TRAIL Receptor and CD95 Signal to Mitochondria via FADD, Caspase-8/10, Bid, and Bax but Differentially Regulate Events Downstream from Truncated Bid*

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The death receptor ligand TRAIL arouses much interest for clinical application. We found that TRAIL receptor could induce cytochrome c (Cyt c) release from mitochondria in cells that failed to respond to CD95. Therefore, we examined whether these two closely related death receptors use different intermediates to convey the apoptotic signal to mitochondria. Dominant negative FADD, FLIP, or a Bid mutant lacking cleavage sites for caspase-8/10 completely inhibited Cyt c release in response to either receptor. Depletion of Bid from TRAIL- or CD95-activated cytosols blocked their capacity to mediate Cyt c release from mitochondria in vitro, whereas Bax depletion reduced it. We conclude that FADD, caspase-8/10, and caspase-cleaved Bid are required for TRAIL receptor and CD95 signaling to mitochondria, whereas Bax is a common accessory. In vitro, caspase-8 treatment of cytosol from CD95-resistant cells permitted generation of truncated Bid and its association with mitochondria. However, this cytosol impaired the ability of truncated Bid to liberate Cyt c from exogenous mitochondria. We conclude that the TRAIL receptor can bypass or neutralize the activity of cytosolic factor that blocks truncated Bid function. This may benefit the capacity of TRAIL to break apoptosis resistance in tumor cells.

Tumor necrosis factor (TNF) receptor family members share homology in the extracellular domain, which interacts with homotrimeric, TNF-related ligands (1). Within this family, the death receptors (DR) share the capacity to induce apoptosis, which impinges on a cytoplasmic death domain (2). Presently, six human DR are known, including TNF receptor-1, CD95 (APO-1/Fas), and two receptors for TNF-related apoptosis-inducing ligand, also known as TRAIL (3). The mouse genome encodes only one TRAIL receptor, suggesting closely related functions for the two human receptors (4). All DR ligands, with the exception of TNFα, are transmembrane ligand forms. Various soluble recombinant ligand forms retain biological activity and have been explored for clinical use. Toxicity of TNFα and CD95 ligand (CD95L) precludes their use for systemic therapy. TRAIL, however, is very promising. In vitro, it killed the majority of malignantly transformed cell lines tested but not normal cells. Both in mouse and monkey preclinical models, TRAIL was not toxic to normal tissues (5, 6).

DR are interesting candidates for anti-cancer therapy, because they can bypass certain forms of apoptosis resistance that frequently occur in tumor cells. DR-induced apoptosis is not affected by loss of functional alleles of the p53 transcription factor, which is the most common mutation in human cancer (7). Also, in a number of tumor cell lines, CD95 and TRAIL receptors could bypass the inhibitory action of Bel-2 proto-oncogene products (8–10). Moreover, DR are promising candidates for combination therapy with radiation or chemotherapeutic drugs, in the case of tumors with wild-type p53; TRAIL receptor-2, also called DR5, is a bona fide p53 target gene (11, 12). Accordingly, TRAIL proved synergistic with radiation and chemotherapeutic drugs in cell death induction (13, 14).

The majority of apoptotic stimuli act via the intrinsic mitochondrial pathway. In this pathway, proapoptotic Bel-2 family members induce permeability of the mitochondrial outer membrane, permitting release of cytochrome c (Cyt c). Binding of cytosolic Cyt c and dATP to the scaffold protein Apaf-1 allows recruitment and activation of the inducer caspase-9, followed by effector caspase activation and execution of the apoptotic program. In addition, release of Smac/Diablo alleviates blockade of effector caspases by the inhibitor of apoptosis proteins (15). Inhibitory Bel-2 family members prevent mitochondrial permeability (15, 16) and consistently inhibit apoptosis induced by DNA damaging (anti-cancer) regimens (17). DR also signal to mitochondria and induce Cyt c release. However, in various cell types, DR can bypass a blockade at the mitochondria imposed by inhibitory Bel-2 family members. This is explained by their capacity to recruit and activate caspase-8 or -10, which can directly cleave effector caspases (8).

