Bcl-2 Family Member Bfl-1/A1 Sequesters Truncated Bid to Inhibit Its Collaboration with Pro-apoptotic Bak or Bax*

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Following caspase-8 mediated cleavage, a carboxy-terminal fragment of the BH3 domain-only Bcl-2 family member Bid transmits the apoptotic signal from death receptors to mitochondria. In a screen for possible regulators of Bid, we defined Bfl-1/A1 as a potent Bid interacting protein. Bfl-1 is an anti-apoptotic Bcl-2 family member, whose preferential expression in hematopoietic cells and endothelium is controlled by inflammatory stimuli. Its mechanism of action is unknown. We find that Bfl-1 associates with both full-length Bid and truncated (t)Bid, via the Bid BH3 domain. Cellular expression of Bfl-1 confers protection against CD95- and Trail receptor-induced cytochrome c release. In vitro assays, using purified mitochondria and recombinant proteins, demonstrate that Bfl-1 binds full-length Bid, but does not interfere with its processing by caspase-8, or with its mitochondrial association. Confocal microscopy supports that Bfl-1, which at least in part constitutively localizes to mitochondria, does not impede tBid translocation. However, Bfl-1 remains tightly and selectively bound to tBid and blocks collaboration between tBid and Bak or Bax in the plane of the mitochondrial membrane, thereby preventing mitochondrial apoptotic activation. Lack of demonstrable interaction between Bfl-1 and Bak or Bax in the mitochondrial membrane suggests that Bfl-1 generally prevents the formation of a pro-apoptotic complex by sequestering BH3 domain-only proteins.

Whether induced by death receptors or by other stimuli, apoptosis signaling generally involves cytochrome c (Cyt c)1 release from mitochondria. In the cytosol, Cyt c acts as cofactor in formation of a multimeric complex between the Apaf–1 scaffold protein and the initiator caspase-9. The ensuing proteolytic activation of caspase-9 allows for effector caspase processing and apoptotic execution (1, 2). Death receptors, which are apoptosis-inducing members of the tumor necrosis factor (TNF) receptor family, recruit and activate caspase-8 or –10. These ptosis-inducing members of the tumor necrosis factor (TNF) receptor family, recruit and activate caspase-8 or –10. These pro-apoptotic Bax type proteins, and pro-apoptotic BH3 domain-only family members (18, 19). The BH3 domain is a motif of about 16 residues, which forms an amphipathic helix that is necessary for death induction and dimerization among Bcl-2 family members. The BH3 helix of one partner can bind to a groove formed by BH1, BH2, and BH3 domain helices of the other (18). Bfl-1 has conserved BH1 and BH2 domains and is functionally defined as an inhibitory Bcl-2-type protein. Well documented is the protection it provides to TNF-α-induced apoptosis in primary endothelial cells and HeLa carcinoma cells (13, 20, 21). Furthermore, Bfl-1 conferred protection to p53-induced apoptosis in baby rat kidney cells (22, 23), and to apoptosis induced by the anti-cancer drug etoposide in fibrosarcoma cells (17). It also inhibited apoptosis initiated by B cell antigen receptor triggering (14, 16) and interleukin-1β withdrawal (9). Systematic examination of Bfl-1 function in primary cells has not been documented. Germ-line gene inactivation is complicated by the presence of at least four aI genes in the murine genome. Disruption of the aI-a gene accelerated spontaneous neutrophil apoptosis, but did not affect TNF-induced apoptosis of these cells (24).

The Bcl-2 family of apoptotic regulators is characterized by the presence of Bcl-2 homology (BH) domains and can be subdivided into three groups: anti-apoptotic Bcl-2 type proteins, pro-apoptotic Bax type proteins, and pro-apoptotic BH3 domain-only family members (18, 19). The BH3 domain is a motif of about 16 residues, which forms an amphipathic helix that is necessary for death induction and dimerization among Bcl-2 family members. The BH3 helix of one partner can bind to a groove formed by BH1, BH2, and BH3 domain helices of the other (18). Bfl-1 has conserved BH1 and BH2 domains and is functionally defined as an inhibitory Bcl-2-type protein. Well documented is the protection it provides to TNF-α-induced apoptosis in primary endothelial cells and HeLa carcinoma cells (13, 20, 21). Furthermore, Bfl-1 conferred protection to p53-induced apoptosis in baby rat kidney cells (22, 23), and to apoptosis induced by the anti-cancer drug etoposide in fibrosarcoma cells (17). It also inhibited apoptosis initiated by B cell antigen receptor triggering (14, 16) and interleukin-1β withdrawal (9). Systematic examination of Bfl-1 function in primary cells has not been documented. Germ-line gene inactivation is complicated by the presence of at least four aI genes in the murine genome. Disruption of the aI-a gene accelerated spontaneous neutrophil apoptosis, but did not affect TNF-induced apoptosis of these cells (24).

