Bid is a proapoptotic, BH3-domain-only member of the Bcl-2 family. In Fas-induced apoptosis, Bid is activated through cleavage by caspase 8 into a 15.5-kDa C-terminal fragment (tBid) and a 6.5 kDa N-terminal fragment (t"Bid). Following the cleavage, t"Bid translocates to the mitochondria and promotes the release of cytochrome c into the cytosol by a mechanism that is not understood. Here we report that recombinant t"Bid can act as a membrane destabilizing agent. t"Bid induces destabilization and breaking of planar lipid bilayers without appearance of ionic channels; its destabilizing activity is comparable with that of Bax and at least 30-fold higher than that of full-length Bid. Consistently, t"Bid, but not full-length Bid, permeabilizes liposomes at physiological pH. The destabilizing effect of t"Bid on liposomes and planar bilayers is independent of the BH3 domain. In contrast, mutations in the BH3 domain impair t"Bid ability to induce cytochrome c release from mitochondria. The permeabilizing effect of t"Bid on planar bilayers, liposomes, and mitochondria can be inhibited by tBid. In conclusion, our results suggest a dual role for Bid: BH3-independent membrane destabilization and BH3-dependent interaction with other proteins. Moreover, the dissociation of Bid after cleavage by caspase 8 represents an additional step at which apoptosis may be regulated.

Apoptosis is a process by which unnecessary or damaged cells are eliminated in multicellular organisms (1, 2). After the induction of apoptosis, cells enter the effector phase, in which they are either rescued or committed to death. The proteins of the Bcl-2 family regulate this process (3, 4). Members of the Bcl-2 family are pro- or antiapoptotic and possess one or several of the Bcl-2 homology (BH)1 domains. Bid is a proapoptotic, BH3-domain-only member of the Bcl-2 family. When expressed in Jurkat cells or transfected in fibroblasts, it induces apoptosis (5). Recently, Bid-deficient mice have been shown to be resistant to Fas-induced hepatocellular apoptosis (6). Bid is a cytosolic protein in nonapoptotic cells, present in a variety of tissues (5). Upon induction of certain types of apoptosis, Bid is cleaved by caspase 8, and its C-terminal fragment translocates to the mitochondria (7–9). According to the x-ray structure, cleavage of Bid unmasks a large hydrophobic surface in its C-terminal fragment (10, 11). The C-terminal fragment of Bid (called t"Bid) is regarded as the active form. It is far more potent in inducing apoptosis than FL Bid (7, 9), and it can be inhibited by coexpression of the N-terminal fragment (t"Bid) (12).

Bid is believed to exert its proapoptotic effect by inducing the release of proapoptotic factors (cytochrome c, apoptosis-inducing factor, and procaspase 9) from mitochondria. Incubation of subnanomolar concentrations of t"Bid with isolated mitochondria results in a complete release of cytochrome c (7). More recently, it has been shown that Bid partially permeabilizes the outer mitochondrial membrane and causes the release of several proteins from the intermembrane space (13). A major controversy is whether Bid acts by itself or through modulation of other Bcl-2 family proteins.

In vitro binding and mutagenesis experiments support the hypothesis that Bid acts by regulating other Bcl-2 family proteins. FL Bid can bind Bax, Bcl-XL, and Bcl-2 (5). When the BH3 domain of Bid is mutated so that it no longer binds Bax, FL Bid fails to promote apoptosis (5). Consistently, FL Bid is over 10 times less active in releasing cytochrome c from Bax-/− mitochondria than from wild type mitochondria. Moreover, FL Bid induces a conformational change in Bax, which correlates with the release of cytochrome c (14, 15). t"Bid has a higher affinity than FL Bid for Bax2 and for Bcl-XL (7, 9, 12). It has been proposed that once Bid is cleaved, t"Bid binds Bcl-XL and inhibits its proapoptotic activity.

Along with the data suggesting the importance of interaction with other proteins, there is increasing evidence for the possibility that Bid may directly permeabilize mitochondrial membranes. Recently, the channel-forming activity of Bid in synthetic lipid bilayers has been reported (16). Channel-forming activity has already been observed with other Bcl-2 family proteins (for a review see Ref. 17). Surprisingly, the channels formed by pro- and antiapoptotic proteins do not seem to differ significantly. However, in a striking paper, Basanez et al. (18) have shown that Bax, but not Bcl-XL, decreases the lifetime of planar lipid bilayers. They proposed that this effect was due to a decrease of the linear tension of the bilayers. In mitochondria,
this would lead to the formation of lipidic holes, allowing the release of cytochrome c. Here we describe a similar membrane destabilizing effect specific to t’Bid. We also show that t’Bid activity is inhibited by the N-terminal Bid fragment. We discuss the role of the conserved BH3 domain in Bid activity.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—Oligomeric C-terminal truncated Bax was purified as described earlier (19). Full-length His-tagged mouse Bid, wild type and mutants, were purified as described by Desagher et al. (14). VDAC was a kind gift from Dr K. Zeth. Cytochrome c and gramicidin were from Sigma.