The CD95 signaling pathway has been explored by biochemical and genetic approaches. Upon ligand binding, CD95 recruits the FADD adaptor through homotypic death domain interactions. FADD in turn recruits caspase-8 through homotypic death effector domain interactions. Within the death-inducing signaling complex (DISC) thus formed at the receptor tail, caspase-8 zymogens are activated by proteolytic self-processing and released into the cytosol, where they can activate effector caspases (18). The second death effector domain-containing caspase, caspase-10, has also been demonstrated in the endogenous CD95 DISC (19). Recently, FADD, caspase-8, and caspase-10 were all demonstrated in native TRAIL receptor complexes (19–22). That FADD is required for apoptosis induction by both TRAIL receptors followed from the use of FADD-deficient cells (23). Involvement of caspase-8 or -10 was first

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§ The abbreviations used are: TNF, tumor necrosis factor; Cyt c, cytochrome c; DISC, death-inducing signaling complex; DR, death receptor; mAb, monochonal antibody; FLIP, FLICE-inhibitory protein; cFLIP, cellular FLIP; tBid, truncated Bid; dnFADD, dominant negative FADD; PIPES, 1,4-piperazinediethanesulfonic acid; MIB, mitochondrial incubation buffer.
suggested by the finding that TRAIL receptor-induced apoptosis is blocked by FLICE-inhibitory proteins (FLIPs), which have a death effector domain and compete with caspase-8/10 for FADD binding (24). Moreover, in caspase-8-deficient cells, either caspase-8 or -10 could restore TRAIL receptor and CD95 signaling (19). These data indicate that both CD95 and TRAIL receptors induce apoptosis via FADD and either caspase-8 or -10.

Caspase-8 cleaves and activates effector caspses but also processes the BH3 domain-only Bcl-2 family member Bid to generate a proapoptotic carboxy-terminal fragment termed truncated Bid (tBid) (25, 26). tBid translocates to mitochondria, inserts in the outer membrane, and, in cooperation with Bax or Bak, brings about Cyt c release, by a yet incompletely resolved mechanism (27, 28). It has been established that CD95 conveys the apoptotic signal to mitochondria via Bid (26). TRAIL receptors also signal to mitochondria, as evidenced by Cyt c release and mitochondrial depolarization (9, 29, 30). However, the exact sequence of events in the TRAIL receptor pathway has not been established. Bid processing has been observed, but it is not clear whether it is instrumental in TRAIL-induced Cyt c release (30–32).

TRAIL receptor signaling pathways are far from elucidated. TRAIL receptors have been reported to recruit the TRADD adaptor, either downstream or independent of FADD, as well as the RIP kinase (33, 34). Both TRAIL receptors activate NF-kB (33, 34) and c-Jun kinase. According to certain authors, this proceeds through two distinct cascades emerging from the TRAF2 adaptor protein (35), whereas others link RIP to the TRAIL receptor (36). DAP3, a GTP-binding adaptor protein, associates with the TRAIL receptor death domains in the yeast two-hybrid system. DAP3 was suggested to link FADD to the receptor tails (37). However, all of these findings are based on use of transient overexpression systems and may not reflect the endogenous situation. For instance, endogenous TRAIL receptor complexes in BJAB cells did not contain TRADD or RIP, in contrast to TNF receptor-1 complexes (21).

Our studies were prompted by the observation that variant Jurkat T cells, which failed to display Cyt c release in response to CD95 stimulation, did so in response to TRAIL. We delineate here the pathway employed by TRAIL receptor-2 to convey the death signal to mitochondria. The pathway requires FADD, NF-kB, and caspase-cleaved Bid, whereas Bax facilitates the process. We find that in this respect, the TRAIL receptor is indiscernible from CD95 in its mechanism of action. However, TRAIL receptor signaling to the mitochondria is not affected by a cytosolic factor, which impedes the CD95 pathway downstream from tBid.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Mouse anti-human CD95 monoclonal antibody (mAb) 7C11 was obtained from Immunotech (Marseille, France), and soluble human recombinant TRAIL and enhancer were from Alexis (Laufelfingen, Switzerland). Recombinant human caspase-8 and anti-Cyt c mAb 7H8.2C12 were obtained from PharMingen. Anti-actin mAb C4 was from DAKO A/S (Glostrup, Denmark). Protein A- and G-Sepharose conjugated rabbit anti-mouse Ig and swine anti-rabbit Ig were obtained and anti-Bax mAb B8429 was from Sigma. Horseradish peroxidase-conjugated rabbit anti-mouse Ig and swine anti-rabbit Ig were obtained from DAKO A/S (Glostrup, Denmark). Protein A- and G-Sepharose beads and the ECL kit for standard immunoblotting procedures were purchased from Amersham Biosciences. SuperSignal West Dura extended duration substrate for chemiluminescence was from Pierce.

**Cells and Stimulation**—The J16 clone was derived from the human T-acute lymphoblastic leukemia cell line Jurkat by limiting dilution and selected for CD95 sensitivity (39, 40). CD95-resistant JA variant clones were derived by limiting dilution from the parental Jurkat line after 5 weeks of selection for cells resistant to anti-CD95 mAb 7C11. Cells were cultured in Iscove’s modified Dulbecco’s medium, supplemented with 8% FCS, 2 mM glutamine, and antibiotics. Before stimulation, cells were transferred to serum-free Yssel medium (41) and seeded at 1 × 10^6/ml, 200 μl/well, in round-bottom 96-well plates for apoptosis assays and at 5–10 × 10^6/ml in 24-well culture plates for Cyt c release assays. Cells were stimulated with medium, anti-CD95 mAb, recombinant TRAIL plus a 10-fold excess of enhancer, or coated anti-CD3 mAb at the indicated concentrations and incubated for various time periods at 37 °C, 5% CO₂.

