The lymphoid protein T-cell ubiquitin ligand (TULA)/suppressor of T-cell receptor signaling (Sts)-2 is associated with c-Cbl and ubiquitylated proteins and has been implicated in the regulation of signaling mediated by protein-tyrosine kinases. The results presented in this report indicate that TULA facilitates T-cell apoptosis independent of either T-cell receptor/CD3-mediated signaling or caspase activity. Mass spectrometry-based analysis of protein-protein interactions of TULA demonstrates that TULA binds to the apoptosis-inducing protein AIF, which has previously been shown to function as a key factor of caspase-independent apoptosis. Using RNA interference, we demonstrate that AIF is essential for the apoptotic effect of TULA. Analysis of the subcellular localization of TULA and AIF together with the functional analysis of TULA mutants is consistent with the idea that TULA enhances the apoptotic effect of AIF by facilitating the interactions of AIF with its apoptotic co-factors, which remain to be identified. Overall, our results shed new light on the biological functions of TULA, a recently discovered protein, describing its role as one of very few known functional interactors of AIF.

We recently identified TULA among multiple proteins that co-purified with c-Cbl from T-lymphoblastoid cells (1). TULA contains an N-terminal UBA domain, a centrally positioned SH3 domain, and a region of homology to phosphoglyceromutases, which was initially termed HCD (Fig. 1) (1, 2). TULA binds to c-Cbl through its SH3 domain and to ubiquitin and ubiquitylated proteins through its UBA domain (1, 3). Dimerization of TULA through its phosphoglyceromutase domain has also been shown (3). Analysis of cell and tissue expression of TULA demonstrates that this protein is expressed primarily in T and B lymphocytes and is localized both in the cytoplasm and nucleus (1, 4).

A mouse orthologue of TULA (Sts-2) was recently identified (4), as was a second member of the family, Sts-1 (5). Unlike TULA, Sts-1 is expressed ubiquitously (4, 5). (In this report we will use the term TULA for consistency.)

TULA has been implicated in the regulation of cell signaling mediated by protein-tyrosine kinases. On the one hand, TULA was reported to increase activity of receptor protein-tyrosine kinases by inhibiting c-Cbl-driven down-regulation of their activated forms. This appears to be mediated by preventing interactions between ubiquitylated forms of activated protein-tyrosine kinases and proteins recruiting them to the degradation pathway and, possibly, by decreasing the level of c-Cbl (1, 3). On the other, the lack of both proteins of the TULA/Sts family resulted in hyper-reactivity of T lymphocytes correlated with an increase in the activity of Zap-70, the molecular basis of which remained unclear (4). These results implied that the effect of TULA on protein-tyrosine kinases might not be the only mechanism through which TULA exerts its biological effect. Indeed, the presence in TULA of multiple functional domains and extensive stretches of amino acid sequences with unknown functions suggested that TULA might exert effects unrelated to either c-Cbl or protein-tyrosine kinases.

In an effort to discover novel functions of TULA, we purified proteins that interact with TULA and identified among them apoptosis-inducing factor (AIF). AIF is a key factor of caspase-independent apoptosis (6–8). In the absence of cellular stress signals, AIF is localized to the internal mitochondrial membrane, where it functions as a FAD-dependent NADH oxidase, which is required for normal oxidative phosphorylation (9) and maintenance of mitochondrial structure (10). Under conditions inducing apoptosis, AIF is released from mitochondria (11–14) and translocated to the nucleus, where it induces caspase-independent apoptotic events through binding to
DNA (15). These two functions of AIF are mediated by distinct structural domains (15, 16) and can be dissociated (6, 10, 17).

