Survival Factor Withdrawal-induced Apoptosis of TF-1 Cells Involves a TRB2-Mcl-1 Axis-dependent Pathway*

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Tribbles, an atypical protein kinase superfamily member, coordinates cell proliferation, migration, and morphogenesis during the development of Drosophila and Xenopus embryos. Although Tribbles are highly conserved throughout evolution, the physiological functions of mammalian Tribbles family remain largely unclear. Here we report that human TRB2 is a pro-apoptotic molecule that induces apoptosis of cells mainly of the hematopoietic origin. TRB2 mRNA is selectively induced by removal of granulocyte macrophage colony-stimulating factor (GM-CSF) or interleukin-2 from human erythroleukemia-derived TF-1 cell line or activated primary CD4⁺ T cells, respectively. It is, however, not induced by many other treatments that trigger apoptosis of these two cell types. Overexpression of TRB2 activates many apoptotic events observed in GM-CSF-deprived TF-1 cells, including loss of mitochondrial membrane potential, Mcl-1 cleavage/degradation, and activation of Bax and a number of caspases. Specific knockdown of TRB2 significantly suppresses GM-CSF deprivation-induced apoptosis and all apoptotic events mentioned above. Finally, we demonstrate that TRB2-induced cleavage and degradation of Mcl-1 are mediated via a caspase-dependent but proteasome-independent mechanism, and overexpression of Mcl-1 or its upstream activator Akt can markedly overcome the apoptogenic effect of TRB2. Altogether, these results suggest that the TRB2-Mcl-1 axis plays an important role in survival factor withdrawal-induced apoptosis of TF-1 cells.

Involves a TRB2-Mcl-1 Axis-dependent Pathway*

Tribbles has been shown to coordinate cell proliferation, migration, and morphogenesis during the development of Drosophila and Xenopus embryos (1–5). The protein sequence of Tribbles suggests that it is a member of the protein kinase superfamily, but its sequence diverges from the conventional kinase consensus in subdomains I and II, which are essential for ATP binding (6). The mammalian orthologs of Tribbles, TRB1, TRB2, and TRB3, all appear to contain the consensus serine/threonine kinase catalytic core, but lack a conserved ATP-binding pocket. Accordingly, no kinase activity has been demonstrated for these proteins by in vitro kinase assay (1, 7). Recent studies on mammalian Tribbles proteins have revealed that they may play some important roles in metabolism and growth regulation. In 293 cells, TRB3 is induced by NF-κB and functions to negatively regulate NF-κB-dependent transcription (8). Under fasting conditions, TRB3 is induced through the PCG-1/peroxisome proliferator-activated receptor-α pathway and inhibits Akt/PKB activation in liver (7, 9). TRB1 and TRB3 were shown to either inhibit or activate the activities of mitochondrial membrane protein kinase kinase via protein-protein interaction (10). During endoplasmic reticulum stress-induced apoptosis, human TRB3 expression was up-regulated, and knockdown expression of TRB3 by RNA interference rescued cell viability (11). Conversely, whereas TRB3 mRNA was induced by nutrient starvation, overexpression of TRB3 prevented nutrient starvation-induced cell death (12). trb2 is recently reported to be an oncogene, which induces acute myelocytic leukemia in the chimeric mice bearing TRB2-transduced bone marrow, a result likely because of conversion of C/EBPa p42 to C/EBPa p30 in TRB2-transduced cells (13).

In the absence of a specific cytokine, cytokine-dependent hematopoietic cells actively undergo apoptosis, which can be blocked by treatment with actinomycin D or cycloheximide, implying a requirement for de novo RNA/protein synthesis in this apoptotic pathway (14, 15). Several genes have been reported to be induced in mRNA levels following cytokine deprivation and may play some important roles in the cell-death pathway. For example, hrk, the BH3-only Bcl-2 family gene, is up-regulated during interleukin 3 (IL-3) deprivation of hematopoietic progenitors and is involved in the regulation of apoptosis (16). 24p3, which encodes a secreted lipocalin, is induced by IL-3 deprivation in several IL-3-dependent cell lines and primary bone marrow cells and is able to induce apoptosis of a

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2 The abbreviations used are: PKB, protein kinase B; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; BM, bone marrow; siRNA, short interfering RNA; EGFP, enhanced green fluorescent protein; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; HA, hemagglutinin; TNF, tumor necrosis factor; DSS, ducinimidyl deoxynucleotide-terminated dUTP nick-end labeling; IRES, internal ribosome-entering site; MMP, mitochondrial membrane potential; An-V, annexin V; μF, microfarads.
variety of leukocytes through an autocrine mechanism (17). RC3, a calcium/calmodulin-binding protein, is induced by IL-2 deprivation in several IL-2-dependent cell lines and activated T cells, which is part of the apoptosis pathway (18). We also launched a gene expedition study by transcription profiling of a human granulocyte macrophage colony-stimulating factor (GM-CSF)-dependent cell line TF-1 cultured in the absence of GM-CSF. Here we report the identification of TRB2 as an important modulator of apoptosis in TF-1 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—TF-1 is a cytokine-dependent hematopoietic cell line whose normal growth in vitro requires culture medium supplemented with human GM-CSF or IL-3. TF1-bcl-2 is a TF-1 derivative ectopically overexpressing Bcl-2 (19). Ba/F3, 32D, and FDCP-1 are all murine IL3-dependent cell lines. Cytokine-independent suspension cell lines (Jurkat, WEHI-3, U937, and K562) were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 55 μM β-mercaptoethanol. All cytokine-dependent suspension cell lines were cultured in the same medium but supplemented with specific survival cytokines. Adherent cell lines (HeLa, 293, H1299, and HepG2) and MEL were all maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.