**Plasmids**—pET15b/Bid vector containing human full-length Bid cDNA was a gift from Dr. X. Wang (Howard Hughes Medical Institute, New York, NY). pET15b/Bid (dnFADD), lacking amino acids 2–588, was made by inserting suitable restriction sites by PCR into this Bid cDNA. Point mutations in the caspase-8 and Granzyme-B cleavage sites of Bid (pET15b/Bid592/77E) were introduced by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) as described (38). Human FADD and mouse cellular FLIPL (cFLIP), cFLIPL, and Bid592/77E cDNAs were kindly provided by Dr. J. Tschopp (Institute of Biochemistry, Epalinges, Switzerland). A cDNA encoding dominant negative FADD (dnFADD), lacking amino acids 2–77, was generated by PCR. All sequences were verified by dideoxynucleotide sequencing. dnFADD, cFLIPL, and Bid592/77E cDNAs were cloned into the retroviral vector LZRS-MS-ires-eGFP, and human Bcl-2 cDNA was cloned into LZR-Bcl-ires-eGFP, or empty vectors were transfected into the 293T human embryonic kidney cell-derived packaging line Phoenix Amphi (42), using Pusgene-6 transfection reagent, according to instructions of the manufacturer (Roche Molecular Biochemicals). Transfected cells were selected with 1 μg/ml puromycin (Clontech). Virus-containing supernatants were harvested after 2–5 days and stored at –80 °C until further use. J16 cells were seeded on 96-well plates (Becton Dickinson, San Jose, CA) and transduced with RetroNectin (Takara) and transductions of 10^8 virus-containing supernatant/5 × 10^5 cells. Supernatants were removed after overnight incubation, and cells were cultured in fresh medium. Transduced cells were selected for enhanced green fluorescent protein expression using a MoFlo high speed cell sorter (CytoVation, Fort Collins, CO) or selected from 48 h after transduction for growth in the presence of 200 μg/ml Zeocin (Invivogen).

**Apoptosis Assay**—To measure nuclear fragmentation (subdiploid DNA content), cells were lysed in 0.1% Triton X-100, 0.1% sodium citrate, 50 μg/ml propidium iodide (43) as described earlier (39, 40). Fluorescence intensity of propidium iodide-stained DNA was determined on a FACScan flow cytometer (Becton Dickinson, San Jose, CA), and data were analyzed using CellQuest software.

**Production of Recombinant Proteins**—Full-length Bid and tBid were expressed from the pET15b vector in *Escherichia coli*. Recombinant proteins, containing an amino-terminal tag of six histidines encoded by the pET15b vector, were recovered from bacteria lysed in 50 mM Tris-HCl, pH 8.0, 20% sucrose, 10 mM β-mercaptoethanol, 0.2 mM sodium metabisulfite, with protease inhibitors. Proteins were purified by Q-Sepharose column chromatography, followed by Talon metal affinity resin, according to the instructions of the manufacturer (Clontech). Recombinant protein was eluted from this column with 100 mM imidazole in 50 mM Tris-HCl, pH 8.0, 10% sucrose, 300 mM KCl, 10 mM sodium metabisulfite and stored at –80 °C. His-tagged full-length Bid was cleaved from its expression vector by *in vitro* transcription and translation in the presence of [35S]methionine/cysteine. They were then purified from Escherichia coli. The E. coli cultures were disrupted by ultrasonic treatment, and the resulting supernatant was passed through a 25-gauge needle (10 strokes). Homogenates were centrifuged in a Beckman Airfuge at 100,000 × g for 15 min at 4 °C, and supernatants were harvested and stored at –80 °C. Protein content in cytosols was determined by the Bio-Rad protein.
FIG. 1. TRAIL induces apoptosis and Cyt c release in CD95-resistant Jurkat cells. Wild-type J16 Jurkat cells and CD95-resistant variant JA cells were left untreated (−) or stimulated (+) with anti-CD95 mAb (αCD95) (A), coiled anti-CD3 mAb (αCD3) (B), or recombinant TRAIL (C) for the indicated time periods. D, J16 cells, transduced with empty vector or Bcl-2 cDNA, were stimulated with anti-CD95 mAb or TRAIL. Propidium iodide-stained nuclei were prepared, and percentage apoptosis was determined by fluorescence microscopy as described (43). Cyt c release was monitored in mitochondria-free cytosols of J16 and JA cells, which had been stimulated with anti-CD95 mAb or TRAIL. Equal amounts of protein were loaded per lane and probed with anti-Cyt c antibody. Anti-actin immunoblotting was included as an additional loading control (E).