t’Bid (mouse Bid residues 60–195) with a tag of six histidines at the N terminus was expressed in the pET23d vector in Escherichia coli. The protein was recovered in the soluble bacteria fraction and purified by chromatography on nickel-nitrilotriacetic acid-agarose followed by Q-Sepharose. The protein was stored in 25 mM Tris-HCl, 0.2 mM DTT, 30% glycerol, pH 7.5, at −80 °C.

t’Bid (mouse Bid residues 1–59) with a tag of six histidines at the C terminus was expressed in the pET23a vector in E. coli. The protein was recovered in the soluble bacteria fraction and purified by chromatography on nickel-nitrilotriacetic acid-agarose followed by MonoQ and gel filtration on Superdex 200. The protein was over 95% pure and was stored in 25 mM Tris-HCl, 0.2 mM DTT, 30% glycerol, pH 7.5, at −80 °C.

**Cutting Bid with Caspase 8**—200 µl of recombinant wild type or mutated full-length (FL) Bid in 25 mM Tris, 0.2 mM DTT, 30% glycerol, pH 7.5, 25 000 µM DTT, 30% glycerol, pH 7.5, was diluted with 200 µl of cutting buffer (50 mM Hepes, 100 mM NaCl, 10 mM Tris, 1 mM NaN3, pH 7.4) to 6.2 mg/ml. 1 µl of recombinant caspase 8 at 5.6 mg/ml was added, and the sample was incubated at room temperature for 2 h. The cutting efficiency was estimated to be over 95% by SDS-polyacrylamide gel electrophoresis offline at 2 kHz. The membrane potential refers to the potential of the cis side minus the potential of the trans side.

**Liposome Permeabilization Assay**—5,6-Carboxyfluorescein (CF) containing liposomes were prepared as described earlier (21). Briefly, 400 µg of phosphatidylcholine, 400 µg of phosphatidylserine, and 230 µg cholesterol were solubilized in PBS, pH 7.2, containing 20 mM CF and 30 mg/ml OG through incubation at room temperature for 3 h. The liposomes were subsequently isolated by passage over a Sephadex G-25 column (1.5 × 20 cm) and dialyzed in PBS. The liposomes were diluted in PBS to give a suitable fluorescence measurement, then Bid or control proteins were added as indicated in the figures, and the change in fluorescence was recorded over time with excitation at 488 nm and emission at 520 nm.

**Gel Filtration Analysis**—Gel filtrations were performed on a Superdex 200 (10/60) column from Amersham Pharmacia Biotech giving the following elution volumes: ferritin, 440 kDa, 60.7 ml; catalase, 232 kDa, 70.5 ml; aldolase, 158 kDa, 72.0 ml; bovine serum albumin, 67 kDa, 80.9 ml; ovalbumin, 43 kDa, 86.6 ml; chymotrypsigen A, 25 kDa, 95.5 ml; and ribonuclease A, 13 kDa, 97.7 ml. A 500-µl sample was loaded onto the column, and the eluate was monitored at 280 nm. Fractions of 2 ml were collected and analyzed by silver staining using the SilverXpress kit from Novex.

**In Vitro Assay for Cytochrome c Release**—The subcellular fractionation was performed as described earlier (15). Mitochondria (100 µg) were incubated in the presence or absence of various recombinant proteins in 100 µl of MBC buffer (210 mM mannitol, 70 mM sucrose, 10 mM Hepes, 0.5 mM EGTA, 4 mM MgCl2, 5 mM Na2HPO4, 5 mM succinate, 1 mM gramicidin, 10 nM ETG, and 100 nM NaN3). Mitochondria were punctured at 25 mV. The membrane potential was recorded using an Axon 200B patch clamp amplifier and stored on digital audio tape (Biologic DTR 1200 recorder). Records were subsequently filtered at 1 kHz through a 4-pole Bessel filter and digitized offline at 2 kHz. The membrane potential refers to the potential of the cis side minus the potential of the trans side.