Human and mouse Bfl-1 share a BH1, BH2, and somewhat less conserved BH3 domain, but the limited homology to a BH4 domain found in human Bfl-1 is not present in the mouse. Protection by Bfl-1 impinges on intact BH1 and BH2 domains (23, 25). Whereas it is undisputed that Bfl-1 is a protective Bcl-2 type protein, its mechanism of action is unknown. The prototype members of this subgroup localize constitutively to membranes of mitochondria, endoplasmic reticulum, and nu-
clues, but Bfl-1 does not have a well defined hydrophobic carboxyl-terminal region, implicated in membrane anchoring. Whereas certain authors find Bfl-1 in mitochondria (21), others find it in the cytoplasm of resting cells and the nucleus of apoptotic cells (26).

A model for the mechanism of action of Bcl-2 family members, prominent until recently, predicts that Bcl-2 type proteins autonomously inhibit apoptosis. Heterodimerization with pro-apoptotic family members would abrogate their protective function and thereby provoke apoptosis (19, 27). However, new findings indicate that death induction is independent of Bcl-2 type proteins, but involves collaboration between Bax-type proteins and BH3 domain-only family members. The BH3 domain-only group has many members, which differ strikingly in their responsiveness to stimuli and thereby in the pathways they regulate (19). For instance, Noxa and Puma are regulated by p53 and implicated in DNA damage pathways (28, 29). Bmf binds to the cytoskeleton and is activated upon cell detachment (30), whereas Bid is processed and activated by caspase-8, downstream from death receptors (3, 4). The Bax group consists of three members, Bax, Bak, and Bok. The availability of Bax and Bak knockout mice has recently allowed work that illuminates how pro-apoptotic Bcl-2 family members induce cell death. Cells lacking both Bax and Bak did not die upon expression of BH3 domain-only proteins, in contrast to cells expressing either one of these molecules. This provided the insight that BH3 domain-only proteins require at least one Bax-type partner to induce cell death (31, 32). Both Bax and Bak undergo a conformational change in response to apoptotic stimuli (33, 34, 35). Moreover, they assemble into homodimers with presumed channel forming properties in the mitochondrial membrane (36, 37). The conformational change and multimerization of Bak or Bax is inducible by Bid (34, 36) and inhibitable by Bcl-2 (37, 38). The novel model emerging from these findings is that Cyt c release depends on interaction between a BH3 domain-only protein and a Bax-type partner, which allows formation of a Bax-type transmembrane pore.

We show here that Bfl-1 acts by inhibiting the collaboration between the BH3 domain-only protein Bid and its pro-apoptotic partners Bax or Bak in the induction of Cyt c release. Bfl-1 does so by binding to full-length Bid via the Bid BH3 domain. It does not interfere with proteolytic activation of Bid, nor with its mitochondrial insertion, but remains selectively complexed to tBid in the mitochondrial membrane where it prevents the activity of a pro-apoptotic complex.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Anti-human CD95 (APO-1/Fas) mAb 7C11 was purchased from Immunotech (Marseille, France). Rabbit anti-Bak pAb was from Sigma. Rabbit anti-Bcl-2 (N-20) and anti-BID-1/1A (FL-175) pAb were purchased from Santa Cruz Biotechnology. Anti-human CD95 (APO-1/Fas) mAb 7H8.2C12 from PharMingen. Protein A- and G-Sepharose and the ECL Western blotting system were from Amersham Biosciences. Soluble human recombinant TRAIL and enhancer were from R&D Systems. Fluorescein isothiocyanate-conjugated goat anti-rat Ig was from Rockland, PA. Mouse anti-human CD95 (7C11), rabbit anti-Bax (N-20) and anti-Bfl-1/A1 (FL-175) pAb were purchased from Immu- noTech (Marseille, France). Rabbit anti-Bak pAb was from Stress Mar (Gibbstown, NJ). Texas Red-conjugated rabbit anti-rat Ig, horseradish peroxidase-conjugated rabbit anti-mouse Ig, or swine anti-rabbit Ig were from DAKO A/S (Glostrup, Denmark). Etoposide was from Sigma. Soluble human recombinant Trail and enhancer were purchased from Alexios (Lauffenring, Germany). Recombinant human caspase-8 was obtained from Pharmingen. Protein A- and G-Sepharose and the ECL Western blotting system were purchased from BioRad.