RESULTS

TRAIL Receptor Induces Apoptosis and Cyt c Release in CD95-resistant Jurkat Cells—Jurkat T leukemic cells are a useful model system for apoptosis, since they are sensitive to a variety of stimuli, including CD95 triggering and DNA damage (39, 40). They are also sensitive to TRAIL, which acts via TRAIL receptor-2 (DR5) in these cells (20). We have previously characterized CD95-resistant clones selected from the Jurkat cell line. These variant cells displayed cross-resistance to peroxidase-conjugated rabbit anti-mouse Ig or swine anti-rabbit Ig (1:7500), as appropriate. Immunostained proteins were visualized by ECL.

Isolation of Mitochondria—Mouse liver cells were lysed by Dounce homogenization in mitochondrion incubation buffer (MIB): 250 mM mannitol, 0.5 mM EGTA, 5 mM HEPES, pH 7.2, 0.1% (w/v) bovine serum albumin, 1 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride (45). Nuclei and debris were removed by centrifugation at 600 × g for 5 min at 4 °C. Mitochondria were pelleted by centrifugation at 10,000 × g for 10 min at 4 °C. The pellet was suspended in MIB and layered on a gradient, consisting of layers of 10, 18, 30, and 70% Percoll in 25 mM HEPES, pH 7.2, 225 mM mannitol, 0.5 mM EGTA, and 0.1% (w/v) bovine serum albumin. Purified mitochondria were collected at the 30%/70% interface after centrifugation in an SW-41 rotor at 13,000 × g for 35 min at 4 °C. The harvested fraction was diluted in MIB, at least 5-fold, and centrifuged at 6,300 × g for 10 min at 4 °C. After two more washes in MIB, mitochondria were suspended to a protein concentration of 5 mg/mg in Wang buffer B (20 mM HEPES, pH 7.5, 220 mM mannitol, 68 mM sucrose, 100 mM KCl, 1.5 mM MgCl2, 1 mM Na2EDTA, 1 mM Na2EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) (30).

In Vitro Mitochondrion Assays—Purified mitochondria (25 μg/sample) were incubated in the presence or absence of defined protein amounts of cytosol or recombinant proteins in a final volume of 30 μl of Wang buffer B at 30 °C for 1 h or for the time periods indicated and then centrifuged for 10 min at 10,000 × g at 4 °C. Mitochondrial pellets, corresponding to 12.5 μg of protein, and the corresponding volume of supernatant fractions were solubilized in SDS sample buffer and separated on 13% SDS-PAGE gels. Cyt c immunoblotting was performed as described above. Where appropriate, ECL signals were quantified using a Fluorchem 8000 chemiluminescence imager (Alpha Innotech Corp., San Leandro, CA).
levels (Fig. 1C). Both CD95- and TRAIL-induced apoptosis were severely impeded by retrovirus-mediated overexpression of Bcl-2 in J16 cells (Fig. 1D). This indicates that, in these cells, apoptotic execution downstream from either receptor is strongly facilitated by a mitochondrial contribution.

Immunoblot analysis of mitochondrion-free cytosols prepared from control and stimulated cells showed that CD95 stimulation could give rise to Cyt c release in wild-type J16 cells but failed to do so in JA cells (Fig. 1E). TRAIL, however, could induce Cyt c release in variant JA cells as effectively as in wild-type J16 cells. In conclusion, the TRAIL receptor, but not CD95, can activate mitochondria in JA cells, which subsequently allows for apoptotic execution.

**CD95 and TRAIL Receptor Both Signal to Mitochondria via FADD and Death Effector Domain-caspases.** Wild-type J16 Jurkat cells were transduced with empty vector or vectors containing dnFADD or cFLIP, cDNAs. Cells were left untreated (−) or stimulated (+) with anti-CD95 mAb or recombinant TRAIL for the indicated time periods. For the apoptosis assays shown in A and B, propidium iodide-stained nuclei were prepared, and percentage apoptosis was determined by flow cytometry. For the Cyt c release assay shown in C, cells were sheared after stimulation and mitochondrion-free cytosols were prepared. Equal amounts of protein were loaded, and immunoblotting was performed for Cyt c as well as for actin.

