Acyl Coenzyme A-binding Protein Augments Bid-induced Mitochondrial Damage and Cell Death by Activating μ-Calpain

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Activation of calpain has been shown to occur in some contexts of cell injury and to be essential for loss of cell viability. Part of this may be mediated at the mitochondrial level. It has been demonstrated that calpain activity is necessary for the complete discharge of apoptosis-inducing factor from the mitochondrial intermembrane space and can cause the cleavage of full-length Bid to a more potent truncated form (Polster, B. M., Basanez, G., Etxebarria, A., Hardwick, J. M., and Nicholls, D. G. (2005) J. Biol. Chem. 280, 6447–6454). In this study, we identify acyl-CoA-binding protein (ACBP) as playing a critical role in the activation of calpain upon exposure of mitochondria to both full-length Bid and truncated Bid (t-Bid). Suppression of ACBP levels by small interfering RNA inhibited the t-Bid-induced activation of mitochondrial μ-calpain and release of apoptosis-inducing factor from the mitochondrial intermembrane space and the cleavage of full-length Bid to t-Bid. Moreover, ACBP required the presence of the peripheral benzodiazepine receptor (for which ACBP is a ligand) to be retained at the mitochondria, to activate μ-calpain, and to amplify Bid-induced mitochondrial damage.

Mitochondria are central integrators of the cell death process. The Bcl-2 family proteins have emerged as critical components that regulate mitochondrial alterations elicited when cells undergo apoptosis or necrosis (2, 3). Bid is a pro-apoptotic Bcl-2 homology 3 (BH3)2 domain-only protein, whose activity is actuated by proteolytic cleavage mediated by either caspase-8 or calpains (4–6). Bid is capable of mobilizing proteins sequestered in the mitochondrial intermembrane space and unleashing them into the cytosol, where they provoke the requisite alterations in cellular activity necessary for the consummation of cell death. In particular, Bid is capable of mediating the release of cytochrome c and apoptosis-inducing factor (AIF). Cytochrome c can trigger the caspase cascade, whereas AIF autonomously possesses proteolytic activity and can cause DNA fragmentation by itself (7).

Calpains are calcium-dependent cysteine proteases and have been implicated in performing a role in apoptosis and necrosis (8–11). In particular, calpain can cleave Bid, leading to enhancement of its ability to cause mitochondrial outer membrane disruption (6). Calpain can also cleave AIF, resulting in its full release from the mitochondrial intermembrane space to the cytosol (1). The two most plentiful calpain require micromolar or millimolar concentrations of calcium for maximal activity and are known as μ- and m-calpains, respectively (8). Calpain activity is regulated by calpastatin, which is an inhibitor of calpain activity. In contrast, acyl-CoA-binding protein (ACBP) has been identified as an activator of μ- and m-calpains, lowering their requirement for calcium by 6- and 50-fold, respectively (12). ACBP is found in the cytosol and nucleus and has also been localized to the mitochondrial intermembrane space, where it is released when the integrity of the outer mitochondrial membrane is compromised (13).

In this study, we identify ACBP as capable of augmenting Bid-induced mitochondrial injury by a 2-fold process. First, ACBP is necessary for activation of μ-calpain activity associated with the mitochondrial fraction when mitochondria are treated with truncated Bid (t-Bid) or full-length Bid. In turn, calpain activation by ACBP promotes the release of AIF from the mitochondrial intermembrane space and the cleavage of full-length Bid to its truncated and more potent form. The importance of ACBP in activating calpain and augmenting mitochondrial injury is demonstrated by the ability of ACBP suppression to ameliorate the cell killing brought about by t-Bid expression and treatment with Fas ligand. Notably, the ability of ACBP to promote the activation of μ-calpain requires its localization to the mitochondria after its release from the mitochondrial intermembrane space through interaction with the peripheral benzodiazepine receptor (PBR).

EXPERIMENTAL PROCEDURES

Materials—Recombinant human full-length Bid and t-Bid were purchased from R&D Systems (Minneapolis, MN). Calpeptin was from Calbiochem. Percoll™ was purchased from Amersham Biosciences. Calpain activity in mitochondrial lysates following exposure to various conditions was measured using a calpain activity assay kit from BIOSOURCE (Camarillo, CA). The fluorometric assay is based on the detection of cleavage of the calpain substrate Ac-LLY-7-amido-4-trifluoromethylcoumarin. Ac-LLY-7-amido-4-trifluoromethylcoumarin emits blue light, but upon cleavage of the substrate by calpain, free 7-amido-4-trifluoromethylcoumarin emits a yel-

* This work was supported by National Institute on Alcohol Abuse and Alcoholism Grants SK01 AA000330-05 and IR01 AA012897-05. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: BH3, Bcl-2 homology 3; AIF, apoptosis-inducing factor; ACBP, acyl-CoA-binding protein; t-Bid, truncated Bid; PBR, peripheral benzodiazepine receptor; siRNAs, small interfering RNAs; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N′,N′,N′-tetraacetic acid acetoxyethyl ester.
low-green fluorescence that can be quantified using a fluorescence plate reader.