Overall, the molecular mechanism of the apoptotic effect of AIF remains poorly understood, and in particular, few functional interaction partners of AIF have been identified (18–21). Our work, presented here, demonstrates that TULA and AIF are interaction partners and establishes a functional link between them in inducing caspase-independent apoptosis. These results shed new light on the mechanism of the apoptotic effect of AIF and reveal a novel biological function of TULA.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and Mutagenesis**—cDNA encoding the full-length TULA or its N-terminal half (TULA-N1/2) was subcloned into the pFLAG 5a vector (Sigma) using the Advantage-HF2 polymerase (Clontech). The forward primer (5'-CAGGATATCATGGCAGGGGGAGG-3') annealed to nucleotides at the N-terminal end of TULA and included a unique EcoRV restriction site. The reverse primers (5'-TAGGGTACCATCCGTTAGTTTTCC-3' and 5'-TAGGGTACCCGTTGCTTAGATCAGGATT-3') annealed to nucleotides 893 to 908 (TULA-N1/2) or 1863 to 1880 (full-length TULA) within the TULA short (1) protein sequence and included a unique KpnI restriction site. These restriction sites were included to create compatible ends for ligating the fragments into the pFLAG 5a vector. The obtained constructs were confirmed by sequencing.

To introduce mutations, two synthetic oligonucleotides complementary to the opposite strands of double-stranded DNA containing the sequence to be mutated were designed to contain 15–18 nucleotides on either side of the mutation site. The oligonucleotides were gel purified (IDT Technologies, Coralville, IA). The mutagenesis reactions were performed using the QuikChange site-directed mutagenesis kit according to the manufacturer's recommendations (Stratagene, La Jolla, CA).

**Cells**—HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) (complete medium). HEK293T cells were plated 24 h before transfection to be 80% confluent on the fetal bovine serum (FBS) (complete medium). HEK293T cells with 20 mM HEPES, 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (complete medium). These cells were grown in antibiotic-free medium for 24 h prior to electroporation. Cells were centrifuged and resuspended at a final density of 2 × 10^7 cells/ml in antibiotic-free medium. DNA (10 μg) was added to a 4-mm cuvette followed by addition of 1 × 10^7 cells in 500 μl of medium. The mixture was pulsed at 310 V for 10 ms in an electroporator (ECM 830 from BTX, Holliston, MA). After electroporation, cells were cultured in complete medium for 48 h. The efficiency of electroporation was ~70%.

In several experiments Jurkat tag cells were transfected using DMRIE-C (3 μg of DNA/5 × 10^6 cells) according to the manufacturer's recommendations. Because the efficiency of DMRIE-C-mediated transfection did not exceed 10%, a GFP-encoding expression plasmid (pEGFP-C2, Clontech) was co-transfected in each sample at a ratio of 1:15 to the total DNA, and only GFP^+ cells were analyzed using flow cytometry. Stable Jurkat cells with a reduced TULA expression level and the corresponding control cells were generated using the shRNA-encoding or empty control lentiviral vector (1).

**Z-VAD-fmk and Z-IETD-fmk** (Biomol, Plymouth Meeting, PA) and camptothecin and etoposide (Sigma) were added to final concentrations of 100, 4, 5, and 10 μM, respectively. Growth factor withdrawal of Jurkat tag cells was carried out in medium supplemented with 0.5% FBS. For anti-CD3 stimulation, wells of a 24-well plate were pre-coated with the mouse monoclonal antibody OKT3 at 10 μg/ml in PBS overnight at 4°C.

**Isolation of TULA-associated Proteins**—1–3 μg of total protein from FLAG-TULA-expressing or vector-transfected HEK293T cells was incubated with 20 μl of anti-FLAG M2 affinity gel (Sigma) and incubated at 4°C for 4 h. The beads were washed three times with lysis buffer, and anti-FLAG-bound proteins were eluted from the beads with 0.1 M glycine (pH 3). Proteins eluted from the anti-FLAG beads were separated on a one-dimensional BisTris minigel and stained in Simply Blue Coomassie (Invitrogen). Each gel lane was divided and cut into 10 equal-sized gel slices. Proteins contained within each slice were equilibrated in 100 mM ammonium bicarbonate and reduced, alkylated, and digested with trypsin as previously described (22). One-tenth of each unfractionated tryptic digest was analyzed by LC-ES MS/MS using a micro-column (Zorbax C18, 75 mm × 12 cm) reverse-phased HPLC interfaced with an Agilent LC-MSD Ion Trap MS. ES MS/MS-based sequencing was performed on-line in a data-dependent manner, and two tandem mass spectra were taken per survey scan as peptides eluted from the HPLC (23). Uninterpreted mass spectra from each of the 10 individual liquid chromatography-tandem mass spectrometry (LC-MS/MS) runs were collated and searched as a single file against a human nonredundant protein data base using the Mascot search engine (Matrix Science) (24). Errors used were 2.0 Da on MS data and 0.8 Da on MS/MS data.