**Cell Culture**—The J16 clone was derived from the human T-acute lymphoblastic leukemia cell line Jurkat by limiting dilution and selected for CD95 sensitivity (40, 41). J16 cells were cultured in Iscove’s modified Dulbecco’s medium, HeLa, COS-7, and Phoenix-Ampho cells in Dulbecco’s modified medium, both supplemented with 8% fetal calf serum, 2 mM glutamine, and antibiotics. Prior to stimulation, J16 cells were suspended in serum-free Yssel’s medium (49) and seeded at 1 3 10^5 per ml, 200 ml/well in round-bottom 96-well plates for apoptosis assays, and at 5–10 3 10^5 per ml in 24-well culture plates for Cyt c release assays. After addition of stimuli, cells were incubated for the indicated time periods at 37°C, 5% CO2. Irradiation was performed with 1000 rad from a 137Ca source (415 Ci; General Atomic, B.V.). HeLa cells, plated on coverslips, were preincubated with 10 mL/ml cycloheximide for 2 h at 37°C, 5% CO2 and subsequently stimulated with 10 ng/ml human recombinant TNFα (Sigma) in the continued presence of cycloheximide.

**Plasmids**—Human Bcl-2 DNA was cloned into the retroviral vector LZR5-MS-IRE-Sea-zoob-PBR, a derivative of LZR5-pBMIN-lacZ, which was a generous gift from Dr. G. Nolan (Stanford University School of Medicine, Stanford, CA). The retroviral vector pEYFz-FmA1 and the empty equivalent pEYFz-MCS were kindly provided by Dr. I. Berberich (Institut für Virologie und Immunobiologie, Universität Würzburg, Germany). pET15B/Bid containing human full-length Bid eDNA was a gift from Dr. X. Wang (Howard Hughes Medical Institute, Dallas, TX). pMT25M-Mye-Bid, pMD4-Bid, pEFGF-N1-Bid, and pET15b-Bid (nucleotides 151–588) vectors were made by introducing suitable restriction sites by PCR into this Bid eDNA. Modified pMT2 vectors with multiple cloning site and upstream Myc or HA tag sequence were constructed in our laboratory by Dr. M. Gembink. pEFGF-N1 is a commercial vector (CLONTECH), which allows fusion of EGF at the carboxy terminus of the eDNA product of interest. Point mutations in the BH3 domain of the different constructs were described (50) by site-directed mutagenesis using the QuikChange Site-directed Mutagenesis Kit (Stratagene). pCR3.1-Bak (mouse) was kindly provided by Dr. U. Arma (Institute of Biotechnology, University of Helsinki, Finland) and pcDNA3-HA-Bax (mouse) by Dr. H. Brady (National Institute for Medical Research, London, UK). Human Bfl-1 eDNA was cloned from the yeast expression vector pACT into pET15b (in vitro transcription/translation) and pMT25M-HA (for confocal microscopy) by PCR. All sequences were verified by dideoxynucleotide sequencing.

**Yeast Two-hybrid System**—For yeast two-hybrid protein interaction screens (51), pMD4-Bid was used as bait to screen a cDNA library of Epstein-Barr virus-transformed human B lymphocytes, according to earlier described procedures (52).

cDNA Transfer into Mouse Cells—COS-7 cells were transfected with combinations of pMT25M-Mye-Bid, pMT25M-HA-Bid-1, and/or empty vector by the standard DEAE-dextran transfection method, using 2.5 mg of plasmid DNA per 10^6 cells. HeLa cells (1 3 10^5) were grown overnight on a coverslip and transfected with 0.4 mg of DNA using Effectene according to the manufacturer’s instructions (Qiagen). To produce retrovirus, LZR5-Bcl-2-IRE-Sea-zoob-PBR, pEYFz-FmA1/Bid-1, or empty vector or pEYFz-MCS vectors were transfected into the 293T human embryonic kidney cell-derived packaging cell line Phoenix-Ampho (Ref. 53; provided by Dr. G. P. Nolan), using FuGENE 6 transfection reagent according to instructions of the manufacturer (Roche Molecular Biochemicals). Transfected cells were selected with 1 mg/ml puromycin (CLONTECH). Virus-containing supernatant was harvested after 48 h and stored at –80°C until further use. J16 cells were cultured with 200 mg/ml mAb 12CA5 (Takara) and transduced at a density of 0.5 3 10^7/ml of virus-containing supernatant. Supernatants were removed after overnight incubation, and cells were cultured in fresh medium. Selection of transduced cells was initiated after 48 h by the addition of 200 mg/ml Zeocin (Invitrogen). The selection process was completed within 2 weeks. Transduced cells were further cultured in the presence of 200 mg/ml Zeocin (Invitrogen).

**Apoptosis Assay**—To measure nuclear fragmentation, cells were lysed in 0.1% sodium citrate, 0.1% Triton X-100, 50 mg/mL propidium iodide (54) as described earlier (40, 41). Fluorescence intensity of propidium iodide-stained DNA was determined on a FACScan (Becton Dickinson, San Jose, CA) and data were analyzed using CellQuest software.

**Production of Recombinant Proteins**—Full-length Bid, tBid, Bfl-1, Bax, and Bak were in vitro transcribed and translated in the presence of [35S]methionine/cysteine from the appropriate cDNA vectors with the TNT Quick Coupled Transcription/Translation for genes cloned downstream from the T7 RNA polymerase promoter, according to instructions supplied by the manufacturer (Promega).