**Chemicals**—Dimethyl sulfoxide (Me₂SO) and digitonin were purchased from Sigma. Z-VAD-fmk was purchased from Bachem. TNF-α, actinomycin D, camptothecin, cycloheximide, dexamethasone, etoposide, and staurosporine were all purchased from BioVision. Disuccinimidyl suberate (DSS) and MG132 were purchased from Pierce and Calbiochem, respectively.

**Cytokine Deprivation and Re-stimulation**—For cytokine depletion experiments, factor-dependent cells were grown to a saturation density, washed three times in medium without cytokine, and seeded in cytokine-free medium with 0.5% fetal bovine serum for the indicated length of time. For cytokine re-stimulation experiments, GM-CSF was added to the starved TF-1 cells to a concentration of 20 ng/ml.

**Isolation and Activation of Mouse Primary CD4+ T Cells**— Splenic CD4+ T cells were isolated by incubating splenocytes with anti-CD4 (L3T4) microbeads followed by purification with an LS-positive selection column and a MACS separator (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s recommendations. Purified CD4+ cells were activated in plates coated with anti-CD3 (2C11, 5 μg/ml) and anti-CD28 antibody (37.51, 2.5 μg/ml) for 3 days and then transferred to plates without antibody coating for further cultivation in the presence or absence of IL-2.

**Expression Vectors**—pTRB2-IRE5-EGFP was generated by subcloning mouse trb2 cDNA into the BamHI site of the pIRE5-EGFP vector (Clontech). For construction of pEGFP-TRB2 (T2E), the coding region of human TRB2 cDNA without a stop codon was ligated into the BamHI sites of the pEGFP-N3 vector (Clontech). To construct expression plasmids encoding the TRB2-EGFP mutants, standard PCR-assisted mutagenesis-coupled cloning methods were carried out using primer 5'-GCAAGGTTTATTATCAGC-3' in combination with the following primers (underlined nucleotides differ from the wild-type sequence): TRB2-K177A, 5'-CTACTTTTTCCGCAG-CGCCAGGTCCGCACGA-3', and TRB2-K177R, 5'-CTACTTTTTCCGCAGCTGTCGCCGCA-3'. These PCR products and a third primer 5'-AGCTTTTTCCGCAGCATGG-3' were used in a second PCR using pEGFP-TRB2 as template. The resultant PCR products were restricted with EcoRV and BstXI before they were used to replace the corresponding fragment of the pEGFP-TRB2. All mutated nucleotides were confirmed by sequencing. Expression plasmids encoding HA-tagged mouse mcl-1 (20) and HA-tagged myr-Akt were as described previously (21).

**Gene Transfer**—Gene transfers into hematopoietic cells were carried out by electroporation using a Bio-Rad Gene pulser set at 180 V and 975 μF (for TF-1 and TF-1-bcl-2 cells) or at 200 V and 975 μF (for other hematopoietic cells) or as otherwise indicated. Transfections of adhesion cells were performed by using Lipofectamine (Invitrogen) according to the manufacturer’s protocol.

**Northern and Western Blot Analyses**—For Northern blot analysis, total RNA was prepared using the TRizol reagent kit (Invitrogen) and analyzed by the standard protocol using cDNA probes specific to TRB1, TRB2, TRB3, and GSPDH. For Western blot analysis, cells to be analyzed were lysed in a buffer containing 25 mM Tris (pH 7.6), 150 mM NaCl, 2.5 mM MgCl₂, 0.5 mM EDTA, 0.5% Nonidet P-40, 5 mM β-glycerophosphate, 1 mM dithiothreitol, 5% glycerol, and protease inhibitors (7), and 100 μg of cell lysates, unless otherwise indicated, were analyzed by Western blotting using antibodies as indicated in each figure.

**Antibodies**—Polyclonal antibody specifically recognizing TRB2 was generated by immunizing rabbits with bacterially produced recombinant TRB2 protein by a standard protocol, and was affinity-purified using specific antigen cross-linked to CNBr-activated Sepharose (GE Healthcare). The specificity of this antibody was confirmed by Western blot analysis with or without TRB2 antigen competition (data not shown). This antibody can recognize both human and murine TRB2. Other antibodies used in this study include those specific to Bcl-2, Bcl-XL, Bax, GFP, human Mcl-1, cytochrome c, p38 (all from Santa Cruz Biotechnology), activated Bax (Clontech), caspase-3, caspase-8, caspase-9, cleaved poly(ADP-ribose) polymerase (all from Cell Signaling Technologies, Boston), hemagglutinin (HA) tag (Roche Diagnostics), and actin (Sigma). Antibody specific to mouse mcl-1 (20) or mitochondrial import receptor Tom70 was as described previously (22). Antibody specific to p53 was a gift from Sheau-Yann Shieh, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan.

**Annexin V and TUNEL Staining and Caspase Activity Assays**—To analyze cells that have undergone apoptosis, annexin V staining and terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assays were carried out using the annexin V-cy3 apoptosis detection kit (BioVision, CA) and the in situ cell death detection kit (TMR red, Roche Diagnostics), respectively. In some cases, cells were stained with 4',6-diamidino-2-phenylindole to visualize nuclei and examined by confocal microscopy. The activated caspase-3, caspase-8, and caspase-9 were evaluated with the CaspGLO™ Red Active caspase-3, caspase-8, and caspase-9 staining kits (BioVision, Boston).
**TRB2 Modulates Apoptosis in TF-1 Cells**

![Figure 1. Induction of TRB2 following GM-CSF and IL-2 deprivation.](image)

CA) by flow cytometry. Flow cytometric analysis was performed with a BD FACSCalibur system.

