Rechsteiner, 1991; Hershco and Ciechanover, 1992). In addition, previous studies using antisense approaches have documented a requirement for ubiquitin for apoptosis induced by γ-radiation in thymocytes (Delic et al., 1993). Inasmuch as this ubiquitin-like domain may be involved in targeting BAG-1 for degradation, it may be relevant that Jurkat cells cotransfected with Bcl-2 and BAG-1 consistently contained higher relative levels of BAG-1 protein than cells transfected with BAG-1 alone, suggesting that the interaction of BAG-1 with Bcl-2 potentially could stabilize the BAG-1 protein (Figure 3; data not shown). Unlike the protein ubiquitin, the function of the ubiquitin-like domains found in Gdx-1, Nedd-8, and other ubiquitin-like proteins is unknown. It has been speculated, however, that they may mediate protein–protein recognition (Toniolo et al., 1988), and thus, the ubiquitin-like domain in BAG-1 theoretically may participate in the binding of BAG-1 to Bcl-2 or other proteins. Recent studies indicate that proteases

can be essential for induction of programmed cell death and apoptosis in a variety of physiological and pathological circumstances (Yuan et al., 1993; Miura et al., 1993; Sarin et al., 1994; Gagliardini et al., 1994). By analogy to the known role of covalent addition of ubiquitin in rendering target proteins recognizable by subunits of a large 26S proteasome, it is tempting to speculate that the BAG-1 protein plays a role in bringing Bcl-2 or other Bcl-2-binding proteins into contact with a protease-containing protein complex that participates in cell death regulation.

Previously it has been suggested that there exist both Bcl-2-dependent and Bcl-2-independent pathways for suppression of apoptosis, largely on the basis of gene transfer experiments where elevated levels of Bcl-2 protein production were shown to be insufficient to protect cells from induction of cell death by some kinds of stimuli. In particular, it has been controversial whether high levels of Bcl-2 protein correlate with resistance to apoptosis induced by anti-Fas antibodies and cytolytic T cells, with Bcl-2 apparently providing protection in some cell lines but not others (Itoh et al., 1993; Owen-Schaub et al., 1994; Vaux et al., 1992a; Strasser et al., 1991; Torigoe et al., 1994b). The gene transfer experiments presented here, however, suggest that the failure of Bcl-2 to promote cell survival in at least some of these scenarios may have an explanation other than invoking the presence of a Bcl-2-independent pathway. In Jurkat T cells, for example, cotransfection of Bcl-2 and BAG-1 expression plasmids rendered these cells relatively more resistant to induction of cell death by staurosporine, anti-Fas antibody, and cytolytic T cells, whereas either Bcl-2 or BAG-1 alone was comparatively ineffective at providing protection from cell death. Clearly, therefore, the context in which Bcl-2 is overexpressed is vitally important to its ability to block cell death. To the extent that partner proteins such as BAG-1 are present at insufficient levels or dominant inhibitors such as Bax or Bcl-x_S are produced at excessive levels, a mere increase in Bcl-2 protein levels may be inadequate to protect some types of cells from some kinds of cell death stimuli. In these cases, the cell death pathway utilized may nevertheless directly involve a Bcl-2-regulable step.

Experimental Procedures

cDNA Library Screening

A mouse embryo DNA library in λEX-lox (Novagen, Incorporated) was plated on BL21(DE3) cells and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) according to the protocol of the manufacturer. Filters were preblocked in Hyb75 solution (20 mM HEPES [pH 7.7], 75 mM KCl, 2.5 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol [DTT], 0.1% Triton X-100) containing 5% nonfat milk for 30 min and then incubated overnight at 4°C in Hyb75 containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% milk, and 1% lysate from *bcl-2*–baculovirus-infected Sf9 cells (Reed et al., 1992; Newmeyer et al., 1994). After one wash for 5 min in Hyb75, filters were incubated for 2 hr at 4°C in the same solution containing 1% nonfat milk and 0.1% (v/v) ascites from the anti-Bcl-2 antibody–producing hybridoma 4D7.4 (Reed et al., 1992). Antibody detection was accomplished by using 0.14 μg/ml alkaline phosphatase–conjugated rabbit anti-mouse IgG (Promega, Incorporated) in Hyb75 with 1% milk followed by 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium chloride (BCIP–NBT). To convert this phage to plasmid, BM25.8 cells that contain P1 *cre* recombinase (Palazzolo et al., 1990) were infected and plated on ampicillin-containing LB plates.

In addition, a mouse kidney cDNA library in λgt10 (Clontech, Incorporated) was screened using the original *bag-1* cDNA clone (pS33–1) as a ^{32}P-labeled hybridization probe to obtain additional cDNA clones, of which λSN-245-9 contained nucleotides −82 to +843 bp.