**FIG. 1.** t’Bid induces ruptures of planar lipid bilayers without appearance of channels. t’Bid or control proteins were added to the cis side of the bilayer chamber, and conductance changes were recorded. The membrane potential was −25 mV. A, 100 nM t’Bid; B, 100 nM VDAC; C, 1 nM gramicidin. The membrane was broken only after addition of t’Bid. Data on t’Bid are representative of over 30 experiments.
RESULTS

Destabilizing of Planar Lipid Bilayers by Bid—The permeabilizing properties of tBid were investigated using planar lipid bilayers. Addition of recombinant tBid (10–300 nM) to the cis compartment of a bilayer chamber invariably resulted in the rupture of the bilayer within minutes (Fig. 1, curve a). Membrane ruptures occurred at low membrane potentials, from 20 to 80 mV, at which the bilayer was normally stable without addition of tBid. Membrane breaking could only be induced by tBid; the dialysis buffer and FL Bid had no effect. However, Bid gained the ability to break lipid bilayers at nanomolar concentrations after cutting with recombinant caspase 8.

When using the highest concentrations of tBid, we occasionally saw voltage-dependent channel activity, which had all the characteristics of bacterial porins. However, this was also observed after adding FL Bid to the bilayer chamber at similar concentrations and seems to be an inevitable consequence of working with high concentrations of bacteria-expressed proteins. Aside from the occasional porin activity, no other channel activity could be recorded prior to the rupture of the membrane. The bilayer ruptures induced by tBid were not preceded by any discernible increases in conductance, suggesting that they were not simply due to a rapid insertion of a large number of channels. In contrast, addition of channel-forming proteins like VDAC or gramicidin to the bilayer chamber caused gradual increases of membrane conductance that did not lead to the rupture of the bilayer (Fig. 1, curves b and c). Lowering the concentration of tBid to 1–3 nM decreased the frequency of the bilayer breaking events without the appearance of channel activity. At still lower protein concentrations, the bilayers were electrically silent. Varying salt concentration between 50 and 400 mM or doping the bilayer with cholesterol did not induce formation of channels by tBid. In studying Bax channel properties, we observed that the insertion of channels was often followed by rupture of the bilayer, an effect that has been recently analyzed in detail by Basanes et al. (18). We found that preincubation of Bax with azolectin liposomes and fusion of these liposomes to the planar bilayer under asymmetrical conditions, as described by Schlesinger et al. (22), allowed the recording of sustained channel activity at low Bax concentrations without rupture of the bilayer (data not shown). Using this procedure, we were unable to detect tBid channel activity.

To quantify the destabilizing effect of tBid, we compared the lifetime of lipid bilayers in the presence and absence of tBid or control proteins in the bilayer chamber. At low membrane potential, lipid bilayers are very stable in the absence of tBid, and their lifetime could not be meaningfully measured. We therefore used the procedure described by Basanes et al. (18) and worked at a very high membrane potential (250 mV) at which the bilayers ruptured spontaneously within tens of seconds. Under these conditions, addition of 1 nM tBid decreased the lifetime more than 2-fold, and 10 nM tBid decreased the lifetime approximately 30-fold (Fig. 2). The activity of Bid cut with caspase 8 was 10-fold weaker, whereas FL Bid and lipid-interacting proteins (bovine serum albumin, cytochrome c, and mitochondrial channel VDAC)
had no effect on the bilayers’ lifetime at concentrations comparable to tcBid. In general, at 160 or 250 mV membrane potential, tcBid was over 30 times more active in inducing bilayer rupture than FL Bid (Fig. 2 and data not shown). Bax had an activity comparable with that of tcBid.

Another method used to investigate protein-lipid interactions is based on the release of a fluorescent dye from liposomes. Addition of 100 nM tcBid to a solution containing phosphatidylcholine-phosphatidylserine-cholesterol (39%:39%:22%) liposomes filled with CF resulted in a strong release of CF at pH 7.2. Bid cut with caspase 8 was much less efficient in inducing CF release, and FL Bid did not induce any release at all (Fig. 3A). Fig. 3B shows the release of CF from liposomes after 3 min of incubation with tcBid, Bid cut with caspase 8 and FL Bid as a function of protein concentration.

The finding that Bid cut with caspase 8 was much less active than tcBid in permeabilizing planar bilayers and liposomes suggested that tcBid may have an inhibitory function. To test this hypothesis, we produced recombinant tcBid and tested its activity on liposomes. As expected, tcBid did not induce any CF release by itself (data not shown). However, when incubated with tcBid, it inhibited its activity in a concentration-dependent manner (Fig. 3C). The activity of 100 nM tcBid was reduced by half with concentrations of tcBid as low as 50 nM. The effect of tcBid was specific for tcBid, because it did not inhibit the permeabilization of liposomes induced by Bax (data not shown).