**Cytochrome c Release and Detection of Oligomerized Bax**—Staining of cytochrome c was carried out essentially as described by Willis et al. (23). In some cases, cells to be analyzed were fractionated into cytosolic and mitochondrial fractions (24), and cytochrome c partitioned in these two fractions was analyzed by Western blotting using anti-cytochrome c antibody. To detect oligomerized Bax, cells to be analyzed were cross-linked with disuccinimidyl suberate essentially as described by Sundararajan and White (25). Following cross-linking, cells were lysed and cell lysates analyzed by Western blotting using Bax-specific antibody.

**RNA Interference**—Double-stranded RNA duplex corresponding to human TRB2 (5′-gctgagaagcgcgagac-3′) and nontargeting (nontargeting number 1, D-001210-01-20) siRNA were purchased from Dharmacon (Chicago). Cells to be analyzed (5 × 10⁶) were mixed with 1 nmol of siRNA in 0.4 ml of Optimal MEM and subjected to electroporation at 250 V, 400 μF with Bio-Rad GenePulser apparatus.

**RESULTS**

**TRB2 Is an Immediate Early Gene Induced by GM-CSF Deprivation**—In the TF-1 cell line, deprivation of human GM-CSF induces profound apoptosis within 24 h. This apoptotic process can be suppressed significantly by the protein synthesis inhibitor cycloheximide, suggesting that *de novo* gene expression is required to mediate this death program. To identify genes whose expression is up-regulated after cytokine deprivation, we studied the expression profiles of 8000 genes using a cDNA microarray (Taiwan Genome Sciences, Inc., Taipei) with mRNA isolated from TF-1 cells cultured in medium with or without GM-CSF. Among several candidates, we confirmed by Northern blot analysis that the TRB2 gene was highly inducible upon GM-CSF deprivation. After removal of GM-CSF, the mRNA levels of TRB2 were up-regulated by 3 h and reached a peak at 24 h (Fig. 1A, lanes 2–5).

However, when the cytokine-deprived cells were re-stimulated with GM-CSF, the TRB2 mRNA returned to a base-line level at 3 h (Fig. 1B, lanes 3–5). Furthermore, the induction of TRB2 mRNA by GM-CSF deprivation did not require new protein synthesis, as cycloheximide could not suppress this induction (Fig. 1C, compare lanes 6 and 9). In fact, cycloheximide alone slightly induced TRB2 expression (Fig. 1C, lane 3). This result suggests that TRB2 is an immediate early gene activated by GM-CSF deprivation. Consistent with the increased expression of TRB2 mRNA, the increased TRB2 protein level following GM-CSF deprivation of TF-1 was also observed (Fig. 1D, lanes 2 and 3). Induction of TRB2 expression was also observed in TF-1 cells that had been previously maintained in another survival cytokine IL-3 or in TF-1 derivatives stably overexpressing IL5R α chain (19) maintained in IL-5 (data not shown). We next examined whether TRB2 could also be induced in other cytokine-dependent cells upon removal of their dependent cytokines. To our surprise, most cell lines examined, including murine GM-CSF-dependent C2GM cells, IL-2-dependent HT-2 cell line, and IL-3-dependent Ba/F3, 32D, and FDCP-1 cells do not manifest this property (data not shown). However, the *trb2* mRNA and the TRB2 protein were clearly induced in the activated primary murine (Fig. 1, E and F, respectively) or human (data not shown) CD4⁺ T cells upon IL-2 deprivation.

**TRB2 Induction Is Highly Selective**—To explore whether TRB2 is a general apoptosis-responsive gene, TF-1 cells were treated with various apoptotic stimuli, including actinomycin D, camptothecin, cycloheximide, dexamethasone, etoposide, staurosporine, and UV irradiation (Fig. 2A). These apoptotic agents, except cycloheximide and dexamethasone, could induce a substantial degree of apoptosis, which was comparable with that induced by GM-CSF deprivation (−GM, Fig. 2A). Intriguingly, the results of Northern blot analysis demonstrated that TRB2 expression was induced profoundly by GM-CSF starvation, and induced slightly by cycloheximide and dexamethasone, but not by other apoptotic treatments (Fig. 2A, lanes 5–19). Because another human tribbles ortholog, TRB3 (also named NIPK or SKIP3), could be induced in PC6-3 cells by nerve growth factor deprivation (26), we investigated whether other members of *tribbles* are inducible in our experiments.

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**FIGURE 1. Induction of TRB2 following GM-CSF and IL-2 deprivation.** A, induction of human TRB2 mRNA upon GM-CSF deprivation of TF-1 cells. TF-1 cells were deprived of GM-CSF for the indicated length of time, and their total RNA was isolated and analyzed by Northern blotting using probes specific to TRB2 or G3PDH (as an RNA loading control). B, repression of TRB2 mRNA expression following re-stimulation of GM-CSF. Same as in A, except that cells were deprived of GM-CSF for 24 h and then re-stimulated with GM-CSF for the indicated length of time. C, expression of TRB2 mRNA does not require new protein synthesis. Same as in A, and cells were treated as indicated. CHX, 100 μM cycloheximide; DMBSO (Me2SO), vehicle control. D, induction of endogenous human TRB2 protein in TF-1 cells following GM-CSF starvation. Same as in A except that protein lysates were analyzed by Western blot using antibodies specific to TRB2 or β-actin. E, induction of murine *trb2* mRNA; *F*, protein by removal of IL-2 from activated mouse primary CD4⁺ T cells. Activated splenic CD4⁺ T cells (see “Experimental Procedures”) were deprived of IL-2 for the indicated times before total RNA or protein lysates were analyzed by Northern (E) or Western blotting (F), respectively. Molecular weight markers and ribosomal RNAs are as indicated.
Northern blot analysis showed that in the absence of GM-CSF TRB1 mRNA was only slightly induced (Fig. 2A, top panel), whereas the mRNA of TRB3 was depressed (Fig. 2A, 3rd panel, compare lanes 1 and 2, and 3 and 4). Likewise, the RNA level of TRB1 could be induced slightly by camptothecin (Fig. 2A, 3rd panel, lanes 7 and 8), cycloheximide (lanes 9 and 10), and etoposide (lanes 13 and 14). On the other hand, TRB3 was clearly induced by dexamethasone (Fig. 2A, 3rd panel, lanes 11 and 12) but not by other treatments. In contrast, camptothecin and staurosporine could reduce the expression of TRB3, like the effect of GM-CSF deprivation (Fig. 2A, lanes 3, 4, 7, 8, 15, and 16).