**Immunoprecipitation and Immunoblotting**—1–3 μg of total protein from whole cell lysate was immunoprecipitated with 1–3 μg of anti-TULA-N (GETQLYAKVSNKLKSRSSPS) (Proteintech Group Inc., Chicago, IL) in a total volume of 1 ml as described previously (1). Then proteins were separated using SDS-PAGE, transferred to nitrocellulose, and probed with 1:1000 anti-FLAG M2 (Sigma), 1:1000 anti-TULA-N, or 1:500 anti-AIF (Santa Cruz Biotechnology, Santa Cruz, CA). After blots were washed, the appropriate peroxidase-conjugated secondary antibody was added, and proteins were visualized using the ECL Plus Kit and the Typhoon Fluorescent Imager (GE Healthcare).
Annexin-V Staining—Electroporated Jurkat tag cells were washed and resuspended in 100 μl of annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4). Then 5 μl of 0.1 mg/ml propidium iodide and 5 μl annexin-Vallophycocyanin conjugate (Molecular Probes, Eugene, OR) were added to the cells. After cells were incubated for 15 min at room temperature, 400 μl of annexin binding buffer was added, and cells were analyzed using flow cytometry. DMRIE-C-transfected Jurkat tag cells and TULA-knockdown Jurkat cells were analyzed using an annexin V-Cy5 apoptosis kit from Biovison (Mountain View, CA).

Transfection of Small Interfering RNAs (siRNAs)—To deplete endogenous AIF and simultaneously overexpress TULA, a 21-mer annealed AIF-targeting siRNA and scrambled control (Ambion, Austin, TX) were resuspended in water at a final concentration of 100 μM. The sense sequence of the AIF-specific siRNA corresponded to nucleotides 1540–1558 in the AIF sequence. (Several AIF-specific siRNAs were tested in pilot experiments, and this siRNA was selected as the most efficient one.) siRNA was electroporated into Jurkat tag cells (100 nM siRNA and 1 × 10⁵ cells in 75 μl of Opti-MEM (Invitrogen)) using 1-mm cuvettes in the BTX Electroporator at 150 V for 100 μs. To simultaneously electroporate siRNA and DNA, FLAG-TULA expression or control plasmid (2 μg) was added to siRNA. After recovery in complete medium for 48 h, transfected cells were either cultured in complete RPMI1640 medium or subjected to serum deprivation in RPMI1640 supplemented with 0.5% FBS for an additional 24 h. At that time overall cell death was measured using trypan blue exclusion. To deplete endogenous TULA, the same electroporation procedure was done using TULA-specific siRNA SMARTpool L-008616-00 from Dharmacon (Lafayette, CO).

Subcellular Distribution—To obtain immunofluorescence images, HeLa cells were seeded onto fibronectin-coated coverslips (BD Biocoat) at a confluence of 50% in Dulbecco’s modified Eagle’s medium containing 10% FBS without antibiotics. On the following day, the cells were transfected to express FLAG-TULA and/or Myc-AIF (3 μg of each construct per coverslip) using FuGENE 6 as per the manufacturer’s recommendations. Forty-eight hours post-transfection the cells were washed, fixed with 4% paraformaldehyde in PBS, washed again, and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. Cells were blocked with 1% bovine serum albumin and washed twice with PBS. Fluorescein isothiocyanate-conjugated anti-FLAG (5–10 μg/ml) and Cy3-conjugated anti-Myc (1 μg/ml) (Sigma) were added as appropriate. The antibodies were incubated with the cells overnight at 4 °C in the dark. The cells were washed three times with PBS before mounting the coverslips onto a slide with anti-fade mounting solution including 4',6-diamidino-2-phenylindole stain (Molecular Probes). Cell images were obtained using the Leica DM IRE2 confocal microscope with a ×100 objective.