**Immunoprecipitation and Western blotting**—COS-7 cells were lysed with 1% NP-40 in 50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 5 mg/mL leupeptin, and 2.5 mg/mL aprotinin at 48 h after transfection. Cell lysates were clarified by centrifugation for 10 min at 14,000 rpm at 4°C. Lysates were incubated for at least 2 h at 4°C with either 12CA5 mAb or polyclonal anti-Bid serum. Immune complexes were incubated with protein A-Sepharose beads for an additional 2 h. Precipitated proteins were suspended in serum-free Yssel’s medium (49) and seeded at 1 3 10^6 per ml, 200 ml/well in round-bottom 96-well plates for apoptosis assays, and at 5–10 3 10^5 per ml in 24-well culture plates for Cyt c release assays. After addition of stimuli, cells were incubated for the indicated time periods at 37°C, 5% CO2. Irradiation was performed with 1000 rad from a 137Ca source (415 Ci; General Atomic, B.V.). HeLa cells, plated on coverslips, were preincubated with 10 mL/ml cycloheximide for 2 h at 37°C, 5% CO2 and subsequently stimulated with 10 ng/ml human recombinant TNFα (Sigma) in the continued presence of cycloheximide.
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were subjected to 15% SDS-PAGE and transferred to nitrocellulose. Blots were blocked in PBS containing 0.05% Tween 20 and 5% non-fat dry milk (Nutricia) and subsequently incubated with the appropriate primary and secondary antibodies. Immunostained proteins were visualized by ECL.

Immunoblot Analysis for in Vivo Cyt c Release—After incubation with the appropriate stimuli, cells were washed twice with ice-cold PBS and resuspended in 100 μl of extraction buffer (50 mM PIPES-KOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM MgCl_2, 1 mM dithiothreitol and protease inhibitors) and allowed to swell on ice for 30 min (55). Cells were homogenized by passing the suspension through a 25-gauge needle (10 strokes). Homogenates were centrifuged at 10,000 g at 4 °C and supernatants were harvested and stored at −70 °C until analysis by gel electrophoresis. Ten μg of cytosolic protein, as determined by the Bio-Rad protein assay (Bio-Rad, München), were loaded per lane on 12% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose sheets, which were blocked for 1 h in PBS, 0.05% Tween with 5% non-fat dry milk, and probed in PBS, 0.05% Tween with anti-Cyt c mAb (1:1000 dilution). Blots were washed in PBS, 0.05% Tween 20 and incubated with either Texas Red- or fluorescein isothiocyanate-conjugated anti-rat Ig for 30 min. Cells were washed and resuspended in 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, Na_2EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) were precipitated with appropriate antibodies and Protein A-Sepharose (Merck, Burlingham, CA) and viewed under a Leica TCS NT confocal laser-scanning microscope (Leica Microsystems, Germany). Confocal images were taken from a basal plane of the cells, just above the basal membrane, unless indicated otherwise.

RESULTS

Bfl-1 Interacts with Full-length Bid—To obtain more insight into the regulation of Bid function, we searched for Bid-interacting proteins using the yeast two-hybrid system. A cDNA library of Epstein-Barr virus-transformed human B-lymphocytes was screened with full-length Bid as bait. The screen revealed eight positive clones, which all represented Bfl-1 (data not shown). To confirm the interaction between Bid and Bfl-1, COS-7 cells were transiently transfected with Myc-tagged full-length Bid and HA-tagged Bfl-1 alone, or in combination. Bfl-1 protein expression was easily detectable in the total lysate and in anti-HA immunoprecipitates (Fig. 1). Anti-HA probing of anti-Bid immunoprecipitates showed that Bfl-1 and Bid interact specifically, since absence of either Myc-Bid or HA-Bfl-1 in the lysates gave no signal (Fig. 1). Thus, Bfl-1 interacts with full-length Bid, as shown by coexpression in yeast and mammalian cells.

Bfl-1 Inhibits CD95- and Trail Receptor-induced Apoptosis and Cyt c Release in Jurkat Cells—Given its interaction with Bid, we expected Bfl-1 to impact on signaling by death receptors such as CD95 (APO-1/Fas), TNF receptor-1, and the Trail receptors. Previously, Bfl-1 was found to inhibit TNF-induced apoptosis in human microvascular endothelial cells and HeLa cells, but its impact on CD95 and Trail receptor signaling was unexplored. To examine the effect of Bfl-1 on death receptor-mediated apoptosis, we used Jurkat T-leukemic cells, because they are defined as Type II cells, in which Bel-2 overexpression inhibits apoptosis induced by CD95 (39). The J16 Jurkat clone, used routinely in our studies (40, 41), was transduced with retroviral vectors containing Bel-2 or Bfl-1 and stimulated with anti-CD95 mAb or recombinant Trail. In agreement with the definition of Jurkat cells as Type II cells, Bel-2 inhibited both CD95- and Trail receptor-induced apoptosis, as read out by nuclear fragmentation. Bfl-1 was fully comparable with Bel-2 in its inhibitory effect on both CD95 and Trail receptor-mediated apoptosis (Fig. 2). After long periods of stimulation and at a high dose of stimulus, neither Bfl-1 nor Bel-2 could fully inhibit apoptosis, which can be explained by mitochondrion independent effector caspase activation by CD95 and Trail receptor. Interestingly, in the same cells tested for death receptor sensitivity, Bfl-1 also inhibited apoptosis as induced by DNA damaging stimuli, i.e. the anti-cancer drug etoposide and γ-radiation, but was much less effective than Bel-2 in doing so (Fig. 2). This result suggests that Bfl-1 is more selective for the death receptor pathway.