**Fig. 2.** CD95 and TRAIL induce Cyt c release via FADD and death effector domain-caspases. Wild-type J16 Jurkat cells were transduced with empty vector or vectors containing dnFADD or cFLIP, cDNAs. Cells were left untreated (−) or stimulated (+) with anti-CD95 mAb or recombinant TRAIL for the indicated time periods. For the apoptosis assays shown in A and B, propidium iodide-stained nuclei were prepared, and percentage apoptosis was determined by flow cytometry. For the Cyt c release assay shown in C, cells were sheared after stimulation and mitochondrion-free cytosols were prepared. Equal amounts of protein were loaded, and immunoblotting was performed for Cyt c as well as for actin.

**Fig. 3.** CD95 and TRAIL induce Cyt c release via caspase-cleaved Bid. J16 and JA cells were transduced with empty vector (closed symbol) or BidD95E/D75E (open symbol) cDNA, in which caspase-8 and granzyme B cleavage sites were mutated. Cells were stimulated with anti-CD95 mAb (A) or recombinant TRAIL (B) at the indicated concentrations for 4 or 6 h, respectively. Apoptosis was read out as nuclear fragmentation. For C, empty vector- or BidD95E/D75E-transduced J16 cells were stimulated with anti-CD95 mAb or recombinant TRAIL for the indicated time periods. Mitochondrion-free cytosols were prepared and assayed for the presence of Cyt c and actin.

**Induce Cyt c release.** J16 cells were retrovirally transduced with vectors encoding either a dominant negative FADD mutant or cFLIP, and tested for Cyt c release and apoptosis. In both dnFADD- and cFLIP-overexpressing cells, CD95- and TRAIL-induced apoptosis were completely blocked (Fig. 2, A and B), indicating the effectiveness of these proteins in preventing caspase-8/10 activation. Inhibition of caspase-8, -3, and -7 processing was demonstrated by immunoblotting (data not shown). Fig. 2C shows that Cyt c release was also completely blocked in dnFADD- and cFLIP-overexpressing cells, whereas it proceeded effectively in J16 cells transduced with empty vector. We conclude that the TRAIL receptor, like CD95, uses FADD and caspase-8/10 to signal to mitochondria.

Next, we explored whether Bid is involved in the TRAIL receptor pathway upstream from mitochondria. J16 and JA cells were retrovirally transduced with a Bid cDNA containing point mutations that delete the caspase-8 (D59E) and granzyme B (D75E) cleavage sites (26). Overexpression of this non-cleavable Bid mutant inhibited both CD95- and TRAIL-induced apoptosis in J16 wild-type cells. The effect of this Bid mutant on TRAIL-induced apoptosis was comparable in J16 and JA cells (Fig. 3, A and B). Immunoblotting of mitochondrion-free cytosols, derived from CD95- and TRAIL-stimulated J16 cells, showed that the BidD95E/D75E mutant impeded Cyt c release...
in both cases (Fig. 3C). In conclusion, like CD95, the TRAIL receptor requires FADD, caspase-8/10, and caspase-cleaved Bid to convey the apoptotic signal to mitochondria.

TRAIL Receptor Can Inactivate or Bypass a Cytosolic Factor That Impedes tBid Function—We used an in vitro assay to examine whether lack of Cyt c release in CD95-resistant JA cells was due to an impediment in the cytosol or intrinsic to mitochondria. Cytosols derived from wild-type J16 cells or variant JA cells were activated in vitro with 2 μg/ml recombinant caspase-8 for the indicated time periods or with the indicated concentrations of recombinant caspase-8 for 3 h at 30 °C. They were subsequently incubated with mouse liver mitochondria for 1 h at 30 °C. Mitochondria were isolated by centrifugation and assayed for the presence of Cyt c by immunoblotting and ECL according to standard procedures. Blots were stripped, reprobed with antibody specific for Bid, and developed using SuperSignal West Dura extended duration chemiluminescence substrate.

Dura extended duration chemiluminescence substrate.

Mitochondria were isolated by centrifugation and assayed for the presence of Cyt c. Mouse liver mitochondria were incubated at 30 °C for the indicated time periods with mitochondrion-free cytosols of J16 or JA cells and purified, bacterially expressed tBid. Cyt c content was assayed in the mitochondrial fractions by immunoblotting. C, mouse liver mitochondria were incubated with 35S-labeled in vitro transcribed/translated full-length (fl) Bid or tBid for 1 h at 30 °C. Mitochondria and supernatant fractions were separated by SDS-PAGE. Cyt c content of the mitochondrial fraction was assayed by immunoblotting (Blot). Autoradiography revealed the presence of 35S-labeled recombinant proteins in both fractions (35S-Met/Cys).

