Tumor necrosis factor receptor-associated factors (TRAFs) were first identified as intracellular proteins that were associated with TNF-R2, a member of the TNF receptor (TNF-R) superfamily (1, 2). There are currently known to be six mammalian TRAF family members (TRAF1–6) that play essential roles in signal transduction by TNF-R family members as well as some members of the Toll-like receptor (TLR) family and the IL-1R. In addition, a distantly related protein, TRAF7, was recently described that was able to activate AP1 activity and induce apoptosis (3). Therefore, TRAFs play important roles in a variety of essential aspects of host defense. TRAF family members (except TRAF1 and -7) all contain a C-terminal receptor-binding TRAF domain, which is composed of a coiled-coil domain, TRAF-N, and a highly conserved TRAF-C domain. Additionally, all TRAFs except TRAF1 have an N-terminal RING domain followed by one (TRAF7) or more (TRAF1–6) zinc fingers. TRAFs function as adaptor proteins, linking membrane receptors to downstream signaling events. TRAFs are able to interact with a series of kinases, such as TAK1 and MEKK2, which leads to activation of the transcription factors NF-kB and AP1, respectively. The N-terminal RING domain is essential for the activation of downstream signaling cascades, and deletion of this domain causes TRAFs to become inhibitors of receptor signaling (4–6).

Human (h)TRAF6 was initially identified by a screen for TRAF2-like sequences and subsequently cloned from a cDNA library (7). Murine (m)TRAF6 was discovered simultaneously by its capacity to bind CD40 via its TRAF domain (8). Murine and human TRAF6 are largely homologous in amino acid sequence except in the connecting region between zinc finger 5 and the TRAF domain. Reciprocal transfer of this connecting region completely exchanged the ability of human and murine TRAF6 to induce apoptosis and activate NF-kB. Unique regions of TRAF6 therefore play an important role in determining cell fate.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and supplemental Fig. 1.

1 To whom correspondence should be addressed. E-mail: Liusheng.He@Stjude.org.
2 The abbreviations used are: TRAF, tumor necrosis factor receptor-associated factor; TNF, tumor necrosis factor; TNF-R, TNF receptor; IL, interleukin; h, human; m, murine; YFP, yellow fluorescent protein; GFP, green fluorescent protein; CFP, cyan fluorescent protein; Z-, benzyloxycarbonyl-; mAb, monoclonal antibody; PARP, poly(ADP-ribose) polymerase; FITC, fluorescein isothiocyanate; shRNA, small-hairpin RNA; TLR, Toll-like receptor; PBS, phosphate-buffered saline; RFP, red fluorescent protein; DAPI, 4,6-diamidino-2-phenylindole; STS, staurosporine; fmk, fluoromethyl ketone; FLICA, fluorochrome-labeled inhibitor of caspase; FRET, fluorescence resonance energy transfer; DED, death-effector-domain; E3, ubiquitin-protein isopeptide ligase.
3 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
4 The current experiments examined this possibility. Not only was evidence found that hTRAF6 is degraded by a pathway initiated by its own E3 ubiquitin ligase activity of hTRAF6.
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ligase activity and in a RING domain- and caspase-dependent manner, but also a novel role of hTRAF6 in induction of apoptosis was found. Apoptosis induction depends on an interaction of the TRAF domain of hTRAF6 and the DED domain of pro-caspase 8, followed by activation of caspase 8 by a RING domain-dependent mechanism. Importantly, the connecting region between the fifth zinc finger and the TRAF domain governed the magnitude of TRAF6 degradation as well as its ability to activate NF-κB or induce apoptosis. These results indicate a novel and complex role of TRAF6 in cell fate decisions that differs significantly between human and mouse.

MATERIALS AND METHODS

Reagents—The proteasome inhibitors, MG132 and lactacystin, and pan-caspase inhibitor, Z-VAD, were purchased from Calbiochem. A rabbit horseradish peroxidase-conjugated anti-GFP antibody and a monoclonal antibody (mAb) against GFP were purchased from ABCam (Cambridge, MA) and Roche Applied Science, respectively. The mAbs against TRAF6, caspase 2, 3, and 8, and actin were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The mAbs against ubiquitin and PARP were purchased from Affinity Bioreagents (Golden, CO) and Enzymes Systems Products (Livermore, CA), respectively. Mammalian expression vectors pECPF-C1, pEYPF-C1, dsRed1-C1, and pcDNA4/HisMax were purchased from Clontech and Invitrogen, respectively. The mAb against Express-tag in the mammalian express vector pcDNA4/HisMax was purchased from Invitrogen. The random shRNA IMG805 and IMG 800 vector that was used to harbor hTRAF6 shRNA was purchased from Imgenex Corp. (San Diego, CA). A mitochondrial indicator CFP-Mito was purchased from Clontech. The human cervical carcinoma cell line HeLa and 293 cells were obtained from the American Type Culture Collection (Manassas, VA). Annexin V-FITC and its APC-conjugated dyes were purchased from Pharmingen. A fluorogenic substrate D2R and FLICA-DEVDase for measuring apoptosis activity were purchased from Oncogene Research Products (Boston, MA) and Chemicon (Temecula, CA), respectively.