For subcellular fractionation, 293T cells were transfected with either empty or TULA expression vector (10 μg/75-cm² flask) using Lipofectamine 2000. Subcellular fractions were obtained from transfected cells at 48 h post-transfection using a Qproteome Cell Compartment kit (Qiagen).

RESULTS

AIF Is a Novel TULA Interacting Protein—To search for novel functions of TULA we sought to identify TULA interaction partners via a proteomics approach. For this purpose, FLAG-tagged full-length TULA and TULA-N1/2 (1–299), a truncation mutant lacking the C-terminal half, but containing both binding domains of TULA (UBA and SH3) (see Fig. 1), were transiently overexpressed in HEK293T cells and immunoprecipitated with anti-FLAG antibody. The eluted immune complexes were separated by SDS-PAGE and proteins associated with these forms of TULA were identified using LC-ES MS/MS. Several proteins were identified in the TULA and TULA-N1/2 immunoprecipitates and not in the vector control, and one of these was c-Cbl (8 unique peptides), a previously characterized TULA-interacting protein (1, 3). A second protein identified with 15 unique peptides was AIF. Originally, we identified AIF only in the TULA-N1/2 immunoprecipitates. However, the molecular mass of AIF suggested that it co-migrates with full-length TULA, which is a very large band on the SDS-PAGE gel. Because co-migration with TULA was likely to hinder identification of AIF in this system, we targeted four unique AIF peptides for mass spectrometry-based sequencing in the gel band corresponding to full-length TULA and identified AIF from all four peptide sequencing events. We performed these experiments using TULA and TULA-N1/2 in triplicate, and AIF was identified each time with more than 10 peptides in each trial (supplemental Table S1). Interestingly, c-Cbl was only identified in the immune complexes with full-length TULA.

To verify association of TULA and AIF and to identify the region of TULA involved in AIF binding, we transiently overexpressed TULA and TULA mutants in HEK293T cells, immunoprecipitated them, and analyzed the obtained immune complexes using Western blotting. Consistent with the mass spectrometry results, co-immunoprecipitation of AIF was clearly detectable (Fig. 2A). Immunoblotting also showed that TULA-N1/2 binds to AIF better than full-length TULA. To assure that the difference in the amount of co-immunoprecipitated AIF was not due to differences in the cellular levels of AIF in cells overexpressing full-length TULA and TULA-N1/2 (as well as other TULA mutant forms, see below), we immuno-
AIF has several putative SH3-binding motifs (PXXXP including PSTPAVPQAP, we hypothesized that TULA binds to AIF through the SH3 domain. However, the mutant form of TULA lacking a functional SH3 domain as a result of the W279L point mutation bound AIF with the same efficiency as wild-type TULA did (Fig. 2A). Furthermore, the SH3-deleted forms of both TULA-N1/2 and full-length TULA bound AIF to the extent characteristic of the binding of AIF by TULA-N1/2 (data not shown). Likewise, deletion of the UBA domain had no effect on AIF binding (Fig. 2A). Finally, the TULA-C1/2 truncated form (amino acids 300–623) did not bind to AIF (data not shown). Taken together these findings indicate that the N-terminal half of TULA is necessary for AIF binding, but that neither the SH3 nor the UBA domain is critical.

Because c-Cbl is well characterized as a TULA binding partner in T cells, we examined its binding to TULA relative to AIF. Interestingly, high binding of c-Cbl to various forms of TULA was invariably linked to the low AIF binding to them and vice versa (Fig. 3), thus being in agreement with the findings of our mass spectrometry based experiments (see above). This mutual exclusion is unlikely to be due to a direct competition of c-Cbl and AIF for the same binding site, because c-Cbl binds to the SH3 domain of TULA (1), which is dispensable for AIF binding (see Fig. 2). It is more likely that c-Cbl and AIF bind to alternative conformation states of TULA or induce such states upon binding.