In the context of cytosol of CD95-resistant cells, tBid can be formed and associate with mitochondria but cannot efficiently liberate Cyt c. A, mitochondrion-free cytosols of wild-type J16 and variant JA cells were activated in vitro with 2 μg/ml recombinant caspase-8 for the indicated time periods or with the indicated concentrations of recombinant caspase-8 for 3 h at 30 °C. They were subsequently incubated with mouse liver mitochondria for 1 h at 30 °C. Mitochondria were isolated by centrifugation and assayed for the presence of Cyt c by immunoblotting and ECL according to standard procedures. Blots were stripped, reprobed with antibody specific for Bid, and developed using SuperSignal West Dura extended duration chemiluminescence substrate. B, mouse liver mitochondria were incubated for 1 h at 30 °C with mitochondrion-free cytosols of J16 or JA cells in the presence of the indicated concentrations of recombinant caspase-8 and purified, bacterially expressed full-length (fl) Bid as indicated. Alternatively, mouse liver mitochondria were incubated at 30 °C for the indicated time periods with mitochondrion-free cytosols of J16 or JA cells and purified, bacterially expressed tBid. Cyt c content was assayed in the mitochondrial fractions by immunoblotting. C, mouse liver mitochondria were incubated with 35S-labeled in vitro transcribed/translated full-length (fl) Bid or tBid for 1 h at 30 °C. Mitochondria and supernatant fractions were separated by SDS-PAGE. Cyt c content of the mitochondrial fraction was assayed by immunoblotting (Blot). Autoradiography revealed the presence of 35S-labeled recombinant proteins in both fractions (35S-Met/Cys).

Fig. 4. In the context of cytosol of CD95-resistant cells, tBid can be formed and associate with mitochondria but cannot efficiently liberate Cyt c. A, mitochondrion-free cytosols of wild-type J16 and variant JA cells were activated in vitro with 2 μg/ml recombinant caspase-8 for the indicated time periods or with the indicated concentrations of recombinant caspase-8 for 3 h at 30 °C. They were subsequently incubated with mouse liver mitochondria for 1 h at 30 °C. Mitochondria were isolated by centrifugation and assayed for the presence of Cyt c by immunoblotting and ECL according to standard procedures. Blots were stripped, reprobed with antibody specific for Bid, and developed using SuperSignal West Dura extended duration chemiluminescence substrate. B, mouse liver mitochondria were incubated for 1 h at 30 °C with mitochondrion-free cytosols of J16 or JA cells in the presence of the indicated concentrations of recombinant caspase-8 and purified, bacterially expressed full-length (fl) Bid as indicated. Alternatively, mouse liver mitochondria were incubated at 30 °C for the indicated time periods with mitochondrion-free cytosols of J16 or JA cells and purified, bacterially expressed tBid. Cyt c content was assayed in the mitochondrial fractions by immunoblotting. C, mouse liver mitochondria were incubated with 35S-labeled in vitro transcribed/translated full-length (fl) Bid or tBid for 1 h at 30 °C. Mitochondria and supernatant fractions were separated by SDS-PAGE. Cyt c content of the mitochondrial fraction was assayed by immunoblotting (Blot). Autoradiography revealed the presence of 35S-labeled recombinant proteins in both fractions (35S-Met/Cys).

To assess directly whether tBid function is inhibited in the context of JA cytosol, we tested the response of mitochondria to recombinant Bid. Recombinant full-length Bid significantly enhanced Cyt c release induced by caspase-8-activated cytosol of J16 cells (Fig. 4B). This is consistent with increased generation of functional tBid. Caspase-8-activated cytosol of JA cells was again much less effective in liberating Cyt c. Moreover, the addition of full-length Bid did not enhance the activity of this JA cytosol (Fig. 4B). Recombinant tBid, added to cytosol of wild-type J16 cells, efficiently induced Cyt c release from endogenous mitochondria (Fig. 4B). However, it could not do so in the context of JA cytosol. The inhibition was not absolute. Upon prolonged incubation or in the presence of increased amounts of recombinant tBid, Cyt c release was observed (results not shown). This experiment indicates that a factor, present in the cytosol of JA cells, impedes tBid function.

To further examine the association of tBid with mitochondria, we made use of recombinant Bid, synthesized in an in vitro transcription/translation system. In this way, we could label full-length and tBid with [35S]methionine and [35S]cysteine, which facilitated their detection. The immunoblot shown in Fig. 4C again demonstrates that tBid (but not full-length Bid) effectively liberated Cyt c from mitochondria in the presence of buffer or J16 cytosol but not in presence of JA cytosol.
with tBid in a caspase-8-independent manner).

In conclusion, inhibition of Cyt c release in JA cells is not intrinsic to mitochondria. In JA cells, a cytosolic component interferes with the capacity of tBid to liberate Cyt c after its generation and association with mitochondria. Since CD95 and TRAIL receptor pathways use tBid as a common intermediate in both J16 and JA cells (Fig. 3) but differ in their capacity to induce Cyt c release in JA cells, we conclude that the TRAIL receptor is able to bypass or suppress the inhibition of tBid function.