To explore whether the inhibitory effect of Bfl-1 on CD95- and Trail receptor-mediated apoptosis was consistent with an effect on Bid function, we examined Cyt c release in Bfl-1-transduced cells. Fig. 3 shows that CD95 and Trail receptor effectively induce Cyt c release in control-J16 cells, transduced with empty vector. Kinetics of Trail receptor-induced Cyt c release are consistently slower in these cells than kinetics of CD95-induced Cyt c release, which is consistent with a slower
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onset of nuclear fragmentation (Fig. 2). Cyt c release in response to both stimuli was severely reduced in cells expressing Bfl-1. In fact, Bfl-1 inhibited Cyt c release to a similar extent as Bcl-2 (Fig. 3).

**Fig. 3.** Bfl-1 inhibits Cyt c release in vivo. J16 Jurkat cells, transduced with empty vector, vector with Bfl-1 or Bcl-2 cDNAs, were left untreated (medium), or stimulated with anti-CD95 mAb 7C11 at 100 ng/ml, or recombinant Trail at 200 ng/ml plus enhancer for 4 h, with human recombinant soluble Trail plus enhancer for 6 h, or with etoposide or γ-radiation for 16 h. Propidium iodide-stained nuclei were prepared and apoptosis was read out as subdiploid DNA content on a FACSscan. Data are representative of multiple independent experiments.

Bfl-1 Blocks tBid-induced Cyt c Release, But Allows Generation of tBid and Its Association with Mitochondria—In J16 cells, CD95- and Trail receptor-induced Cyt c release is inhibited by a Bid mutant, which lacks the caspase-8 cleavage site, proving that tBid generated by caspase-8 is a crucial mediator in these responses. However, caspase-8-processed Bid is difficult to detect, possibly because of the small pool of Bid molecules involved, or because of its rapid turnover. Bid fragments can be detected a few hours after receptor stimulation, but these are generated downstream from the mitochondria (results not shown). To define the impact of Bfl-1 on Bid processing and function, we therefore resorted to an in vitro system, in which mouse liver mitochondria are incubated with recombinant proteins for 1 h at 30 °C. Subsequently, mitochondrial and cytosolic protein fractions were electrophoresed and immunoblotted for the presence of Cyt c. Whereas full-length Bid or caspase-8 alone did not affect mitochondrial permeability, the combination allowed effective Cyt c release, in line with the generation of active tBid (Fig. 4A). Similarly, the recombinant tBid fragment induced Cyt c release. These results show that the system faithfully mimicks the in vivo situation. In both cases, Cyt c release was completely blocked by simultaneous addition of Bfl-1.

Autoradiography of the blots allowed us to examine the presence of radiolabeled recombinant proteins in the mitochondrial and soluble fractions (Fig. 4B). This revealed that recombinant caspase-8 had effectively generated tBid. In contrast to full-length Bid, which was found predominantly in the soluble fraction, tBid generated from full-length Bid by caspase-8, as well as recombinant tBid preferentially associated with the mitochondria. Note that recombinant tBid has a somewhat higher molecular mass than tBid processed from full-length Bid, because it has a His tag. In the presence of Bfl-1, proteolytic processing of full-length Bid by caspase-8 was not influenced at all. Moreover, tBid generated by caspase-8, as well as recombinant tBid associated with the mitochondria with exactly the same efficiency as in the absence of Bfl-1 (Fig. 4B).

The proportion of Bfl-1 molecules associating with the mitochondria in the absence of tBid was in the order of 50%, which was not dramatically changed in the presence of tBid. Apparently, insertion of tBid into the mitochondrial membrane is not the driving force for association of Bfl-1 with these organelles.

In conclusion, Bfl-1 associates with full-length Bid, but does not affect its processing by caspase-8, nor association of tBid with the mitochondria. However, Bfl-1 dramatically inhibits tBid-mediated Cyt c release.