We also evaluated the possibility that AIF binds to the TULA homologue Sts-1/TULA-2. Both proteins were co-expressed in 293T cells, Sts-1 was immunoprecipitated and the obtained immune complexes were analyzed using Western blotting. Co-immunoprecipitation of AIF and TULA-2 was not detected (data not shown), despite their high levels of expression, indicating that the interaction of TULA with AIF is specific for this particular family member.

**TULA Facilitates T-cell Apoptosis**—Our multiple attempts to generate stable TULA-overexpressing T- or B-cell lines using lentiviral transduction have failed, despite the generally high success rate for the lentiviral system used (25, 26) and the fact that vector control stable transductants were consistently generated (data not shown), suggesting that constitutive high expression of TULA is detrimental for cell viability. Because AIF has been shown to be a key factor of caspase-independent apoptosis, we decided to explore the effect of TULA expression on T-cell apoptosis and to analyze the functional link between TULA and AIF in this event.

First, we analyzed the effect of reducing endogenous levels of TULA on T-cell apoptosis. A stable variant of Jurkat cells with a reduced level of TULA ("TULA-knockdown") was generated using a shRNA-encoding lentiviral vector (1). In these cells, the level of TULA protein was reduced ~4-fold as compared with the parental cells and cells expressing a control shRNA (Fig. 4A). (A decrease in TULA mRNA in these cells has been shown in our previous report (1).) Apoptosis in the TULA knockdown and control cells was induced using anti-CD3 (a T cell-specific apoptotic stimulus mimicking biological TCR/CD3-mediated signaling), etoposide (an apoptosis-inducing drug), and growth

![FIGURE 2. Co-immunoprecipitation of TULA and AIF. 293T cells were transfected with wild-type (WT) and various mutant forms of TULA (10 μg of each construct per 75-cm² flask, except for TULA-N1/2, which was transfected at a dose of 20 μg/75-cm² flask) using Lipofectamine 2000. Control cells were transfected with the empty vector at a dose equal that of the expression construct per 75-cm² flask, except for TULA-N1/2, which was transfected at a dose equal that of the expression vector per 75-cm² flask. Cells were lysed 48 h after transfection. Cell lysates were subjected to immunoprecipitation (IP) with an indicated antibody (NRS, normal rabbit serum) (A) or analyzed as whole cell lysates (WCL) (B). Antibodies used for Western blotting (WB) are indicated. The proteins detected are indicated by arrowheads at the right. The molecular weight markers are indicated at the left.](image1)

![FIGURE 3. Binding of TULA to AIF and c-Cbl. 293T cells were transfected with various mutants of TULA as described in the legend to Fig. 2. Cells were lysed 48 h after transfection. Cell lysates were subjected to immunoprecipitation with anti-TULA. Immunoprecipitates were analyzed using Western blotting (WB) with the antibodies indicated. The proteins detected are indicated by arrowheads at the right. The molecular weight markers are indicated at the left.](image2)
factor withdrawal (serum deprivation was used, because Jurkat cells are interleukin-2-independent). Early apoptosis of Jurkat cells was assessed using annexin-V staining indicative of phosphatidylserine exposure on the outer leaf of the plasma membrane. Results from these experiments show that TULA expression is critical for apoptosis induced by serum deprivation, but not by anti-CD3 or etoposide treatment (Fig. 4B).

To ascertain that the effect of TULA shRNA was not due simply to clonal variability, we determined whether depletion of TULA achieved using transient transfection of TULA-targeting siRNA (Fig. 4C) would produce a similar effect. These experiments indicated that TULA-specific siRNA substantially reduces serum deprivation-induced cell death (Fig. 4D). The finding that the effect of transient transfection is somewhat lower than that of stable TULA-specific shRNA expression is likely to be explained by a higher residual level of TULA in siRNA-treated cells as compared with shRNA-expressing cells (Fig. 4, A versus C).