To extend our observation in TF-1 cells, activated mouse primary CD4⁺ T cells were treated with various apoptotic inducers as indicated. Cells in lanes 5–19 are in the presence of GM-CSF. Twelve or 24 h after treatment, total RNA from these cells was analyzed by Northern blotting using probes specific to human TRB1, TRB2, and TRB3 (A) or murine trb2 mRNA (B). ActD, 10 μM actinomycin D; Camp, 1 μM camptothecin; CHK, 100 μM cycloheximide; DEX, 20 μM dexamethasone; Etop, 10 μM etoposide; STS, 500 nM staurosporine; UV, 100 mJ/cm². C, lack of TRB2 mRNA super-induction by TNFα. Same as in A, and cells were treated as indicated. TNFα, 1 ng/ml of TNFα. D, lack of induction of trb2 mRNA by activation-induced-cell death in CD4⁺ T cells. Same as in A, except that activated CD4⁺ T cells were re-stimulated with anti-CD3 (5 μg/ml) plus IL-2. For all treatments, percentage of apoptotic cells was determined by staining with annexin V. Ethidium bromide staining of ribosomal RNA was used as an RNA loading control for all panels.

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TRB2 Modulates Apoptosis in TF-1 Cells

We next examined whether a similar apoptosis-enhancing effect could be observed for the TRB2-EGFP fusion protein (T2E, Fig. 3D, lane 1) which is encoded by the plasmid pTRB2-EGFP (Fig. 3A, panel b). In the IL-3-containing medium, transfection of Ba/F3 cells with 30 μg of pEGFP-N3 vector usually resulted in 20–30% GFP(+) cells (12 or 20 h post-transfection), whereas under the same conditions, transfection with the pTRB2-EGFP plasmid resulted in only 5–15% GFP(+) cells (see one example shown in supplemental Fig. S1). Of note, although transfection of cells with lower amounts of EGFP or T2E expression vectors (5–15 μg) resulted in lower percentages of GFP(+)-positive cells, the same trend was always observed, i.e. significantly less GFP(+) cells were observed in cells transfected with T2E than with EGFP expression vector (supplemental Fig. S1). Furthermore, we noticed that for cells transfected with the control vector, a majority of GFP(+) cells were TUNEL(–) under confocal microscopy. However, the TUNEL(+) / GFP(+) population in pTRB2-EGFP-transfected cells was significantly increased, even when cells were cultured in the presence of IL-3 (Fig. 3E). Flow cytometric analysis revealed that in one representative experiment, expression of T2E in Ba/F3 cells resulted in 61% of GFP(+) cells to be TUNEL-positive, whereas overexpression of EGFP proteins resulted in only 3% cell death (Fig. 3F, top panels, T2E versus E). Similar percentages of cell death were observed when the apoptotic cells were quantified by the annexin V staining assay (Fig. 3F, bottom panels). Furthermore, T2E-induced cell death was also consistently observed in experiments performed with TF-1 cells (Fig. 3G).

Next, we examined whether the apoptotic effect of T2E was mediated through caspase-dependent pathways. As shown in Fig. 3G, T2E-induced apoptosis of either Ba/F3 or TF-1 cells cultured in cytokine-containing medium could be markedly suppressed when cells were treated with the pan-caspase inhibitor Z-VAD-fmk (Fig. 3G, white versus grey columns). These data suggest that overexpression of the TRB2-EGFP fusion protein could cause cell death with typical apoptotic characteristics.

Because TRB2 is a kinase-like protein, we wondered whether any potential kinase activity of TRB2 would be required for its pro-apoptotic function. To address this issue, the lysine residue at the putative catalytic center of subdomain VIB of TRB2, which has the motif RDLKL, was mutated into either arginine (K177R) or alanine (K177A) to create a kinase-dead mutant, which has the motif RDLKL, was mutated into either arginine (K177R) or alanine (K177A) to create a kinase-dead mutant, and the apoptogenic activity of these two kinase-dead mutants was then compared with that of the wild-type protein. As shown in Fig. 4, H and I, under the same experimental conditions when wild-type and both mutants (all expressed as EGFP fusion protein) were transfected, TRB2 expression and the percentage of apoptotic cells were determined by immunoblotting with p38 MAPK antibodies. The expression of p38 MAPK was included as an internal control. The same immunoblot was reprobed with β-actin antibody to serve as a protein loading control. D-F, expression of the TRB2-EGFP (T2E) fusion protein promoted apoptosis of Ba/F3 cells in the presence of IL-3. Ba/F3 cells expressing T2E (lane 2) or EGFP (E lane 1) alone were analyzed by immunoblot essentially as described in B except that cells were cultured in IL-3-containing medium and harvested 8 h after transfection (D). The apoptotic cells detected by TUNEL staining were visualized by confocal microscopy (E) or quantified by flow cytometry (F, top panel). Part of apoptotic cells was quantified by annexin V (Ann-V) staining (F, lower panel). Nuclei were stained with 6-diamidino-2-phenylindole (DAPI). G, T2E-induced apoptosis is blocked by Z-VAD-fmk. Cells transfected with EGFP or T2E-expressing vector (electroporation using Bio-Rad Gene Pulser) were set at 220 V and 975 μF for Ba/F3 cells, and 220 V and 975 μF for TF-1 cells or vehicle control (MeSO2) plus IL-3 or GM-CSF for 24 h before the percentage of apoptotic cells was quantified by annexin V staining. H and I, kinase-dead mutants of TRB2 (K177A and K177R) retain apoptogenic activity of the wild-type protein. These data suggest that overexpression of the TRB2-EGFP fusion protein could cause cell death with typical apoptotic characteristics.
fusion proteins, i.e. T2E, K177A-E and K177R-E) were transiently expressed at a similar level in Ba/F cells (Fig. 3H), they all resulted in a similar degree of apoptosis (Fig. 3I), suggesting that the pro-apoptotic function of TRB2 does not require its putative kinase activity.