Bax Is a Component of CD95 and TRAIL Receptor Signaling Pathways—The only cytosolic protein known to participate in mitochondrial activation downstream from BH3 domain-only Bcl-2 family members such as Bid is Bax. To examine whether CD95 and TRAIL receptor use Bax as a signaling intermediate, we again performed in vitro assays. Cytosols were derived from J16 wild-type cells, which had been stimulated with anti-CD95 mAb or TRAIL for various periods of time. These in vitro activated cytossols were used as such or after depletion of Bid or Bax by repeated immunoprecipitation. Depletion of Bid was effective, as shown in Fig. 5B; Bid-depleted cytosol from unstimulated J16 cells could not be activated by recombinant caspase-8 to induce Cyt c release, while mock-depleted cytosol was highly effective. Depletion of Bax was successful, as determined by immunoblotting (Fig. 5B). CD95- and TRAIL-activated cytossols were effective in liberating Cyt c from mouse liver mitochondria. However, they reproducibly lost their activity upon Bid depletion (Fig. 5A). These findings are consistent with the results depicted in Fig. 3 and emphasize that TRAIL receptor, like CD95, requires Bid to signal to mitochondria. In addition, we find that both pathways involve Bax; depletion of Bax greatly reduced the activity of CD95-activated cytosol and completely blocked the activity of TRAIL-activated cytosol in these assays (Fig. 5A). In repeated experiments, the effect of Bax depletion was consistently less absolute than the effect of Bid depletion, suggesting that Bax is involved but not absolutely required in conveying the death signal to mitochondria downstream from these receptors. Interestingly, Bax depletion did not influence the capacity to induce Cyt c release of cytosol, which had been activated by recombinant caspase-8 in vitro (Fig. 5B). This indicates that TRAIL receptor and CD95 invoke Bax function (i.e. activate Bax to allow its collaboration with tBid in a caspase-8-independent manner).

In conclusion, we find that TRAIL receptor and CD95 both employ Bax to signal to mitochondria. Differential use of Bax by TRAIL receptor and CD95 is therefore not the explanation for the capacity of TRAIL to free Cyt c from mitochondria in JA cells.

DISCUSSION

It was originally found by some, but not other authors that TRAIL receptors could induce apoptosis independent of FADD (47). In addition, TRADD and RIP were found to associate with TRAIL receptors (33, 34). These findings suggested that CD95 and TRAIL receptor signaling pathways were distinct. However, recent experiments have shown that FADD takes part in the TRAIL receptor pathway leading to apoptosis; FADD is present in the native TRAIL receptor DISC (20–22). Moreover, FADD reconstitution into FADD-deficient mouse embryo fibroblasts sensitized them to TRAIL (23). The involvement of FADD in TRAIL receptor signaling to mitochondria has not been examined previously. Our data establish that, also in this pathway, there is an absolute requirement for FADD.

The involvement of death effector domain caspasess in the TRAIL receptor pathway leading to mitochondrial activation,
we found that their substrate Bid is instrumental in this pathway as well. We established this by depletion of Bid from receptor-activated cytosol as well as by use of a noncleavable Bid mutant in vivo. Although proteolytic cleavage of Bid in response to TRAIL has been observed in several studies, it was not investigated earlier whether this was critical for TRAIL receptor signaling to mitochondria or a secondary event due to effector caspase activation. Expression of the noncleavable Bid mutant did not fully abrogate TRAIL- and CD95-induced apoptosis. This may be explained by the relatively low level overexpression achieved by retroviral transduction in this case. Immunoblotting indicated that levels of the Bid mutant protein are only about 2-fold higher than levels of endogenous wild-type Bid (results not shown). It should be remarked that both receptors can ultimately bypass the mitochondria to activate effector caspsases and induce apoptosis. Although Bcl-2 overexpression significantly impedes the apoptotic response to both receptors in these Jurkat cells (see Fig. 1D), upon prolonged stimulation apoptotic cells accumulate (results not shown). The resistance in JA cells is also not absolute, confirming that upon strong and persistent DR stimulation, the mitochondrial contribution is not required for apoptosis. This is consistent with earlier results (48).

Although the mitochondrial contribution may not be required for the ultimate demise of cells stimulated via DR, it can clearly contribute to the efficiency of the death response. At a minimum, mitochondria facilitate effector caspase activation by allowing Cyt c-mediated activation of the inducer caspase-9 as well as by release of the Smac/Diablo molecule. Smac/Diablo binds to inhibitor of apoptosis proteins and therewith reverses their inhibitory action on caspase molecules (17). In addition, mitochondria are expected to contribute to the apoptotic response in qualitative terms. For instance, release of apoptosis-inducing factor and endonuclease G is expected to affect the nuclear morphology of apoptotic cells (17). Since the ultimate phenotype of apoptotic bodies may well affect the phagocytic process, it is too narrow a view to only take into account the number of dead cells as an outcome of death receptor stimulation. DR target mitochondria in a specific and well regulated manner, strongly suggesting an important physiological significance.