We examined whether tcBid is capable of inhibiting tcBid-induced release of cytochrome c from mitochondria. It has been shown earlier that addition of 1 nM tcBid results in a complete release of cytochrome c. Our experiments confirm this result (Fig. 3D, lane 2). Co-addition of 1 nM tcBid markedly decreased tcBid effect (lane 3), whereas 100 nM tcBid suppressed tcBid effect almost completely (lane 5). tcBid alone did not induce any release of cytochrome c at concentrations up to 100 nM (lanes 6 and 7).

OG Dissociates Bid cut with caspase 8 and Increases Its CF-releasing Activity—Another method used to investigate protein-lipid interactions is based on the release of a fluorescent dye from liposomes. Addition of 100 nM tcBid to a solution containing phosphatidylcholine-phosphatidylserine-cholesterol (39%:39%:22%) liposomes filled with CF resulted in a strong release of CF at pH 7.2. Bid cut with caspase 8 was much less efficient in inducing CF release, and FL Bid did not induce any release at all (Fig. 3A). Fig. 3B shows the release of CF from liposomes after 3 min of incubation with tcBid, Bid cut with caspase 8 and FL Bid as a function of protein concentration.

The finding that Bid cut with caspase 8 was much less active than tcBid in permeabilizing planar bilayers and liposomes suggested that tcBid may have an inhibitory function. To test this hypothesis, we produced recombinant tcBid and tested its activity on liposomes. As expected, tcBid did not induce any CF release by itself (data not shown). However, when incubated with tcBid, it inhibited its activity in a concentration-depend-
concentration with the liposomes, we concluded that the OG effect was due to its direct action on cut Bid rather than on the liposomes.

To investigate the effect of OG on Bid quaternary structure, we measured the elution times of Bid from a Superdex 200 column. In the absence of detergent in the migration buffer, FL Bid migrated at a molecular mass of 24 kDa, which corresponds to its calculated monomeric mass (22,844 Da) (data not shown). After cutting with caspase 8, Bid still migrated in one peak at 24 kDa (Fig. 4B). The proportions of C- and N-terminal fragments were identical in all fractions forming the peak (Fig. 4C). This suggests that Bid is not dissociated in solution after cleavage by caspase 8. Moreover, when recombinant t’Bid and t’Bid are preincubated without detergent, they also migrate together on gel filtration, showing their ability to associate (data not shown). However, when the migration buffer contained 2% OG, the C- and N-terminal fragments of Bid dissociated. A second peak can be detected in the elution profile as a shoulder running before the main peak (Fig. 4C). Recombinant t’Bid migrated in the presence of OG at the same elution time as the C-terminal fragment of Bid cut with caspase 8 (data not shown). It should be noted that the elution time of the proteins in OG did not correspond to their calculated molecular mass, probably because of oligomer formation.

Permeabilizing Properties of Bid BH3 Mutants—The BH3 domain is the only fragment of Bid showing sequence similarity to other Bcl-2 family proteins. It has been shown to be important for the proapoptotic activity of Bid as well as for its binding to Bax, Bcl-XL, and Bcl-2 (5). To test whether the BH3 domain is necessary for the permeabilizing activity of Bid, we produced two BH3 mutants cut by caspase 8: cut Bid mIII-2 (93IGDE96 AAAA) and cut Bid mIII-3 (G 94 AAA). Both mutants were less efficient in inducing cytochrome c release from mitochondria than wild type cut Bid. (Fig. 5A). The mIII-2 mutation decreased Bid activity by a factor of 20. These results support the importance of the BH3 domain for Bid proapoptotic activity. Surprisingly, both mutants had an activity similar to the wild type protein in releasing CF from liposomes (Fig. 5B) and in decreasing the lifetime of planar lipid bilayers (Fig. 5C).

DISCUSSION

Intrinsic Activity of t’Bid—In the present study, we describe a membrane destabilizing activity of t’Bid. Addition of nanomolar concentrations (up to 300 nM) of t’Bid to the bilayer
chamber results in the rupture of lipid bilayers without appearance of ionic channels. tBid also induces a vigorous release of CF from liposomes. Interestingly, both events occur at physiological pH, and both are specific to tBid, because FL Bid is neither active in destabilizing planar bilayers nor in permeabilizing liposomes.