The ability of the TRB2 fusion proteins (T2E) to induce cell death was further investigated in a few other murine and human cell lines. As shown in Fig. 4, T2E induced apoptosis in all cytokine-dependent hematopoietic cell lines tested, including 32D, FDCP-1, and HT-2 (lanes 1–3), although these cell lines did not express TRB2 upon cytokine deprivation (data not shown). Furthermore, the primary murine CD4+ T cells were very sensitive to T2E (Fig. 4, lane 4). On the other hand, some cytokine-independent leukemic cell lines, e.g. WEHI3 and Jurkat (Fig. 4, lanes 5 and 6), were very sensitive to T2E-induced apoptosis, whereas other cell lines, including K562 and U937 (lanes 8 and 9), were highly resistant. Interestingly, most non-hematopoietic cell lines tested, including HeLa, 293, H1299, and HepG2, were highly resistant to T2E-induced apoptosis (Fig. 4, lanes 10–13). These data suggest that the apoptotic activity of T2E is largely restricted to hematopoietic cells.

**TRB2-EGFP Triggers Apoptosis via Mitochondria Dysfunction—**A variety of external and internal signals converge on mitochondria to trigger or inhibit apoptosis (27). To understand the mechanism of the apoptotic effect of T2E, we investigated the ability of T2E to damage mitochondria. The mitochondrial dye MitoTracker Red was used to stain the viable cells, because apoptotic cells lost membrane potential and lost the staining of MitoTracker Red. As shown in one representative result (Fig. 5A), expression of T2E in Ba/F3 cells increased dramatically the proportion of cells that lost the membrane potential, from 10 to 78% (see Δψm,L). Loss of mitochondrial membrane potential (MMP, Δψm) correlated strongly with activation of caspases, including caspase-3, caspase-8, and caspase-9 (Fig. 5A), and the percentage of cells that have undergone apoptosis as revealed by the annexin V (An–V) staining method. T2E-induced activation of caspase-3, caspase-8, and caspase-9, as evident from the generation of specific cleavage products, was also demonstrated in TF-1 cells by Western blotting with antibody specifically recognizing each caspase (Fig. 5B). Of note, under our experimental conditions, although the cleaved form of caspase-3 was only slightly detectable, the production of cleaved poly(ADP-ribose) polymerase, a typical caspase 3 substrate, was quite prominent (Fig. 5B, 2nd to the bottom panel), suggesting that caspase-3 was also highly activated by T2E in TF-1 cells.

We next examined whether T2E could induce activation of Bax, a proapoptotic molecule that, in response to apoptotic stimuli, will change conformation and oligomerize, which leads to mitochondria dysfunction (28, 29). To address this issue, Ba/F3 cells were transfected with vectors expressing control (EGFP) or T2E, and transfected cells (GFP+) were sorted out and treated with DSS before cell lysates were prepared and analyzed by Western blotting using Bax-specific antibody. As shown in Fig. 5C, dimerized Bax was identified only in the presence of DSS in T2E-expressing cells (Fig. 5C, lane 4), but not in cells expressing the control protein EGFP (lane 2). Alternatively, T2E-induced activation of Bax was demonstrated in TF-1 cells using antibody specifically recognizing the active conformation of human Bax (a-Bax). As shown in Fig. 5D, in one representative experiment expression of T2E markedly increased the percentage of cells stained by such an antibody (66% of T2E versus 16% of EGFP-expressing cells). T2E-induced activation of Bax and loss of MMP (Δψm,L) could not be blocked by the pan-caspase inhibitor Z-VAD-fmk (Fig. 5E), albeit such a peptide inhibitor inhibited T2E-induced apoptosis quite well (Fig. 5E, compare bars labeled with An–V). These data indicate that Bax activation and mitochondria dysfunction induced by T2E overexpression do not require prior activation of caspases.

We next examined whether release of cytochrome c from mitochondria into the cytosolic compartment, a hallmark of mitochondria dysfunction, occurred in T2E-expressing cells. We first addressed this issue by comparing the cytochrome c levels in mitochondria and cytosolic fractions prepared from Ba/F3 cells expressing T2E or control protein EGFP. As shown in Fig. 5F, in control cells cytochrome c was only detected in the mitochondrial (pellet) fraction (compare lanes 1 and 3). How-
ever, in T2E-expressing cells, a great portion of cytochrome c was detected in the cytosolic (supernatant) fraction (compare lanes 2 and 4). In another assay carried out in TF-1 cells, we observed that expression of T2E, but not the control protein EGFP, markedly reduced the staining of transfected cells with anti-cytochrome c antibody (Fig. 5G, C.c in TF-1). Together, these two approaches demonstrated that T2E expression caused cytochrome c release from mitochondria to cytosol.