**Tissue Culture**—HeLa cells (ATCC-CC-1, American Type Culture Collection) were maintained in 25-cm² flasks (Corning Costar Corp., Oneonta, NY) with 5 ml of Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum, all incubated under an atmosphere of 95% air and 5% CO₂. Transfections were performed using Trans-IT transfection reagent (PanVera) according to the manufacturer’s instructions. Transfection efficiencies of 50–60% were routinely obtained as assessed by β-galactosidase staining. For experiments, cells were transfected with 0.5 μg of pcDNA-LacZ in combination with pcDNA-t-Bid. In all cases, the plasmid concentrations were kept to a total of 1.5 μg. The cells staining positive for β-galactosidase were assessed for signs of cell death with propidium iodide.

**Measurements of Viability**—HeLa cells were incubated in 500 μl of complete Dulbecco’s modified Eagle’s medium in 1.88-cm² wells of a 24-well plate at a final cell density of 1.0 × 10⁵ cells/well. Prior to treatment with 100 ng/ml Fas ligand, HeLa cells were washed twice with Ca²⁺/Mg²⁺-free phoshate-buffered saline, after which 500 μl of Dulbecco’s modified Eagle’s medium without serum was added to the wells. Cell viability was determined by the ability of the cells to exclude trypan blue. 10 μl of a 0.5% solution of trypan blue was added to the wells. Both viable and nonviable cells were counted for each data point in a total of eight microscopic fields. Cell viability was also measured using the plasma membrane-impermeable dimeric cyanine dye YOYO-1. YOYO-1 fluoresces brightly only when bound to nucleic acids. Cells are incubated with YOYO-1 before and after the addition of digitonin. Fluorescence before digitonin addition originates from dying cells that have lost plasma membrane integrity and take up YOYO-1. HeLa cells were exposed to various conditions in 24-well plates. YOYO-1 was added to a final concentration of 1 μM. At various time points, the fluorescence of the wells was measured on a BioTek microplate reader at 485 nm excitation and 538 nm emission. At the end of the experiments, digitonin was added to a final concentration of 100 μg/ml, which permeabilizes 100% of the cells as determined by trypan blue dye exclusion. Viability was calculated as $V = 100 - \frac{(F_b - F_o)}{(F_a - F_o)}$, where $V$ is the percentage of viable cells in the well, $F_a$ is the fluorescence after the addition of digitonin, $F_b$ is the fluorescence before digitonin, and $F_o$ is the fluorescence of YOYO-1 in medium alone. The YOYO-1 fluorescence measurement of cell viability gave results similar to those of trypan blue dye exclusion. Please note that both of these assays measure only cell viability and are not intended to differentiate between apoptosis and necrosis.

**RNA Interference**—The Dharmacon SMART selection and SMART pooling technologies were utilized for the RNA interference studies. SMART selection uses an algorithm composed of 33 criteria and parameters that attempt to limit nonfunctional small interfering RNAs (siRNAs). SMART pooling combines four SMART-selected siRNA duplexes in a single pool to increase the probability of reducing mRNA levels by at least 75%. In addition, the siRNA duplexes composing the pool were tested individually for their effect on ACBP expression and phenotype of the cells. siRNA-1, siRNA-2, and siRNA-3 all decreased ACBP expression, with siRNA-3 giving the most consistent results. Therefore, siRNA-3 was used in subsequent experiments to assess the effects of decreasing ACBP levels on the sensitivity of mitochondria to Bid. Similarly, preliminary experiments were carried out on suppression of the PBR by siRNA. The expression of the catalytic 80-kDa subunits of μ- and m-calpains was targeted by specific siRNAs, and suppression of the 28-kDa regulatory subunit, which is common to both isoforms, was also suppressed by a specific siRNA against it. A non-targeting siRNA with no known homology to any gene was utilized as a control. The siRNAs were delivered by a lipid-based method (Gene Therapy Systems, Inc.) at a final siRNA concentration of 100 nm. After formation of the siRNA-liposome complexes, the mixture was added to the HeLa cells in serum-free medium for 4 h. Afterward, the medium was aspirated, and complete medium was added back. Transfection of the HeLa cells with Cy3-labeled siRNA verified that transfection efficiencies of 90–95% were routinely obtained. This high efficiency of transfection was due to the small size of the siRNA oligonucleotides (21-mers).

**Isolation of the Cytosol and Mitochondrially Enriched Fractions**—HeLa cells were plated in 25-cm² flasks and used at a final density of 1.35 × 10⁶ cells/flask. The cells were harvested by trypsinization and centrifuged at 600 × g for 10 min at 4 °C. The cell pellets were washed once with phosphate-buffered saline and then resuspended in 3 volumes of isolation buffer (20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM aprotinin) in 250 mM sucrose. After chilling on ice for 3 min, the cells were disrupted by 40 strokes of a glass homogenizer. The homogenate was centrifuged twice at 1500 × g at 4 °C to remove unbroken cells and nuclei. The mitochondrially enriched fraction (heavy membrane fraction) was then pelleted by centrifugation at 12,000 × g for 30 min. The endoplasmic reticulum contained in the top fluffy layer was removed from the high speed pellet. Mitochondrial integrity was determined by the respiratory control ratio as oxygen consumption in states 3 and 4 of respiration using a Clark-type oxygen electrode with glutamate and malate (Sigma) as respiratory substrates.