BAG-1 Fusion Proteins

For GST–BAG-1 fusion protein, the *bag-1* cDNA (pS33–1) was excised from pEX/*lox*–BAG-1 by digestion with EcoRI and HindIII and subcloned into the EcoRI site of pGEX-3X (Pharmacia, Incorporated) by use of a HindIII–EcoRI adapter. The pGEX-3X and pGEX-3X–BAG-1 plasmids were transformed into XL1-blue cells (Stratagene, Incorporated). Overnight cultures were diluted 1:10 into LB medium containing 50 μg/ml ampicillin, and 2 hr later, 0.1–0.2 mM IPTG was added prior to incubating cultures 12–18 hr at 30°C with aeration. Cells were recovered from 250 ml cultures by centrifugation, resuspended in ~10 ml of phosphate-buffered saline (PBS; pH 7.4) containing 1% Triton X-100, 1 mM PMSF, and 1 mg/ml lysozyme, and sonicated on ice by using five bursts of 30 s each from a 1.6 mm tip (Ultrasonicator model XL-2020; Heat Systems, Incorporated). After centrifugation at 16,000 × g for 15–30 min, the supernatant was mixed with ~1 ml of glutathione–Sepharose-4B (Pharmacia, Incorporated) for 1 hr at 4°C. The beads were then washed two times in PBS containing 1% Triton X-100, and the GST–BAG-1 and GST proteins were eluted by using 5 vol of 5 mM glutathione, 50 mM Tris [pH 8.0].

Filter binding assays were performed using ~0.25 μg of purified GST–BAG-1 and GST control proteins. After size fractionation of proteins by SDS–polyacrylamide gel electrophoresis (PAGE) and transfer to nitrocellulose, filters were processed as described above for library screening. For binding of Bcl-2 to GST–BAG-1 protein in solution, GST control or GST–BAG-1 proteins were immobilized on glutathione–Sepharose-4B (~0.25 μg of protein/1 μl of beads) that had been preblocked in 0.5% nonfat milk and 0.05% bovine serum albumin (BSA), and ~10–20 μl was incubated for 1 hr on a rotator at 4°C with 50 μl of Sf9 cell lysates in 0.4 ml of binding buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40 [NP-40], 2 mM DTT, 0.05% BSA, 5% glycerol). Sepharose beads were then washed three times in 1 ml of TENNS buffer (2.5 mM Tris [pH 7.4], 2.5 mM EDTA, 250 mM NaCl, 1% NP-40, 2.5% sucrose), then resuspended in 20 μl of Laemmli buffer, and 10 μl was analyzed by SDS–PAGE/immunoblot assay (12% gels) using 0.2% (v/v) of an anti-Bcl-2 rabbit antiserum (Reed et al., 1991) and alkaline phosphatase–conjugated goat anti-rabbit IgG (Promega, Incorporated), followed by NBT–BCIP in 0.1 M Tris [pH 9.5], 0.1 M NaCl, 5 mM $MgCl_2$.

Antibodies and Immunoblotting

Antisera were prepared in rabbits essentially as described previously (Reed et al., 1991) by using a synthetic peptide corresponding to the C-terminal 16 amino acids of BAG-1 (NH_2–CQETERLQSTNLALAE-COOH) that was conjugated to maleimide-activated keyhole limpet hemocyanin (KLH) (Pierce, Incorporated). For SDS–PAGE/immunoblot assays, blots were preblocked in Tris-buffered saline (TBS; 20 mM Tris [pH 7.5], 150 mM NaCl) containing both 3% BSA and 5% nonfat milk, and washes were performed using TBS containing 0.05% Triton X-100. Primary antibodies were used at 0.1%–0.2% (v/v), and detection was with 0.05% horseradish peroxidase–conjugated goat anti-rabbit IgG (Amersham, Incorporated) in TBS with 3% BSA by the enhanced chemiluminescence (ECL) method (Amersham, Incorporated).

Gene Transfections

For BALB/c 3T3 cells, the *bag-1* cDNA SN-245-9 was subcloned into the HindIII and XbaI sites of pRc/CMV (Invitrogen, Incorporated). ScaI-linearized DNA (25 μg) was mixed with 2.5×10^6 BALB/c 3T3 cells in 0.8 ml of Hanks' balanced salt solution (HBSS; 20 mM HEPES [pH 7.05], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose) on ice. Electroporations were performed in 0.4 cm (diameter) cuvettes with platinum electrodes using 270 V and 1500 μF (Cell Ject; EquiBio, Incorporated). Selections were begun 2 days later using medium containing 1 mg/ml geneticin (GIBCO BRL, Incorporated), and individual clones were isolated using cloning cylinders.

For Jurkat T cells, the *bag-1* cDNA SN-245-9 in pSK-II was excised by use of HindIII and BamHI and subcloned into pCEP (Invitrogen, Incorporated). Jurkat cells (5×10^6) were mixed with 25 μg of plasmid

DNA in 0.8 ml of HBSS and electroporated using 270 V and 900 μF. After 2 days, cells were seeded at 10^5 cells/ml in medium containing 1 mg/ml hygromycin (Calbiochem, Incorporated).

Cytotoxicity Assays

Jurkat cells were cultured for various times at 5 × 10^5 cells/ml in 96-well flat-bottomed plates (Falcon, Incorporated) (0.2 ml/well) containing 1 μg/ml 2D1 antibody (Takahashi et al., 1993), 10 μM staurosporine (Sigma, Incorporated), or none of these reagents. BALB/c 3T3 cells were cultured with or without 1 μM staurosporine at 10^4 cells per flat-bottomed well. Relative numbers or percentages of viable cells were determined by trypan blue dye exclusion or 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay as described previously (Kitada et al., 1994). For CTL experiments, Jurkat cells were labeled with ^{51}Cr and cultured for 4 hr with CTLs at an E/T ratio of 20/1. The percentage specific ^{51}Cr release was then calculated by correction for spontaneous release and normalization for total release induced by 1% NP-40 as described (Torigoe et al., 1994a, 1994b).

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