To further demonstrate the importance of mitochondria in transmitting the death signal of TRB2 and in activating caspases, we next examined whether overexpression of Bcl-2 can inhibit T2E-induced apoptosis, considering the fact that TF-1 cells stably overexpressing Bcl-2 (TF1-bcl-2) are highly resistant to GM-CSF withdrawal-induced apoptosis (19). As shown in Fig. 5G, T2E overexpression-induced apoptosis (An-V) was markedly reduced in TF1-bcl-2, compared with parental TF-1 cells (compare 65.1% (±1.4) (n = 4) in TF-1 versus 28.3% (±1.7) (n = 6) in TF1-bcl-2 cells). This reduction of apoptosis correlated well with the reduction in cytochrome c release (C.c), MMP loss (ΔψmL), and activation of caspase-3 (C-3), caspase-8 (C-8), and caspase-9 (C-9) (Fig. 5G, compare panels labeled TF-1 and TF1-bcl-2). Taken together, these results suggest that mitochondria play an important role in mediating the cell-death signal of T2E.

Involvement of TRB2 in GM-CSF Deprivation-induced Apoptosis of Human TF-1 Cells—Next, we wanted to examine whether TRB2 is involved in GM-CSF deprivation-induced apoptosis of TF-1 cells. To address this issue, we carried out experiments using siRNA that could specifically knock down TRB2 in TF-1 cells (Fig. 6A). As shown in Fig. 6, B–D, in the absence of GM-CSF, knockdown of TRB2 significantly suppressed GM-CSF deprivation-induced Bax activation (Fig. 6B, 31.0% (±2.7) (n = 3) in control versus 18.9% (±0.7) (n = 3) in TRB2 siRNA), loss of MMP (Fig. 6C, ΔψmL), activation of caspase-3, caspase-8, and caspase-9 (Fig. 6C), and apoptosis (~20% reduction at 24 h and ~25% reduction at 36 h, Fig. 6D, p = 0.0005). Altogether, these data suggest that TRB2 is involved in GM-CSF withdrawal-induced apoptosis of TF-1

FIGURE 5. TRB2 activates caspases via mitochondrial dysfunction. A, reduction of mitochondrial membrane potential (ΔψmL), a reduction in cytochrome c, caspase-8, and caspase-9 and apoptosis of T2E-expressing Ba/F3 cells. Cells transfected with the indicated expression vectors were analyzed for mitochondrial membrane potential, caspase activity, and apoptosis by flow cytometry as described under "Experimental Procedures." Shown is one representative result from three independent experiments. An-V, annexin V. B, activation of caspases in T2E-expressing TF-1 cells. The GFP+ population sorted from TF-1 cells transfected with the indicated vector were lysed and analyzed by Western blotting using antibodies specific to each protein as indicated. Arrows indicate the active/cleaved forms of caspases. PARP, poly(ADP-ribose) polymerase. C, T2E promotes oligomerization of Bax in Ba/F3 cells. Same as in A, except that GFP-positive Ba/F3 cells were sorted out and treated with DSS (+) or vehicle control (Me2SO−) before cell lysates were prepared and analyzed by Western blotting using Bax antibody. The arrow indicates the Bax dimer. D, activation of human Bax by T2E overexpression. TF-1 cells transfected with E or T2E expression vector were stained with antibody specific to the human active form of Bax (a-Bax), and analyzed by flow cytometry. Analysis was gated on the GFP population. E, mitochondria damage and Bax activation precede caspase activation in T2E-expressing cells. Same as that described for D except that electroporated cells were treated with Z-VAD-fmk (100 μM) or vehicle control (Me2SO) for 24 h before they were analyzed by flow cytometry for the indicated parameters. The data shown here are the averages (±S.D.) of two experiments done in triplicate. F, T2E induces cytochrome c (Cyto. C) release from mitochondria in Ba/F3 cells. GFP-positive populations sorted from Ba/F3 cells transfected with E or T2E expressing vectors were fractionated into cytosolic (Sup) and mitochondrial (pellet) fractions. Cell lysates from each fraction were then analyzed by Western blotting using antibodies specific to each protein as indicated. G, reduction of T2E-induced apoptosis and related events by Bcl-2. Both TF-1- and Bcl-2-overexpressing TF1-bcl2 cells were transfected and analyzed essentially as described in A. The data shown here are the averages (±S.D.) of three independent experiments. Cc, cytochrome c release; determined by reduction of staining with anti-cytochrome c antibody. The transfection efficiency for TF-1 and its derivatives (judged from GFP+ cell numbers) was 1.5–4% (for T2E) and 10–12% (for E).
cells and that the apoptotic signaling pathway triggered by the T2E fusion protein is to some extent, if not all, reflects that induced by the TRB2 protein itself.

**TRB2 Expression Triggers Mcl-1 Degradation—**Bcl-2 family members are known to be the major players in MMP homeostasis and can intricately regulate mitochondria-dependent apoptosis. GM-CSF-deprivation of TF-1 cells was also shown to down-regulate the expression of pro-survival Mcl-1 protein (30). Therefore, we examined whether TRB2 can modulate the protein levels of any Bcl-2 family proteins. To address this issue, GFP (+)-Ba/F3 cells expressing EGFP or T2E were sorted out by flow cytometry, and their cell lysates were analyzed by Western blot analysis using antibodies specifically recognizing Bcl-2, Bcl-xL, Mcl-1, or Bax. As shown in Fig. 7A, overexpression of T2E reduced the amount of the full-length Mcl-1 protein while generating a truncated product of the Mcl-1 molecule, which was implicated to have a pro-apoptotic activity in previous studies (31, 32). However, under the same conditions, the protein levels of Bcl-2, Bcl-xL, and Bax (Fig. 7A) were not altered.