To further establish a role for TULA in T-cell apoptosis and compare the pro-apoptotic potential of mutant forms of TULA, we employed an independent experimental approach, transiently overexpressing wild-type TULA and its mutants lacking known functional domains in Jurkat cells and comparing sensitivity of these cells to apoptosis induced with anti-CD3 or camptothecin. The percentage of cells undergoing apoptosis in the control and the camptothecin-treated cells increased substantially when wild-type TULA was overexpressed, although no synergism between TULA overexpression and camptothecin treatment was observed. In contrast, overexpression of TULA did not significantly modify sensitivity of Jurkat cells to anti-CD3-induced apoptosis; they were highly sensitive regardless of TULA overexpression (Fig. 5A). These results taken together with those shown in Fig. 4 indicate that the pro-apoptotic effect of TULA is not linked to TCR/CD3-mediated signaling.

These experiments also demonstrated that TULA mutants lacking the UBA domain or a functional SH3 domain were unable to facilitate T-cell apoptosis despite being expressed at a level comparable with that of TULA (Fig. 5A, bottom panel). Considering that proteins of a single family may have similar functions, we evaluated the effect of Sts-1 (TULA-2) on apoptosis of Jurkat cells. Unlike TULA, its ubiquitous homologue did not facilitate apoptosis either in untreated cells or in cells treated with camptothecin (Fig. 5B). This result indicates that the pro-apoptotic effect of TULA is specific for this family member.
TULA Exerts Its Apoptotic Effect through AIF—The results shown in Figs. 4 and 5 indicate that sensitivity of Jurkat cells to TCR/CD3-mediated apoptosis remains unchanged by either knockdown or overexpression of TULA, thus ruling out the possibility that TULA affects apoptosis by regulating TCR/CD3 signaling. Given that TULA and AIF bind in vivo and that AIF induces apoptosis, we considered the possibility that TULA exerts its pro-apoptotic effect through AIF.

First, we addressed this issue by evaluating caspase dependence of the observed effect of TULA, because TCR/CD3-induced apoptosis requires caspase activation (29–32), whereas AIF has an established role in caspase-independent apoptosis (6–8). To determine whether or not caspases play a role in the pro-apoptotic effects of TULA, we determined the effects of Z-VAD, a pan-caspase inhibitor, on cell death in our experimental system. These experiments indicated that inhibition of caspases reduces death of TULA-overexpressing Jurkat cells under basal conditions (Fig. 6 A) or under serum deprivation (Fig. 6 B), whereas no such effects were observed under stressed culture conditions using confocal microscopy. Under normal conditions, no significant co-localization of TULA and AIF was detectable (Fig. 8). However, their co-localization became apparent in the cytoplasm when cells were subjected to staurosporine treatment, which induces apoptosis mediated by the release of pro-apoptotic factors increase. Consistent with a cooperative role of these proteins in apoptosis, a decrease in AIF expression significantly inhibited the effect of TULA overexpression on serum deprivation-induced cell death (Fig. 7). (It should be noted that in this system TULA overexpression exerted only a minor pro-apoptotic effect in complete medium (cell death was 15.8 ± 3.1 and 21.0 ± 1.1% for vector control and TULA-overexpressing cells, respectively), and therefore, serum deprivation was essential for revealing the observed effects. This variation between the results shown in Figs. 5 and 7 is probably due to the differences between the basal levels of cell death in the systems utilized; it is lower for transfected cells (Fig. 5) than for electroporated cells (Fig. 7).)

Taken together, our results indicate that TULA physically interacts with AIF and exerts its pro-apoptotic effect in an AIF-dependent fashion. However, it remains unclear how the interaction of TULA and AIF facilitates apoptosis, because these proteins appear to reside in different compartments inside the cell; AIF is localized to mitochondria under normal physiological conditions (6–8, 15, 33, 34), but after apoptotic stimulation it is released to the cytosol and subsequently translocates to the nucleus (11–14), whereas no mitochondrial localization has been shown for TULA. The latter is localized primarily to the cytoplasm with some amount of it being present in the nucleus (1). To determine whether or not AIF and TULA can co-localize, we transiently co-expressed epitope-tagged constructs of both proteins in HeLa cells and examined their localization under normal and stressed culture conditions using confocal microscopy. Under normal conditions, no significant co-localization of TULA and AIF was detectable (Fig. 8). However, their co-localization became apparent in the cytoplasm when cells were subjected to staurosporine treatment, which induces apoptosis mediated by the release of pro-apoptotic factors...
from mitochondria (Fig. 8). The apoptosis induction under these conditions is visualized by considerable morphological disturbances of the nuclei in staurosporine-treated cells. The lack of co-localization of TULA and AIF prior to stress treatment argues that TULA is unlikely to facilitate apoptosis by promoting the release of AIF from mitochondria.