**Treatment of the Mitochondrially Enriched Fraction**—The mitochondrially enriched fraction (at a final concentration of 0.5 mg/ml in a volume of 250 μl) was incubated in a KCl-based respiratory buffer (150 mM KCl, 25 mM NaHCO₃, 1 mM MgCl₂, 3 mM KH₂PO₄, and 20 mM HEPES (pH 7.4)). Notably, the incubation buffer was treated with Chelex to remove any endogenous calcium. Following this, calcium was added back to give a final free calcium concentration of 250 nM as measured by Fura2. Glutamate and malate (1 mM) were added as respiratory substrates. Following a 15-min preincubation, the mitochondrially enriched fraction was either left untreated or treated with full-length Bid or t-Bid at a final concentration of 100 or 10 nM, respectively. For the determination of cytochrome c and AIF release, the mitochondrially enriched fraction was first pelleted at 12,000 × g for 30 min at 4 °C. The supernatant was removed and filtered through a 0.2-μm and then a 0.1-μm Ultrafree-MC filter (Millipore Corp.). HeLa cells or mitochon-
dria were pretreated for 30 min with the membrane-permeable calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM) at a concentration of 50 \( \mu \)M. After the 30-min preincubation, the remaining BAPTA-AM was removed by washing the cells or mitochondria and adding fresh medium.

**Western Blot Analysis**—Mitochondrial pellets or supernatants were normalized for protein content (25 \( \mu \)g/lane). Samples were separated on 12% SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Mouse anti-cytochrome c primary monoclonal antibody (clone 7H8.2C12) was from Pharmingen and was used at a dilution of 1:5000. Rabbit anti-AIF primary polyclonal antibody (catalog no. AB16501) was from Chemicon International Inc. (Temecula, CA). Full-length Bid and t-Bid were detected with a rabbit polyclonal antibody (BIOSOURCE) that recognizes both at a dilution of 1:1000. ACBP was detected with a monoclonal antibody (Abcam) at a dilution of 1:2000. A polyclonal antibody to the PBR (Trevigen, Inc.) was used at a dilution of 1:1,000. The 80-kDa subunits of \( \mu \)- and m-calpains were detected with antibodies (ABR-Affinity BioReagents) at a dilution of 1:1000. In each case, the relevant protein was visualized by staining with the appropriate horseradish peroxidase-labeled secondary antibody (1:10,000) and detected by enhanced chemiluminescence.

**Preparation of Recombinant ACBP**—The mammalian expression vector pcDNA3.1 with cDNA encoding ACBP was transfected into Chinese hamster ovary cells. After 48 h, the cells were harvested. Cell lysates were prepared and incubated with a nickel-chelating resin. The bound ACBP was eluted with 100 mM imidazole. The released ACBP was further purified on a Mono Q column, and detergent was removed using Extracti-Gel D detergent-removing gel (Pierce). The resulting eluate was concentrated in a Microcon concentrator with a molecular mass cutoff of 30 kDa.

**RESULTS**

Bid Induces Calpain-dependent Release of AIF from the Mitochondrial Intermembrane Space—Mitochondria were isolated from HeLa cells and incubated with 10 nM t-Bid in respiratory buffer. As shown in Fig. 1A (upper panel), t-Bid induced the release of cytochrome c from the mitochondria over a 20-min time course with a resultant accumulation in the supernatant. Incubation with t-Bid also induced the release of AIF from the mitochondrial intermembrane space (Fig. 1A, lower panel). As has been reported (1), the ability of t-Bid to liberate AIF from the mitochondria was dependent on calpain activity. The addition of the calpain inhibitor calpeptin at 25 \( \mu \)M prevented the release of AIF from the mitochondrial intermembrane space brought about by t-Bid, but had no effect on the release of cytochrome c (Fig. 1A, upper and lower panels, fifth lanes). Bad, a pro-apoptotic protein that binds to Bcl-xL and inhibits its anti-apoptotic activity but does not directly permeabilize the outer mitochondrial membrane, did not bring about the release of either AIF or cytochrome c when incubated with mitochondria.

The calpain activity necessary for the release of AIF was triggered in the mitochondrial fraction when it was exposed to t-Bid. As shown in Fig. 1B, treatment of mitochondria with t-Bid led to a progressive increase in calpain activity over a 20-min time course.
ACBP Is Necessary for the t-Bid-induced Activation of Calpain and Release of AIF from the Mitochondrial Intermembrane Space—HeLa cells were transfected with 100 nM non-targeting siRNA with no homology to any known gene or 100 nM siRNA targeting ACBP. As shown in Fig. 2A, suppression of ACBP levels precluded the t-Bid-induced release of AIF from the mitochondrial intermembrane space without affecting the release of cytochrome c. Notably, transfection with a non-targeting control siRNA did not prevent the t-Bid-induced release of AIF. The inability of t-Bid to release AIF from the mitochondrial intermembrane space when ACBP levels were suppressed was accompanied by inhibition of the activation of calpain induced by t-Bid. As shown in Fig. 2B, t-Bid treatment of mitochondria isolated from HeLa cells transfected with the non-targeting siRNA brought about calpain activation, with the activity reaching a maximum of 2.5-fold above basal levels at 20 min. In contrast, the t-Bid-induced activation of calpain was severely inhibited in mitochondria isolated from HeLa cells transfected with the siRNA targeting ACBP, with little increase in activity at 20 min post-treatment (Fig. 2B).