On the other hand, reduction of full-length Mcl-1 and a concomitant increase of its cleavage product were observed in TF-1 cells deprived of GM-CSF (Fig. 7B). However, such a phenomenon was significantly reversed in cells whose TRB2 were specifically knock downed (Fig. 7B, compare lanes 2 and 3).

Mcl-1 degradation has been reported to be mediated by the proteasome-dependent pathway (20, 33). Furthermore, caspase 3 was shown to cleave Mcl-1 at Asp-127 and Asp-157, and the resultant products containing only the C-terminal domain of...
Mcl-1 turned out to be proapoptotic (32). Therefore, we next examined whether proteasome or caspases might be involved in TRB2-induced Mcl-1 degradation and/or cleavage. To address this issue, Ba/F3 cells transiently transfected with EGFP or T2E-expressing vector were cultured in medium containing MG132 (proteasome inhibitor) or Z-VAD-fmk (a pan-caspase inhibitor). As shown in Fig. 7C, T2E-induced degradation of full-length Mcl-1 and generation of the ~22-kDa cleavage product were markedly prevented by treatment of cells with Z-VAD-fmk (compare lanes 1 and 3). In contrast, in the presence of MG132, T2E still efficiently induced the degradation of MG132-stabilized full-length Mcl-1 and enhanced Mcl-1 cleavage (compare lanes 2 and 5 of Fig. 7C). Of note, MG132 alone under our experimental conditions (treatment for 16 h) slightly activated caspase-3, which might lead to the generation of the background level of the ~22-kDa cleavage product of Mcl-1. The T2E-induced degradation/cleavage of Mcl-1 in the presence of MG132 was specific, as such an effect was not observed for the tumor suppressor protein p53, whose stability was also markedly enhanced by MG132 (Fig. 7C, compare lanes 2 and 5 of the p53 panel). Taken together, these results suggest that TRB2 expression leads to cleavage and degradation of Mcl-1 via a caspase-dependent but proteasome-independent mechanism. Finally, we examined whether overexpression of Mcl-1 or a constitutively active form of Akt (myrAkt), which could up-regulate the expression of Mcl-1 (21), would protect cells from TRB2-induced cell death. As shown in Fig. 7D, transfection of pTRB2-EGFP alone into Ba/F3 cells caused apoptosis in 77.6% (±6.7) (n = 6) of GFP(+) cells in IL-3-containing medium. However, under the same conditions co-expression of myrAkt or mMcl-1 reduced the extent of apoptosis to 31.2% (±2.3) (n = 6) (Fig. 7D, lane 3) and 24.0% (±4.0) (n = 6) (lane 4), respectively. These results suggest that enforced overexpression of Mcl-1 can significantly overcome the apoptogenic activity of TRB2.

DISCUSSION

In this study, we demonstrate that the TRB2 gene is induced in TF-1 cells in the absence of their survival cytokines. However, removal of cytokines from most other cytokine-dependent cell lines tested in this study does not induce TRB2, although under such conditions they all undergo apoptosis. This result suggests that the cellular context plays an important role in determining TRB2 induction. It further suggests that other factors functionally similar to TRB2 may be involved in the factor deprivation-induced apoptotic program in other factor-dependent cells. In fact, earlier studies on two factor deprivation-induced pro-apoptotic molecules, 24p3 and RC3 (17, 18), support this notion. Although 24p3 is induced by cytokine deprivation of some IL-3-dependent cells, it is not induced by withdrawal of survival factor from IL-2 dependent HT-2 or IL-7-dependent D1-F4 cells (17). On the other hand, although rc3 is induced preferentially by IL-2 deprivation of IL-2-dependent primary human T lymphocytes and HT-2 and CTLL-2 cell lines, it is not induced upon removal of IL-3 from FL5.12 and 32D or IL-7 from D1-F4 cells (18). These results suggest that expression of TRB2, 24p3, or RC3 is likely to be regulated in a cytokine- and cell context-specific manner.

The induction of TRB2 upon IL-2 withdrawal from the activated primary CD4+ T lymphocytes is intriguing, as it may suggest that TRB2 is involved in the apoptosis regulation of activated T cells and may thus play some roles in homeostasis regulation of some T lymphoid progenitor cells. Along this line of reasoning, the observed induction of TRB2 in TF-1 cells may simply be due to the possibility that TF-1 cells still retain some properties of T lymphoid progenitors, because it was originally isolated from the bone marrow of patients with erythroleukemia (34).

To further address the role of TRB2 in the T cell system, we have generated the TRB2-deficient mice using the conventional gene targeting approach (which will be reported elsewhere). Our initial characterization of these mutant mice revealed that lack of TRB2 expression does not significantly affect the survival of activated T cells following IL-2 deprivation. Several possibilities may account for this negative result. For example, TRB2 does not play a role in T cell apoptosis in vivo or the existence of a redundant apoptosis pathway after IL-2 deprivation of the activated T cells, e.g. the RC3-dependent pathway (17, 18). Alternatively, a compensatory increase of a TRB2-related protein, e.g. TRB3 in the TRB2 knock-out mice, could have accounted for the observed negative result. More experiments are required to distinguish these possibilities.