In Intact Cells, Bfl-1 Localizes in Part to Mitochondria and Allows Death Receptor-induced Translocation of Bid—The in vitro studies indicated that Bfl-1 has a capacity to associate with mitochondria and does not interfere with the mitochondrial association of tBid. To examine the exact intracellular localization of Bfl-1 and its impact on Bid translocation in response to stimulus, confocal microscopy was used. Bfl-1 and a full-length Bid molecule, tagged at its carboxyl terminus with green fluorescent protein (GFP), were expressed in HeLa cells, which are adherent cells with a large cytoplasm and therefore allow proper visualization of mitochondrial translocation. The localization of Bfl-1 relative to mitochondria was examined by immunostaining of Bfl-1, and labeling of mitochondria with MitoTracker. In unstimulated HeLa cells, Bfl-1 was concentrated in the perinuclear region, where it co-localized for a

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large part, but not completely, with mitochondria. Outside the perinuclear region, Bfl-1 was detected at incidental mitochondria, but also in low intensity diffusely throughout the cytoplasm (Fig. 5A). Bfl-1 localization relative to mitochondria did not alter significantly after TNF receptor stimulation, which induces Cyt c release and apoptosis in HeLa cells (Fig. 5A). Co-expression of Bfl-1 and Bid-GFP revealed a diffuse, cytoplasmic localization of Bid-GFP in unstimulated cells, whereas Bfl-1 was again concentrated in the perinuclear region (Fig. 5B). At 2 h after TNF receptor stimulation, Bid-GFP was concentrated in the perinuclear region, reflecting its mitochondrial translocation (as corroborated with MitoTracker staining; not shown). Apparently, Bfl-1 did not impede the mitochondrial translocation of Bid-GFP. Overlay of Bid-GFP and Bfl-1 staining revealed a significant degree of co-localization, consistent with the simultaneous presence of tBid and Bfl-1 at the mitochondria after death receptor stimulation (Fig. 5B).

**Bfl-1 Abrogates Synergism between tBid and Bax or Bak in Bringing about Cyt c Release**—tBid is proposed to bring about Cyt c release by collaborating with Bax or Bak at the mitochondrial outer membrane. Therefore, we examined how Bfl-1 impacts on this collaboration. In isolated mouse liver mitochondria, endogenous Bak, but not Bax protein could be detected (not shown). When they were incubated with recombinant Bak or Bax alone, no Cyt c release was observed (Fig. 6, A and B, upper panels). Increasing amounts of recombinant tBid were added, which in the absence of recombinant Bak or Bax were not sufficient to bring about Cyt c release. However, when a fixed amount of recombinant Bak or Bax was added simultaneously with tBid, synergy between tBid and Bak or Bax allowed for Cyt c release to occur (Fig. 6, A and B, upper panels). In the presence of Bfl-1, no Cyt c release took place, indicating that the synergy between tBid and Bax or Bak was completely inhibited (Fig. 6, A and B, upper panels).

Autoradiography of the blots revealed that association of recombinant Bax with the mitochondria was inefficient and not enhanced by the presence of tBid (Fig. 6A, lower panel). Recombinant Bak on the other hand, preferentially localized to the mitochondria, which was also independent of tBid (Fig. 6B, lower panel). The amounts of recombinant tBid, Bax, Bak, and Bfl-1 as visualized by autoradiography further serve as controls for the Cyt c release assay (Fig. 6, A and B, lower panels).

**Bfl-1 Selectively Binds to tBid, Not Bax or Bak, via the Bid BH3 Domain**—Since Bfl-1 inhibited tBid-induced Cyt c release, we examined whether it might do so by binding and sequestering tBid after its insertion into the mitochondrial outer membrane. Bringing together recombinant tBid, Bax, Bak, and Bfl-1 in vitro in buffer, either in the presence or absence of various detergents, did not allow efficient interaction between any of these Bcl-2 family members. However, a completely different picture emerged when the recombinant proteins were added together in the presence of mouse liver mitochondria. Detergent extraction of mitochondria, incubated with recombinant proteins, followed by immunoprecipitation with anti-Bid antisera revealed that tBid very efficiently complexes with Bfl-1 when they are together in the mitochondrial membrane (Fig. 7A). Bfl-1 and tBid were present in an anti-Bid immunoprecipitate at about equimolar levels. A BH3 domain mutation in tBid (M97A/D98A), which had been described to abrogate...
interaction between Bid and Bcl-2 (50), reduced the efficiency of interaction between tBid and Bfl-1 at least 10-fold, as determined by phosphorimaging of the blot (Fig. 7A). In the same experiment, and others like it, only trace amounts of tBid-Bax or tBid-Bak complexes could be detected, despite the presence of mitochondria (Fig. 7A). This finding suggests that the interaction between tBid and Bfl-1 is of another nature than the interaction between tBid and Bax or Bak, which is proposed to take place in the mitochondrial membrane. The observed, low level of complex formation between tBid and Bak was affected however, by the BH3 domain mutation in tBid (Fig. 7A).