Recently, Schendel et al. (16) have described channel-forming activity of tBid in planar lipid bilayers at acidic and neutral pH, with 1.8–3.6 μM (30–60 μg/ml) tBid in the bilayer chamber. The channels were voltage-dependent and displayed multiple conductance levels ranging from 7.4 pS to 100 pS in 150 mM KCl. Channels were also detected with high concentrations of FL Bid but only at acidic pH. In contrast to our results, Schendel et al. (16) did not describe any lytic effect of tBid in planar lipid bilayers. The channel-forming activity was observed only at micromolar concentrations, whereas in our experiments tBid had a strong membrane destabilizing activity already in the low nanomolar range. Whether differences in experimental procedures or in protein preparation account for these differences is unclear.

Inhibitory Effect of tBid—Previous experiments have shown that Bid is activated through caspase 8 (7, 9). tBid has cytochrome c releasing and proapoptotic activities much stronger than those of FL Bid, whereas tBid is inactive. Furthermore, it has been shown that cleavage of Bid by caspase 8 does not result in its immediate dissociation in vitro (11). This suggested that tBid may be an inhibitor of tBid activity. Consistently, coexpression of tBid with tBid has been shown to reduce the proapoptotic activity of tBid in MCF-7 cells (12).

It has been believed that tBid inhibits tBid by masking its BH3 domain, thereby inhibiting its interaction with other Bcl-2 family proteins (10). Here we report that tBid inhibits tBid permeabilizing activity even in the absence of additional proteins. First, Bid cut with caspase 8 is much less active in liposomes and planar bilayers than tBid. Second, addition of tBid potently inhibits the CF releasing activity of tBid in liposomes. Third, gel filtration experiments confirm that the C- and N-terminal fragments of Bid remain associated after cleavage by caspase 8. Incubation of cut Bid with OG both dissociates the C- and N-terminal fragments and increases Bid activity. Importantly, tBid is also able to inhibit tBid-induced release of cytochrome c from mitochondria. We suggest that tBid acts by masking the large hydrophobic domain of tBid and inhibiting its association with mitochondrial lipids.

The dissociation of tBid and tBid is an additional step at which Bid activity can be regulated. Several mechanisms for this regulation are possible: specific protein-protein or protein-lipid interactions may promote the dissociation; tBid may be proteolysed after cleavage; or phosphorylation of Bid may promote or inhibit dissociation. The hypothesis that a specific interaction with lipids may promote the dissociation of cleaved Bid merits special attention. We have shown that cleaved Bid dissociates in the presence of a nonionic detergent, a condition that could mimic the membrane environment. Selective targeting of Bid to mitochondria could be the consequence of a specific ability of mitochondrial lipids to dissociate the two subunits.

Mechanism of Bid Action—What is the physiological relevance of our findings? Is the bilayer destabilizing activity of tBid sufficient, or necessary, for its proapoptotic function? The proapoptotic effect of Bid seems to rely on its ability to induce cytochrome c release from mitochondria into the cytosol. The mechanism of cytochrome c release is the subject of much controversy. It has been proposed to depend upon PTP opening and mitochondrial swelling (23, 24) or channels formed by Bax (20–22). Here we suggest that the membrane destabilizing activity of tBid could reflect its ability to directly permeabilize mitochondrial membranes in vivo.

Recently, Basanes et al. (18) described a destabilizing effect of Bax on planar lipid bilayers. They proposed that Bax decreases the linear tension of the membranes, which promotes the formation of lipidic holes in mitochondria. Cytochrome c and other proteins could then be released through those holes. The activity of tBid described here strongly suggests that it could play a role similar to Bax. It is of interest to note that whereas Bax induces both channel activity and bilayer rupture, in our experiments with Bid, only the destabilizing effect was observed. This effect could therefore reflect an important physiological role of these proteins.

One important question to be addressed is the physiological role of the conserved BH3 domain of Bid. Some mutations in the BH3 domain (mIII-2 and mIII-3) do not alter the membrane destabilizing activity of cleaved Bid in artificial models, but they decrease the cytochrome c releasing activity. The difference may be explained by the impaired binding of the mutants to Bax (or another protein) (7). The activity of Bid in vivo would then rely on two mechanisms: first, a BH3-independent permeabilization of mitochondrial membranes, and second, a BH3-dependent activation of Bax or inhibition of Bcl-XL. Both activities of Bid are regulated by cleavage with caspase 8. We suggest that the dissociation of the inhibitory N-terminal fragment following the cleavage is an additional step necessary for the activation of Bid. The physiological mechanism regulating this dissociation needs further investigation.

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