Of note, the TRB2-EGFP fusion protein (T2E) appeared to have a better apoptotic activity than the wild-type TRB2 molecule, as the fusion protein can overcome the survival activity of IL-3 and trigger apoptosis of Ba/F3 cells cultured in IL-3-containing medium, whereas the wild-type TRB2 molecule can only enhance IL-3 withdrawal-induced apoptosis of the same cells. Comparison of the expression levels of these two proteins revealed that, under the same transfection conditions, the wild-type TRB2 protein was expressed at a comparable (without IL-3) or slightly higher level (with IL-3) than the EGFP fusion protein (data not shown). Although these results suggest that the T2E fusion protein may have gained some extra functions than the wild-type protein, our TRB2 siRNA knockdown experiments strongly suggest that the majority, if not all, of the observed apoptotic effects of the T2E fusion protein come qualitatively from the TRB2 molecule itself. Based on the fact that wt-TRB2 manifests no activity or marginal apoptotic activity in the presence of survival cytokine, but can enhance apoptosis under conditions when cell death is initiated by removal of these survival cytokines, we propose the following “latency-and-activation” model. In this model, we propose that TRB2 may exist as a latent apoptotic molecule when survival cytokines are present in the growth environment. Upon deprivation of these survival cytokines, the latent molecule then becomes activated via a yet-to-be-identified mechanism. Given that the T2E fusion protein can trigger apoptosis in the presence of survival cytokines, we propose that a conformational alteration of TRB2 has been generated as a result of protein fusion, which prematurely converts the latent state of TRB2 into an active conformation. One possible active conformation of TRB2 is to become a dimer or oligomer, as EGFP has been reported to have

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a dimerization ability (35). More experiments are required to test this model.

Other than the latency-and-activation property as described above, we also noticed that manipulation of the wild-type TRB2 levels does not cause a dramatic effect on cellular apoptosis, i.e. enforced expression of TRB2 in Ba/F3 cells increases cell death about 2-fold, whereas siRNA knockdown of TRB2 in TF-1 cells decreases apoptosis by about 50%. These data suggest that TRB2 is likely to be a modulator of apoptosis, rather than a key component in the induction of the apoptosis cascade.

Bcl-2 (36–38) and Bcl-xL (39) were reported to be cleaved during cell death. However, TRB2 selectively induces degradation/cleavage of Mcl-1 in our system. How TRB2 triggers such a response is not clear. Given that Drosophila Tribbles target String/CDC25 and Slbo for ubiquitin-dependent proteasome degradation (3, 4), it is possible that human TRB2 induces Mcl-1 degradation via a similar pathway. However, in this study we found that TRB-2-induced degradation of full-length Mcl-1 and generation of an ~22-kDa cleavage product is mediated via a caspase-dependent but proteasome-independent pathway. More experiments are needed to delineate the molecular mechanism by which TRB2 expression leads to caspase activation and Mcl-1 cleavage/degradation. On the other hand, one common feature of Tribbles proteins is that many of them have been shown to bind to and inhibit specific kinases, including Akt/PKB (7), MEK-1, M KK4, and M KK7 (10). Akt/PKB and MEK-1 have been shown to regulate Mcl-1 protein levels and play an important role in mediating survival signals of many cytokines in hematopoietic cells (30, 40). Moreover, Du et al. (7) reported that TRB2 can suppress the Akt kinase activity. It is therefore possible that TRB2 may modulate cellular levels of Mcl-1 indirectly via inhibiting a kinase like Akt. More experiments are required to test this possibility.

Intriguingly, although TRB2 has a very potent cytotoxic effect in most factor-dependent cells, it causes no harm to most adherent cells or some leukemic cell lines tested in this study. This cell type-restricted cytotoxicity of TRB2 has a strong resemblance to that of a secreted lipocalin, 24p3 (17), which promotes apoptosis in many leukocytic cell lines, primary lymphocytes, and neutrophils, but not in nonhematopoietic cells or macrophages (17). Given that the oncogenic proteins, including Bcl-2 and the constitutively active form of Akt, are able to alleviate the strong apoptotic signals of TRB2, it is possible that in many cancer cells Bcl-2 and Akt oncoproteins are often over-expressed, which are able to confer a strong anti-apoptotic effect to reverse the adverse effect of TRB2. Likewise, focal adhesion through the β1 integrin provides strong survival signals in fibroblasts via activation of the integrin-linked kinase and Akt/PKB pathway (41) or protects keratinocytic epithelial cells from apoptosis by activation of the focal adhesion kinase and ERK (extracellular signal-regulated kinase) pathway (42). Therefore, in this study, fibroblastic and epithelial cell lines are likely protected from the intrinsic cell death signal of TRB2 by cell adhesion to the extracellular matrix. These data suggest a recessive nature of the apoptosis signal induced by TRB2, as it appears to be suppressed by dominant survival activities generated by intracellular oncogenic proteins or by cell adhesion to various extracellular matrices.

In contrast to the pro-apoptotic property reported in this study, Keeshan et al. (13) demonstrated that trb2 could function as an oncogene that induces acute myelogenous leukemia in hematopoietic progenitors. It is not clear what has caused these two seemingly opposite functions of TRB2, but below is one likely interpretation. In the report by Keeshan et al. (13), wild-type murine TRB2 was expressed from an IRES-EGFP-containing MigR1 vector. In this study, we demonstrate that wt-TRB2 enhances apoptosis only in the absence of survival cytokine as described in our latency and activation model. Bone marrow (BM) is in an environment that is rich in many survival cytokines. BM progenitors may thus be protected from TRB2-induced apoptosis. Under such conditions, other functions of TRB2 may then be revealed. Furthermore, TRB2 may manifest distinct activities in a cellular context-specific manner. In our experimental system, TRB2 down-regulates Mcl-1 levels and induces apoptosis. However, in BM progenitors as used in the study by Keeshan et al. (13), TRB2 may predominantly affect the processing of C/EBPα from the p42 to the p30 form, which may confer proliferation advantage on the TRB2-transduced BM progenitor cells. More experiments are certainly required to test this possibility.

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