ACBP is found in the cytosol, but has also been localized to the mitochondrial intermembrane space, from where it is released when the integrity of the outer mitochondrial membrane is compromised (13). As shown in Fig. 2C (upper panel), exposure of mitochondria isolated from HeLa cells transfected with the non-targeting siRNA to t-Bid resulted in the release of ACBP from the mitochondrial fraction to the supernatant, which occurred over a time course identical to that of cytochrome c and AIF release. In contrast, mitochondria isolated from HeLa cells transfected with the siRNA targeting ACBP, with little to no effect on AIF or μ-calpain expression.

FIGURE 2. Suppression of ACBP prevents t-Bid-induced activation of calpain and release of AIF from the mitochondrial intermembrane space. In A, HeLa cells were transfected with the siRNA targeting ACBP. The cells were then cultured for 48 h and harvested, and mitochondria were isolated and suspended in mitochondrial respiratory buffer at a final concentration of 0.5 mg/ml. t-Bid was added at a concentration of 10 nM. Aliquots were removed at the time points indicated; the mitochondria were spun down; and the release of cytochrome c (Cyt. C) and AIF into the incubation medium was assessed. In B–D, HeLa cells were transfected with the siRNA targeting ACBP or the non-targeting control siRNA. After 48 h, the cells were harvested, and the mitochondria were isolated, suspended in respiratory buffer, and treated with 10 nM t-Bid. At the time points indicated, aliquots were removed. The mitochondria were pelleted, and the mitochondrial lysates and supernatant were assessed for levels of calpain activity and ACBP and the expression of ACBP and the 80-kDa catalytic subunit of μ-calpain (μ-Calpain). The results presented are typical and are the means of three independent experiments. RFU, relative fluorescence units.
ACBP Augments Bid-induced Mitochondrial Injury

If the release of ACBP sequestered in the mitochondrial intermembrane space is responsible for activating calpain, then the addition of exogenous ACBP to the mitochondrial fraction isolated from cells in which ACBP expression is suppressed should restore the ability of t-Bid to release AIF from the mitochondrial intermembrane space and to activate calpain. Therefore, mitochondria isolated from HeLa cells in which ACBP levels were suppressed were incubated with t-Bid in the presence or absence of increasing concentrations of exogenously added recombinant ACBP. As shown in Fig. 3A (first lane), incubation of mitochondria isolated from HeLa cells transfected with the siRNA targeting ACBP was refractory to a t-Bid (10 nM)-induced release of AIF from the mitochondrial intermembrane space even after 20 min of exposure. Recombinant ACBP at increasing concentrations was then added in combination with t-Bid. As demonstrated in Fig. 3A (second through fifth lanes), the addition of recombinant ACBP restored the ability of t-Bid to release AIF from the mitochondrial intermembrane space in a concentration-dependent manner, with 100 nM ACBP in combination with 10 nM t-Bid causing the release of most of the AIF from the mitochondria. The restoration of AIF release from the mitochondrial intermembrane space by the addition of exogenous ACBP was accompanied by the re-establishment of calpain activation by t-Bid. As demonstrated in Fig. 3B, 100 nM ACBP fully restored the ability of t-Bid to activate calpain in mitochondria isolated from HeLa cells when ACBP levels were suppressed. As shown in Fig. 1, mitochondria in which ACBP levels were suppressed were resistant to the t-Bid-induced activation of calpain. However, when the mitochondria were exposed to t-Bid in the presence of exogenous ACBP, the calpain activity reached 2.4-fold above the basal level during a 20-min time course, an increase comparable with that induced by t-Bid in control mitochondria.

Full-length Bid is capable of provoking mitochondrial damage, albeit not as potently as t-Bid (27). Like caspase-8, calpain is able to cleave Bid to a more active form. Therefore, we wanted to determine whether ACBP and the resultant activation of calpain could cause Bid to be cleaved to the more potent truncated form. As shown in Fig. 3C (first lane), incubation of full-length Bid (100 nM) with mitochondria isolated from non-targeting siRNA-transfected cells resulted in the appearance of t-Bid after 20 min. In contrast, there was no t-Bid formed when full-length Bid was incubated with mitochondria isolated from HeLa cells in which ACBP levels were suppressed (Fig. 3C, second lane). However, as with AIF release and calpain activation, the addition of exogenous ACBP (100 nM) in combination with full-length Bid restored the appearance of t-Bid upon incubation with mitochondria in which ACBP levels were suppressed (Fig. 3C, third lane). Notably, the formation of t-Bid in the presence of ACBP was prevented by the calpain inhibitor calpeptin (Fig. 3C, fourth lane).