To address this issue directly, we determined the effect of TULA overexpression on subcellular localization of AIF. HEK293T cells were transfected with wild-type and mutant forms of TULA, and distribution of AIF was examined using subcellular fractionation followed by Western blotting. AIF is largely localized to the membrane in a manner consistent with its mitochondrial targeting, whereas a very small fraction of it is present in the cytosol and in the nucleus (Fig. 9A), probably due to the transfection-induced cell stress, and not to cross-contamination of fractions, which was minimal (Fig. 9B). This pattern of subcellular distribution of AIF was not affected by overexpression of any form of TULA (Fig. 9C). Therefore, the results shown in Figs. 8 and 9 argue that (a) the functional interaction of TULA with AIF occurs when AIF has already been released from mitochondria and (b) translocation of AIF to the nucleus is not the major target of the pro-apoptotic effect of TULA.

**DISCUSSION**

Taken together, our results obtained using several independent approaches demonstrate that TULA is involved in T-cell apoptosis (Figs. 4 and 5). Furthermore, our results indicate that TULA affects apoptosis through a mechanism independent of either TCR/CD3-mediated signaling or caspase activation (Figs. 4–6). Finally, we have shown that TULA binds to AIF (Fig. 2, supplemental Table S1), a key factor of caspase-independent apoptotic events. Binding of AIF to TULA together with the caspase independence of the pro-apoptotic effect of TULA have provided an initial argument in favor of the idea that TULA affects apoptosis through its interaction with AIF.

Indeed, further experiments indicated that AIF is essential for the pro-apoptotic effect of TULA (Fig. 7). Notably, the effect of AIF-specific siRNA on the pro-apoptotic activity of TULA was higher than its effect on the protein level of AIF. This apparent discrepancy may reflect the fact that the pro-apoptotic effect of TULA is highly sensitive to the level of AIF or that siRNA differentially affects the level of AIF in various cellular compartments, i.e. the fraction of AIF that mediates the effect of TULA may be depleted more than others. It should also be noted that AIF depletion reduced not only TULA-facilitated apoptosis, but also apoptosis in the absence of overexpressed TULA. This may be due to the role endogenous TULA plays in the basal apoptosis in Jurkat cells.

The essential role of AIF in TULA-facilitated apoptosis and the physical interaction of TULA with AIF, taken together, strongly suggest that binding of TULA to AIF is crucial for the pro-apoptotic effect of TULA. Indeed, overexpression of the TULA mutant lacking the N-terminal domain (TULA-C1/2) and incapable of binding to AIF does not promote apoptosis in Jurkat cells and even decreases it (data not shown). This finding is consistent with the notion that TULA-AIF binding is essential for the pro-apoptotic effect of TULA, but may also be explained by the lack of TULA UBA and SH3, which are essential for this effect, whereas not required for TULA-AIF binding (Fig. 2). However, because our mutational studies implicated multiple sites within the N-terminal half of TULA in binding to AIF, thus not permitting us to obtain a specific mutant of TULA defective in AIF binding, but fully functional otherwise (data not shown), evidence of the essential role of TULA-AIF interactions in the effect of TULA based on the mutational disruption of these interactions remains to be provided.