Next, we examined whether Bfl-1 was selective in its interaction with tBid, or also formed complexes with Bax or Bak in the presence of mitochondria. tBid was included to simultaneously investigate impact of Bfl-1 on complex formation between tBid and Bax or Bak. As for Fig. 7A, the level of complex formation between tBid and Bax or Bak was very low, as revealed in Fig. 7B by immunoprecipitation via Bid, Bax, or Bak. Therefore, no conclusions could be drawn concerning a possible effect of Bfl-1 on this complex formation (Fig. 7B). Immunoprecipitation with anti-Bid serum again showed very efficient complex formation between Bfl-1 and tBid, as in Fig. 7A. In contrast, neither anti-Bax nor anti-Bak immunoprecipitates contained a significant amount of Bfl-1. Clearly, Bfl-1 is highly selective between Bcl-2 family members and, in the plane of the mitochondrial membrane, only efficiently associates with tBid, not with Bax or Bak. Lack of demonstrable interaction between Bfl-1 and Bax or Bak in the mitochondrial membrane suggests that Bfl-1 may generally prevent the formation of a pro-apoptotic complex by interacting with BH3 domain-only proteins.

**Fig. 6.** Bfl-1 abrogates synergism between tBid and Bax or Bak in induction of Cyt c release. A, isolated mouse liver mitochondria were incubated for 30 min at 30°C with increasing concentrations of in vitro transcribed/translated tBid alone, or together with Bax, or Bak and Bfl-1. The upper panel shows Cyt c release, as assayed by immunoblotting, the lower panel shows the presence of 35S-radiolabeled recombinant proteins in mitochondrial (mito) and soluble (sol) fractions, as determined by autoradiography. B, the same type of experiment as outlined for A, but using Bak instead of Bax. The upper panel shows the result of anti-Cyt c immunoblotting, the lower panel the result of autoradiography.

**Fig. 7.** Bfl-1 binds selectively to the BH3 domain of tBid in the plane of the mitochondrial membrane. A, isolated mouse liver mitochondria were incubated with in vitro transcribed/translated Bfl-1, Bax, or Bak, in combination with recombinant wild type tBid, or with tBid containing M97A/D98A BH3 domain mutations (tBidm) for 30 min at 30°C. Mitochondria were subsequently lysed with CHAPS detergent. The upper panel shows the presence of 35S-radiolabeled recombinant proteins in the lysate. The lower panel reveals which recombinant proteins are present in anti-Bid immunoprecipitates derived from the mitochondrial lysates, as determined by autoradiography. B, isolated mouse liver mitochondria were incubated for 30 min at 30°C with in vitro transcribed/translated recombinant tBid and Bax or Bak, in the presence or absence of Bfl-1. Mitochondria were lysed with CHAPS and lysates, as well as anti-Bid and -Bax, and Bak immunoprecipitates were examined for the presence of radiolabeled recombinant proteins by autoradiography. To note, the band running at ~17 kDa in the upper panel of B is most likely a degradation product of recombinant Bak, since it is also precipitated with anti-Bak serum.

**DISCUSSION**

Bfl-1 has been grouped together with Bcl-2, Bcl-xL, Bcl-w, Boo, and Mcl-1 in the inhibitory Bcl-2 subfamily. However, Bfl-1 lacks a bona fide hydrophobic anchor at the carboxy terminus, which targets prototype inhibitory family members to membranes of endoplasmic reticulum, nucleus, and mitochondria (27). Three charged residues interrupt the potential equivalent of this anchor in Bfl-1 (23), raising the question whether Bfl-1 is freely distributed throughout the cytosol, as opposed to its closest relatives. Reports on the intracellular localization of Bfl-1 are partly conflicting. In transfected baby rat kidney cells, Bfl-1 was present in the perinuclear region, similar to Bcl-2 (22). In transduced endothelial cells, it was found predominantly in the membrane fraction, including mitochondria, but also in cytosol (21). Endogenous Bfl-1 was
found in the cytosol of live macrophages and in the nucleus once they were apoptotic (26). We find that Bfl-1 has a propensity to associate with the mitochondrial membrane and constitutively localizes to mitochondria and other structures in the perinuclear region. Weak staining outside this region suggests that Bfl-1 in part localizes to cytosol. The collective data favor the interpretation that Bfl-1, like the prototype Bcl-2, primarily performs its inhibitory function at intracellular membranes, including those of mitochondria.

Whereas the anchor sequence is necessary for membrane localization of Bcl-2, its anti-apoptotic activity does not consistently depend on it (27). Deletion of the carboxy-terminal residues in Bfl-1 reduced protection to p53-induced apoptosis (23). Whether it affected membrane association was not reported. It will be interesting to examine whether Bfl-1 requires localization to intracellular membranes to confer cell survival. Its predominant interaction with tBid was only revealed in the presence of mitochondria. This suggests that membrane localization of both proteins, and/or the availability of other partners at the mitochondrial membrane, promote complex formation between tBid and Bfl-1 and thereby presumably the anti-apoptotic activity of Bfl-1.