The PBR Is Required for the t-Bid-induced Activation of Calpain in the Presence of ACBP—ACBP is a ligand of the PBR, which is localized to the outer mitochondrial membrane (14). As shown in Fig. 4A, transfection of HeLa cells with the siRNA targeting the PBR resulted in suppression of PBR levels in isolated mitochondria without a concomitant reduction of ACBP expression. Notably, the siRNA targeting ACBP had no effect on the expression of the PBR. Similarly, the siRNAs against ACBP and the PBR had no influence on the expression of μ-calpain or AIF; and conversely, the siRNA targeting the 80-kDa subunit of μ-calpain did not affect the expression of the PBR, ACBP, or AIF.

As shown in Fig. 4B (upper panel, first lane), suppression of the PBR resulted in inhibition of the ability of t-Bid to induce the release of AIF from the mitochondrial intermembrane space, a result identical to that seen in mitochondria in which ACBP levels were suppressed (Fig. 4B, lower panel, first lane).
The inability of exogenous ACBP to restore the sensitivity of mitochondria not expressing the PBR to the t-Bid-induced release of AIF and activation of calpain implies that the PBR may be necessary to localize ACBP released from the mitochondrial intermembrane space to the mitochondrial outer membrane. In Fig. 5A (first and second panels), mitochondria isolated from HeLa cells transfected with the non-targeting siRNA were treated with t-Bid and again displayed a release of ACBP into the supernatant over a 20-min time course. Notably, despite the accumulation of ACBP in the supernatant, the mitochondrial fraction was found to retain a significant proportion of ACBP (Fig. 5A, first panel). However, as shown in Fig. 5A (third and fourth panels), in mitochondria in which PBR expression was inhibited, there was a greater accumulation of ACBP in the supernatant, and no ACBP remained associated with the mitochondria following treatment with t-Bid. Such results imply that ACBP released from the intermembrane space by t-Bid requires the PBR, an outer mitochondrial membrane protein, to remain associated with the mitochondria and cause the activation of calpain and the release of AIF.

**FIGURE 4.** PBR expression is required for the activation of calpain and the release of AIF from the mitochondrial intermembrane space. HeLa cells were transfected with the siRNA targeting ACBP, the PBR, or the 80-kDa catalytic subunit of μ-calpain or with the non-targeting control siRNA. After 48 h of incubation, the cells were harvested, and the mitochondria were isolated. In A, the mitochondrial lysates were assessed for ACBP, PBR, AIF, and μ-calpain (μ-Calpain) expression by Western blotting. In B, the mitochondria from HeLa cells in which PBR or ACBP levels were suppressed were suspended in respiratory buffer and exposed to 10 nM t-Bid, 100 nM ACBP, or both. The mitochondria were pelleted, and the supernatant was assessed for the release of AIF. In C, calpain activity was measured fluorometrically in the mitochondrial lysates as described under “Experimental Procedures.” The results presented are typical and are the means of three independent experiments. RFU, relative fluorescence units.

However, unlike the case with ACBP, mitochondria in which PBR levels were suppressed remained refractory to the t-Bid-induced release of AIF, even in the presence of exogenous ACBP (Fig. 4B, compare upper and lower panels, third lanes). Moreover, the inability of t-Bid to release AIF from mitochondria not expressing the PBR was accompanied by inhibition of the t-Bid activation of calpain. As shown in Fig. 4C, unlike the case when ACBP levels were suppressed (Fig. 3B), the addition of exogenous ACBP did not restore the ability of t-Bid to activate calpain in mitochondria in which PBR expression was prevented.

The inability of exogenous ACBP to restore the sensitivity of mitochondria not expressing the PBR to the t-Bid-induced release of AIF and activation of calpain implies that the PBR may be necessary to localize ACBP released from the mitochondrial intermembrane space to the mitochondrial outer membrane. In Fig. 5A (first and second panels), mitochondria isolated from HeLa cells transfected with the non-targeting siRNA were treated with t-Bid and again displayed a release of ACBP into the supernatant over a 20-min time course. Notably, despite the accumulation of ACBP in the supernatant, the mitochondrial fraction was found to retain a significant proportion of ACBP (Fig. 5A, first panel). However, as shown in Fig. 5A (third and fourth panels), in mitochondria in which PBR expression was inhibited, there was a greater accumulation of ACBP in the supernatant, and no ACBP remained associated with the mitochondria following treatment with t-Bid. Such results imply that ACBP released from the intermembrane space by t-Bid requires the PBR, an outer mitochondrial membrane protein, to remain associated with the mitochondria and cause the activation of calpain and the release of AIF.