Several findings presented in this report allow us to outline the molecular basis of the pro-apoptotic effect of TULA. First of all, the caspase-independent nature of the effects of TULA argues that TULA does not act by permeabilizing mitochondrial membrane, because this would result in the release of multiple apoptotic factors acting through caspases. Therefore, considering that the effect of TULA is mediated by AIF, TULA may facilitate (a) release of AIF from mitochondria, (b) transfer of AIF to the nucleus, and (c) interactions of AIF with its co-factors. Because TULA and AIF are not substantially co-localized in unstressed cells (Fig. 8) and because overexpression of TULA does not alter subcellular distribution of AIF in unstressed cells (Fig. 9), it is unlikely that TULA acts by inducing the release of
AIF from mitochondria. Likewise, the lack of a significant effect of TULA overexpression on the nuclear localization of AIF (Fig. 9) provides no support for the idea that TULA facilitates apoptosis by increasing the nuclear translocation of AIF. Therefore, it is more likely that TULA promotes AIF-dependent apoptosis by facilitating interactions between AIF and its apoptotic co-factors (6). This possibility is supported by the following findings: UBA- and SH3-deficient forms of TULA bind to AIF, but lack a pro-apoptotic effect (Figs. 2 and 5), suggesting that interactions of the UBA and/or SH3 domains of TULA with proteins other than AIF are required for this effect. Although the identity of co-factors whose binding to AIF is facilitated by TULA remains to be elucidated, our results argue that c-Cbl is unlikely to be essential for the AIF-TULA cooperation, because the ability of several forms of TULA to bind to AIF and c-Cbl showed a clear inverse correlation (Fig. 3).

Analysis of the mechanism by which TULA cooperates with AIF is hindered by the lack of clarity regarding the molecular mechanism of the apoptotic effect of AIF. It has been shown that the nuclear transfer of AIF is essential for this effect, that AIF exerts its effect through binding to DNA, and that the initial step of AIF-induced apoptosis is likely to be chromatin condensation (6–8, 15, 33, 34). However, little is known about proteins cooperating with AIF in apoptosis. Although it was suggested that endonuclease G and cyclophilin A might cooperate with AIF in apoptosis (19, 21), the involvement of these two proteins in the effect of AIF remains to be established.

As noted above, the caspase-independent nature of the pro-apoptotic effect of TULA (Fig. 6), which is consistent with the body of data related to AIF, is one argument supporting the functional cooperation between TULA and AIF in apoptosis induction. It remains unclear what needs of the cell are served by the existence of the AIF-dependent apoptotic mechanism alongside the well-characterized caspase-based mechanisms. Possibly, AIF plays a critical role in cell death in response to specific stimuli, including CD2- and CD44-mediated signaling (34, 35) and certain drugs (7, 36–43). For instance, comparison of the sensitivity of wild-type and AIF-null ES cells to various apoptotic stimuli indicated that AIF is essential for some (growth factor withdrawal), but not the other (etoposide, azide, UV), pathways of death induction (44). Similarly, TULA does not interfere with T-cell apoptosis induced by TCR/CD3 ligation, but plays a critical role in growth factor withdrawal-induced apoptosis (Fig. 4), thus suggesting that the pro-apoptotic effect of TULA is specific for certain types of cell death. It is also possible that AIF functionally cooperates with caspases in the cell death cascades, being responsible for the early stages of apoptosis (34, 39). In particular, a recent report indicates that large scale DNA degradation, which was thought to be a hallmark of the apoptotic effect of AIF, may be caspase-dependent, but agrees with the previous studies that AIF-dependent chromatin condensation is independent of caspases and represents an early step of the cell death, which precedes its caspase-dependent steps (45).

To summarize, we have demonstrated, for the first time, that TULA (Sts-2) exerts a pro-apoptotic effect and that this effect is mediated by AIF. The effect of TULA on cell death is specific for particular death stimuli; it is significant for serum deprivation-induced apoptosis, but is negligible for apoptosis induced by TCR/CD3 ligation or several DNA-damaging drugs. The effect of TULA is largely caspase-independent, thus being entirely consistent with the idea that it is mediated by AIF, a key factor of caspase-independent apoptosis. It appears that the role of
TULA Interacts with AIF and Affects Cell Death

TULA is primarily to amplify the apoptotic events dependent on AIF rather than act as an independent inducer of apoptosis. Our results suggest that TULA promotes AIF-dependent apoptosis primarily by facilitating the interactions between AIF and its co-factors. Further studies should reveal the molecular basis of the apoptotic effect of TULA in detail.

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