Bfl-1 has previously been shown to protect from TNF-induced apoptosis (13, 20, 21). TNF induces apoptosis via TNF receptor-1, a member of death receptor family. We show here that Bfl-1 also affects apoptosis signaling by the CD95 and TRAIL death receptors. Our finding that Bfl-1 prevents the pro-apoptotic activity of tBid explains its capacity to regulate death receptor signaling in general. Importantly, Bfl-1 inhibits activation of the mitochondrial pathway downstream from death receptors, which is not always necessary to bring about an apoptotic response. In a number of cell types, death receptors can bypass mitochondria to induce apoptosis, because of the capacity of caspase-8, –10 to directly process effector caspases (39). This may explain why Bfl-1 severely delays TNF-induced apoptosis in microvascular endothelial cells, but not in umbilical cord endothelial cells (21).

Previously, Bfl-1 was found to inhibit mitochondrial Cyt c release and dysfunction in response to TNF, in agreement with our findings. In that study, however, Bid processing appeared to be inhibited by Bfl-1 (21). Most likely, the Bid processing observed was mediated by effector caspases, rather than by caspase-8 upstream from the mitochondria. We have also found such secondary, Bcl-2 inhibitable, Bid processing in Jurkat cells (results not shown).

The question is, whether the inhibitory activity of Bfl-1 in other apoptosis pathways can also be attributed to its effect on Bid function. Given the variety of pathways that are affected by Bfl-1, Bid may not be its only target. Analysis of Bid-deficient mice has thus far not provided evidence that Bid participates in death receptor-independent pathways (42). For instance, eto-poside-induced apoptosis in mouse embryonic cells proceeded normally in the absence of Bid (32). Recent evidence suggests that DNA damage pathways may use Noxa and/or Puma, which are p53-inducible BH3 domain-only proteins (29, 43). Whereas Bfl-1 and Bcl-2 inhibited the death receptor pathways with similar efficiency, we found Bfl-1 to be much less protective than Bcl-2 in DNA damage pathways. Since a variety of BH3 domain-only proteins can collaborate with Bax and Bak (44), a differential capacity of Bfl-1 and Bcl-2 to inhibit certain pathways is best explained by a differential capacity to sequester the BH3 domain-only partner of the pro-apoptotic complex. It will be interesting to examine, therefore, whether Bfl-1 and Bcl-2 have different selectivities in binding BH3 domain-only proteins in the mitochondrial membrane.

Unlike Bcl-2, Bfl-1 is not expressed constitutively, but induced by stimuli that activate NF-κB (13–15, 17). NF-κB transcription factor complexes can induce many other anti-apoptotic gene products, such as Bcl-xL, Flipp, and IAPs (15, 45–47). Many members of the TNF receptor family can activate NF-κB and therein promote cell survival. Intermediate in this response is the TRAF adapter protein. The up-regulation of Bfl-1 by Traf-linked TNF receptor family member CD40 is in line with this general principle (15, 16). Death receptors can couple to the apoptotic pathway via Fadd and caspase-8, whereas most of them can also promote cell survival via TRAFs. Whether a cell survives death receptor signaling may well depend on the anti-apoptotic proteins that are in place before the death signal is irrevocable.

A prevailing thought has been that death signaling involves neutralization of the apoptosis inhibitory activity of Bcl-2-type proteins by their pro-apoptotic family members. However, it has recently become clear that the death signal depends on cooperation between a BH3 domain-only protein and a Bak/Bak-type protein (13, 32). Bax and Bak have been found to change conformation and form homomultimers in response to apoptotic stimuli or BH3 domain-only proteins (34). In a number of independent studies, Bcl-2 and Bcl-xL blocked the mitochondrial change and/or multimerization (32, 33, 37, 38). Apart from our work, one recent study provides evidence that inhibitory Bcl-2-type proteins may inhibit the cooperation between BH3 domain-only proteins and Bax or Bak by sequestering the BH3 domain-only partner of the complex: a Bcl-xL mutant that failed to bind Bim, Bad, or Bid, as well as a Bcl-2 mutant that failed to bind Bid in vivo, could no longer protect from apoptosis induced by overexpression of these BH3 domain-only proteins (44). Earlier work had indicated that Bcl-xL mutants unable to bind Bax or Bak were still protective (45). Future use of mutant Bcl-2 family members in functional assays, combined with analysis of complex formation in vivo should elucidate whether inhibitory Bcl-2 family members generally function by sequestering the BH3 domain only partner of the pro-apoptotic complex.

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Bcl-2 Family Member Bfl-1/A1 Sequesters Truncated Bid to Inhibit Its Collaboration with Pro-apoptotic Bak or Bax

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