**μ-Calpain Activity Associated with the Mitochondria Is Required for Release of AIF Induced by t-Bid and Promotes Cell Death—**As shown in Fig. 5B (upper and lower panels, first lanes), suppressing the levels of the 80-kDa catalytic subunit of μ-calpain by siRNA inhibited the t-Bid-induced release of AIF from the mitochondria, but did not prevent cytochrome c release. In contrast, suppression of the 80-kDa catalytic subunit of m-calpain did not prevent induction of AIF release triggered by t-Bid (Fig. 5B, upper panel, fourth lane). Calpains require calcium for activity. To test the calcium dependence of μ-calpain associated with the mitochondria, BAPTA-AM, a membrane-permeable calcium chelator, was incubated with the mitochondria prior to t-Bid addition. As shown in Fig. 5B (upper and lower panels, second lanes), preincubation of the mitochondria for 30 min with BAPTA-AM prevented the t-Bid-induced release of AIF from the mitochondria, yet exerted no influence on cytochrome c release.
FIGURE 5. PBR expression is required to retain ACBP at the mitochondria, and \( \mu \)-calpain (but not m-calpain) is localized to the mitochondria. In A, the mitochondria were isolated from HeLa cells transfected with the non-targeting siRNA or the siRNA targeting the PBR. The mitochondria were then suspended in respiratory buffer and exposed to 10 nm t-Bid. At the times indicated, aliquots were removed, and the mitochondria were pelleted. The mitochondrial lysate (Mito.) and supernatant (Super.) were assessed for ACBP levels by Western blotting. In B, HeLa cells were transfected with the siRNA targeting the 80-kDa catalytic subunit of \( \mu \)-calpain (u-Calpain) or m-calpain or the non-targeting control siRNA. After 48 h of incubation, the cells were harvested, and the mitochondria were isolated. Alternatively, the mitochondria were preincubated for 30 min with 50 \( \mu \)M BAPTA-AM and then washed. In both instances, the mitochondria were suspended in respiratory buffer and treated with 10 nm t-Bid. The supernatant was removed and filtered through a 0.2-\( \mu \)m and then a 0.1-\( \mu \)m Ultrafree-MC filter. AIF and cytochrome c (Cyt. C) released into the supernatant were assessed by Western blotting. In C, HeLa cells were transfected with the siRNA targeting the 80-kDa catalytic subunit of \( \mu \)- or m-calpain or the 28-kDa regulatory subunit common to both isoforms. Following 48 h of incubation, the cells were harvested, and whole cell lysates were prepared, or alternatively, mitochondria were isolated. The levels of \( \mu \)- and m-calpains were determined by Western blotting using antibodies specific for the 80-kDa catalytic subunits.
Fig. 5C (second and fourth panels, second lanes) demonstrates that the siRNA targeting the 80-kDa catalytic subunit of \( \mu \)-calpain suppressed the expression of \( \mu \)-calpain in whole cell lysates and isolated mitochondria. In contrast, the siRNA targeting the 80-kDa catalytic subunit of m-calpain caused a decrease in the expression of m-calpain associated with the whole cell lysate, but did not affect the expression of \( \mu \)-calpain in whole cell lysates and mitochondria (Fig. 5C, first, second, and fourth panels, first lanes). In contrast, the siRNA targeting the 28-kDa regulatory subunit common to both \( \mu \)- and m-calpains caused a decrease in the levels of the 80-kDa catalytic subunits of \( \mu \)- and m-calpains. Such an observation is consistent with the common 28-kDa regulatory subunit being required for stabilization and inhibiting the degradation of the 80-kDa catalytic subunits (15, 16). Notably, \( \mu \)-calpain was found consistently in the mitochondria, with only low levels of m-calpain detected (Fig. 5C, third and fourth panels). Similarly, as shown in Fig. 6A, suppression of the 80-kDa catalytic subunit of \( \mu \)-calpain prevented t-Bid from stimulating mitochondrial calpain, whereas the siRNA targeting m-calpain had no effect on t-Bid-induced mitochondrial calpain activation.

Inhibition of the t-Bid-induced activation of calpain and release of AIF from the mitochondrial intermembrane space when ACBP or PBR levels were suppressed was attended by amelioration of the cell killing induced by t-Bid expression or exposure to Fas ligand in intact cells. HeLa cells were first transfected with the siRNA targeting ACBP or the PBR or with the non-targeting control siRNA. After 48 h, the cells were cotransfected with an expression construct for t-Bid together with one for \( \beta \)-galactosidase, which was utilized as a marker for transfection. The number of cells staining positive for \( \beta \)-galactosidase was then evaluated for signs of viability at the times indicated. As shown in Fig. 6B (left panel), the expression of t-Bid brought about a loss of viability in 75% of the non-targeting control siRNA-transfected HeLa cells 24 h following transfection with the t-Bid expression vector. Conversely, cells in which the ACBP and PBR levels were suppressed by the siRNAs were refractory to t-Bid-induced cell killing, with the siRNAs against ACBP and the PBR reducing the loss of cell viability at 24 h after t-Bid transfection to 35 and 20%, respectively. Fas ligand promoted the generation of t-Bid upon engagement with the Fas receptor. As shown in Fig. 6B (right panel), suppression of ACBP or the PBR inhibited the cell killing induced by treatment with 100 ng/ml Fas ligand, with only 30 and 12% of the cells losing viability at 24 h post-exposure, respectively, compared with an 80% loss in the non-targeting control siRNA-transfected cells. Notably, suppression of \( \mu \)-calpain also decelerated the cell killing brought about by t-Bid or Fas, as did chelation of calcium by pretreatment of cells with BAPTA-AM.