We find here that CD95 and the TRAIL receptor use Bax as an intermediate to signal to mitochondria. Possible involvement of Bax in DR signaling has not received much attention. Most studies have focused on Bid. However, recently, it has become clear that BH3 domain-only proteins cooperate with Bax/Bak type proteins to induce mitochondrial permeabilization. Evidence has been presented that BH3 domain-only proteins, such as Bid, by transient interaction induce a conformational change in Bax or Bak, which is accompanied by or evokes multimerization and possible pore formation by these molecules. Most illuminating have been studies using mitochondria or cells from Bax/Bak double deficient mice, which proved nonresponsive to BH3 domain-only proteins (27, 28, 49). The presence of either Bax or Bak proved sufficient for Cyt c release to occur, indicating at least partial redundancy between these proteins. Involvement of Bax in the CD95 pathway followed from the observations that CD95 induces a conformational change in Bax and its relocalization to mitochondria in breast cancer cells or hepatocytes (28, 50). This mitochondrial translocation was dependent on Bid and could also be induced by tBid (28). However, a contribution of Bax to Cyt c release had not been demonstrated directly. We find that Bax contributes in the CD95 pathway but is not absolutely required for Cyt c release. This is consistent with a redundancy between Bax and Bak, which is constitutively present in the outer membrane of mouse liver mitochondria (27). Recently, the presence of at least one functional Bax allele was found to be essential for TRAIL-induced apoptosis in a colon cancer cell line (51). Bax translocated to mitochondria in response to TRAIL and tBid. Moreover, Bak was required for release of Cyt c and Smac/Diablo as well as for caspase-9 and caspase-3 activation. Importantly, expression of a cytosolic form of Smac/Diablo sufficed to restore the apoptotic response to TRAIL in Bax-deficient cells. This result indicates that release of Smac/Diablo, rather than Cyt c, is the essential event downstream from Bax in this case. A total dependence on Bax for TRAIL-induced mitochondrial changes in these colon cancer cells can be explained by their lack of detectable Bak expression (51). Interestingly, chemotherapeutic drugs were recently shown to up-regulate Bak and restore TRAIL sensitivity in these cells (52). Our data corroborate that Bax participates in the TRAIL receptor pathway. Depletion of Bax reduced the activity of TRAIL-stimulated cytosols more dramatically than that of CD95-activated cytosols. However, our findings do not indicate that Bax is absolutely required for TRAIL-induced mitochondrial permeability, since Cyt c release was observed upon prolonged incubation.

The accumulated data indicate that CD95 and TRAIL receptor use the same signaling intermediates to communicate with mitochondria: FADD, caspase-8/10, caspase-cleaved Bid, and Bak. However, the TRAIL receptor can bypass or abrogate the function of a cytosolic factor that interferes with the function of tBid after its association with mitochondria. It is highly unlikely that tBid function is impeded by interference with its myristoylation, which facilitates anchoring to the mitochondrial membrane (53). We find no inhibition of mitochondrial association. Moreover, the recombinant tBid protein whose function is inhibited in our studies contains sequences encoded by the pET15b vector at the amino terminus, which mask the Gly residue to which the myristate is normally linked (53). The JA variant cells regain sensitivity to CD95 stimulation upon preincubation with cycloheximide or actinomycin D, indicating that resistance is due to a gain-of-function alteration (results not shown). Differential sensitivity of various cell types to CD95- and TRAIL-induced apoptosis has been observed earlier. Selectivity of inhibition for the CD95 pathway may imply that there are different, unknown, mediators involved in the CD95 and TRAIL receptor pathways downstream from tBid. The inhibitory protein would then have a selective effect on the mediator in the CD95 pathway. An alternative explanation is that TRAIL receptor but not CD95 can alleviate the inhibition, for instance by the induction of proteolytic activity. In such a scenario, the common mediators tBid or Bax may be target of inhibition. Thus far, however, we have not found evidence for association of unique proteins with Bid or Bax in resistant JA cells. Although this issue has not been resolved, the availability of the JA variant cells has allowed us to conclude that the TRAIL receptor and CD95 can differentially regulate events involved in Cyt c release downstream from tBid. Since the apoptotic response to DNA-damaging anti-cancer regimens is fully abrogated in the JA cells (39), this regulatory mechanism may prove to be of broad importance.

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