**DISCUSSION**

The results presented here demonstrate that ACBP accentuates the ability of Bid to activate calpain, thus facilitating the release of AIF from the mitochondrial intermembrane space. Moreover, suppression of ACBP levels results in amelioration of the cell killing induced by t-Bid overexpression or Fas ligand treatment. The PBR (for which ACBP is a ligand) is necessary for ACBP to exert a stimulatory effect on the Bid-induced activation of calpain and release of AIF from the mitochondrial intermembrane space, possibly by localizing ACBP to the outer mitochondrial membrane.

Bid is a pro-apoptotic BH3 domain-only protein. The mechanisms by which Bid is thought to alter mitochondrial function and structure are quite diverse. Of particular importance is the interaction of BH3 domain-only proteins such as Bid with the multidomain pro-apoptotic proteins Bak and Bax; Bid initializes the oligomerization of Bak and/or Bax to facilitate permeabilization of the outer mitochondrial membrane and release of intermembrane space proteins (17–24). Alternatively, evidence also indicates that the interaction of Bid with mitochondrial calcium by pretreatment of cells with BAPTA-AM.

**FIGURE 6. Chelation of mitochondrial calcium prevents t-Bid-induced calpain activation, and suppression of ACBP or the PBR prevents the cell killing induced by t-Bid overexpression and treatment with Fas ligand.** In A, HeLa cells were transfected with the siRNA targeting the 80-kDa catalytic subunit of \( \mu \)- or m-calpain or the non-targeting control siRNA. Alternatively, the mitochondria were preincubated for 30 min with BAPTA-AM, washed, and resuspended in respiratory buffer. Following 48 h in the case of siRNA transfection, the cells were harvested, and the mitochondria were isolated and suspended in respiratory buffer. In both instances, the mitochondria were treated with 10 nM t-Bid. At the time points (minutes) indicated, aliquots were removed. The mitochondria were pelleted, and the mitochondrial lysates were assessed for calpain activity levels. In B (left (L) panel), HeLa cells were transfected with the siRNA targeting ACBP, the PBR, or the 80-kDa catalytic subunit of \( \mu \)-calpain or with the non-targeting control siRNA or pre-treated with BAPTA-AM for 30 min. After 48 h of siRNA transfection or 30 min after BAPTA-AM addition, the cells were cotransfected with pcDNA-t-Bid and \( \beta \)-galactosidase. At the time points indicated, the cells were stained for \( \beta \)-galactosidase activity and assessed for cell viability with propidium iodide. In B (right (R) panel), HeLa cells were transfected with the siRNA targeting ACBP, the PBR, or the 80-kDa catalytic subunit of \( \mu \)-calpain or with the non-targeting control siRNA or pretreated with BAPTA-AM for 30 min. After 48 h of siRNA transfection or 30 min after BAPTA-AM addition, the cells were treated with 100 ng/ml Fas ligand. Cell viability was assessed as described under “Experimental Procedures.” RFU, relative fluorescence units.
ACBP Augments Bid-induced Mitochondrial Injury

lipids such as cardiolipin promotes destabilization of the lipid bilayer of the outer mitochondrial membrane, thus resulting in increased permeability (18, 25, 26).

The activity of Bid is post-translationally controlled by myristoylation, phosphorylation, and proteolytic cleavage (5). Full-length Bid has been demonstrated to possess the ability to increase mitochondrial membrane permeability and to induce apoptosis; however, proteolytic cleavage results in the production of t-Bid, which is much more potent in bringing about mitochondrial injury (1, 27). Bid can be cleaved by the action of caspase-3, caspase-8, cathepsins, granzyme B, and calpains. Bid is susceptible to cleavage by μ- or m-calpain between Gly70 and Arg71 in the loop region of Bid (6). The cleavage of Bid by calpains has been demonstrated to be important in the development of apoptosis induced by ischemia/reperfusion injury, ionomycin, and cisplatin. In addition to Bid, calpains can also cleave Bax to a more potent form, thus demonstrating the disruptive potential of calpains (28).

The calpains are heterodimers composed of a distinct 80-kDa catalytic subunit and a common 28-kDa regulatory subunit (8). In unstimulated cells, μ- and m-calpains are widely distributed in the cytoplasm, and in platelets, they translocate to the plasma membrane upon stimulation. The calpains have also been localized to subcellular organelles such as the endoplasmic reticulum, Golgi apparatus, and mitochondria. In particular, μ-calpain has been localized to the mitochondria (29).

Similarly, we found the 80-kDa catalytic subunit of μ-calpain predominately in the mitochondria, with little m-calpain present.

The regulation of calpain activity has been the subject of intensive investigation. Calpastatin is a stoichiometric inhibitor of both μ- and m-calpains, with one molecule of calpastatin inhibiting four molecules of calpain (10). Paradoxically, the concentration of calcium required for activation of either μ- or m-calpain in vitro is greater than the 50–300 nM basal Ca2+ concentrations measured in living cells. In this respect, activators of calpain activity have also been identified. ACBP has been shown to decrease the Ca2+ requirement for μ- and m-calpain activation by 6- and 50-fold, respectively (12). However, the ability of ACBP to actually modulate calpain activity in a physiological or pathophysiological setting has not been demonstrated to date.

ACBP (also known as the diazepam-binding inhibitor) is a 10-kDa protein that binds medium- and long-chain acyl-CoA esters with high affinity (30, 31). Interestingly, the binding of acyl-CoA to ACBP is not necessary for it to stimulate calpain activity. A pool of ACBP localized to the mitochondrial intermembrane space is released upon permeabilization of the outer mitochondrial membrane (13). Indeed, Fig. 2C shows that exposure of mitochondria to t-Bid caused the release of ACBP from isolated mitochondria into the supernatant. The liberation of ACBP was accompanied by the activation of calpain and the release of AIF from the mitochondrial intermembrane space. AIF has been shown to be a substrate of calpain and to require its activity for full discharge from the mitochondria (1).

Suppression of ACBP levels through the use of siRNA resulted in inhibition of both the t-Bid-induced mitochondrial activation of calpain and release of AIF from the mitochondria. In addition to its widely known role as a fatty acyl-CoA-binding protein, ACBP has been found to interact with proteins at diverse subcellular locations, where it alters their function and activity. ACBP can be found in association with hepatocyte nuclear factor-4α in the nucleus, where it enhances hepatocyte nuclear factor-4α-mediated transactivation (32). In addition, ACBP in complex with fatty acyl-CoA can activate the Ca2+ release channel of skeletal muscle sarcoplasmic reticulum, which is controlled by the ryanodine receptor (33). ACBP is also an endogenous ligand of the PBR (14, 34).

The PBR is an outer mitochondrial membrane protein expressed in a wide range of tissues such as the liver (especially those involved in steroid biosynthesis) and in transformed cells. The PBR mediates the transportation of cholesterol and porphyrin into the mitochondria. In this study, we have shown that exposure of mitochondria to t-Bid induced the accumulation of ACBP in the supernatant. However, following treatment with t-Bid, a substantial portion of ACBP remained associated with the mitochondrial fraction. Notably, this was abolished when PBR expression was inhibited. Such a result suggests that the PBR is necessary to retain ACBP at the mitochondria, thus positioning ACBP at a site conducive for calpain activation. Indeed, it was demonstrated that calpeptin completely inhibits AIF release upon induction of permeability transition (1).

As shown under “Results,” suppression of ACBP inhibited but did not fully prevent cell death brought about by overexpression of t-Bid and treatment with Fas ligand. In addition, t-Bid was still able to release cytochrome c from the mitochondrial intermembrane space in the absence of ACBP expression. Such results suggest that ACBP may serve to augment and amplify the mitochondrial damage induced by Bid through provoking calpain activation and release of AIF from the mitochondrial intermembrane space. Additionally, in some instances, full-length Bid has been implicated as a initial trigger for cell killing (18, 27). Under such circumstances, the activation of calpain by ACBP at the mitochondria may serve to intensify the mitochondrial damage brought about by Bid through cleaving it. As shown in Fig. 3C, full-length Bid was cleaved to t-Bid when added to isolated mitochondria. Moreover, this was prevented by the abolition of ACBP expression. Thus, a positive amplification loop can be set up wherein full-length Bid can release limited amounts of intermembrane space proteins such as cytochrome c and ACBP. In turn, ACBP localized to the mitochondrial membrane through binding with the PBR can bring about calpain activation with the resultant cleavage of Bid to a more potent form (Fig. 7).

A number of questions remain concerning the regulation of calpain activation by ACBP and the role played by the PBR. For instance, it is unknown if cytosolic ACBP has a role in controlling calpain activity or if there is something unique about ACBP located in the intermembrane space that enables it to be retained at the mitochondrial outer membrane through an interaction with the PBR. Also whether the activation of calpain by ACBP in an intact cell is confined to a zone in proximity to the mitochondria or is a more generalized and dispersed phenomenon is not known. Indeed, it may be that initial calpain activation promoted by ACBP at the mitochondria can lead to a more widespread wave-like activation of cellular calpains. As
shown above, the calcium chelator BAPTA-AM prevented t-Bid-induced mitochondrial calpain activation and AIF release without affecting cytochrome c discharge. The Ca\(^{2+}\) concentration was 250 nM in the incubation medium, within the range of basal intracellular concentrations. Even though ACBP has been shown to lower the Ca\(^{2+}\) concentration required for \(\mu\)- and m-calpain activation to 1.8 and 6 \(\mu\)M, respectively, this is still higher than what was required in the incubation medium. It is likely that calcium sequestered by mitochondria may contribute to the activation of \(\mu\)-calpain as evidenced by the ability of pretreatment with BAPTA-AM to prevent the t-Bid-induced stimulation of mitochondrial \(\mu\)-calpain and release of AIF. Whatever the mechanism, the results reported indicate that ACBP is necessary for calpain activation and the resulting release of AIF from the mitochondrial intermembrane space. Moreover, an interaction between ACBP and the PBR is necessary to retain ACBP at the mitochondria for calpain activation.

In summary, as outlined in Fig. 7, ACBP can help provoke the activation of \(\mu\)-calpain at the mitochondria through interaction with the PBR. In turn, \(\mu\)-calpain can release AIF from the mitochondrial intermembrane space and cleave full-length Bid, thus helping to amplify mitochondrial injury and ultimately leading to a loss of cell viability.

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