Expression Plasmids and Cloning—Mammalian expression vectors encoding hTRAF2 and hTRAF6 were described previously (7, 33). CFP-hTRAF2 and YFP-hTRAF2 and YFP-hTRAF6 fusion constructs were described previously (28). pDsRFP-TRAF6 and His-TRAF6 were generated similarly. The YFP-hTRAF6 RING (YFP-TRAF6R) and YFP-hTRAF6TRAF (YFP-TRAF6TD) constructs were generated by standard cloning techniques (see supplemental Table 1 for primers). All other hTRAF6 deletion YFP chimeric protein constructs were generated by ligation of appropriate sites in order to delete the designated fragment from a parental YFP-hTRAF6 plasmid. The YFP-and His-hTRAF6C70A constructs were generated by using a standard mutagenesis kit provided by Stratagene (La Jolla, CA). Murine TRAF6 plasmid was described previously (19), and its YFP fusion construct was generated by standard cloning techniques with pEYPF-C1 vector and PCR technique. The hTRAF6 shRNA constructs against three different regions, from nucleotide 131 to 152, 550 to 571, or 572 to 593, which were predicted by the Imgenex web-based shRNA designer program, were generated in the IMG-800 vector (Imgenex, San Diego, CA) using the annealed oligonucleotides shown in supplemental Table 1. The C-terminal-tagged pro-caspase 8-GFP and its inactive mutant (C360S) caspase 8m-GFP plasmids were described previously (34). To generate an N-terminal CFP-tagged inactive pro-caspase 8 construct (CFP-caspase 8c360s), a PCR technique was used with the vector caspase 8m-GFP as a template and the paired primers shown in supplemental Table 1. The caspase 8 deletion constructs were generated similarly. In order to generate an artificial chimera encoding the RING finger of human TRAF6 and the zinc fingers and the TRAF domain of human TRAF2, the fragments encoding the zinc fingers and TRAF domain of human TRAF2 (amino acids 124–498) were amplified using the primers shown in supplemental Table 1.

The FRET-based caspase activity indicator CFP-LEVD-YFP was described previously (20). Other FRET-based caspase indicators CFP-DEVd-YFP (caspase 3), CFP-IETD-YFP (caspase 8), and CFP-LEHD-YFP (caspase 9) were generated using standard cloning techniques with pECPF-C1 and PCR using the primers shown in the supplemental Table 1. The plasmid for hTRAF6 with a murine connecting region between the zinc fingers and TRAF domain, designated as hTRAF6-m20, was generated by an insertion of PCR fragments using mTRAF6 as a template and the primers shown in supplemental Table 1. Similarly, the plasmid containing mTRAF6 and a human connecting region between zinc fingers and TRAF, designated as mTRAF6-h12, was generated with standard PCR techniques. All plasmids were confirmed with DNA sequence analysis.

Cell Culture, Transfection, Immunoprecipitation, and Immunoblotting—Cell cultures of HeLa cells and 293 cells, immunoprecipitation using GFP mAb and the subsequent GFP, ubiquitin, and TRAF6 immunoblotting were performed as described (35). Similar conditions were used to incubate A20 mouse lymphoma cells. Immunoblotting methods using antibodies against PARP and actin were described previously (20).

Chemical Treatment and TRAF6 shRNA Assay—HeLa cells co-transfected with distinct hTRAF6 shRNA vectors or transfected with control IMG 805 vectors alone were selected using the antibiotic G418 at a concentration of 50 μg/ml for 2 days and then treated with 12.5 or 50 μM MG132 (proteasome inhibitor) for an additional 3 h. These treated cells were loaded with the caspase fluorescent substrate D2R to detect caspase activity following the procedure provided by Pharmingen or directly lysed in RIPA buffer and assessed by TRAF6 Western blotting.

Immunofluorescence and Confocal Microscopy—HeLa cells grown in exponential phase on glass coverslips in 6-well plates were transfected with YFP chimeric constructs as described above. At 2 and 16 h post-transfection, cells were fixed with 4% paraformaldehyde in PBS at least for 20 min, washed three times in PBS, then stained with DAPI at a concentration of 5 μg/ml for 20 min, and mounted with anti-fade reagent (Molecular Probes). These fixed and DAPI-stained cells mounted on the slides were then photographed for YFP images and nuclear DAPI images that would identify apoptosis-related nuclear fragmentation.

When used for immunostaining, cells fixed at 2 h after transfection were blocked with 5% fetal calf serum at room temperature for 1 h, then incubated with 1:200 diluted primary antibody for 1 h (mAb against caspase 2 and polyclonal antibodies against caspase 3 or 8), washed twice in PBS, and finally incubated with 1:500 diluted mouse or rabbit IgG-specific antibodies conjugated with rhodamine for an additional 1 h at room temperature. After washing three times, cells were finally mounted onto microscope slides. YFP images and red rhodamine fluorescence images were taken using confocal microscopy excited with 488 and 543 nm argon-ion laser lines, respectively.

The Measurement of Caspase Activity by Caspase-sensitive FRET Probes, Fluorogenic Fluorescence Substrate D2R, and FLICA Using Flow Cytometry—A caspase 6, 8, and 4-sensitive FRET probe CFP-LEVD-YFP was described previously (23). The expression of this intact fusion protein results in intense FRET signals, whereas its cleavage at the LEVD linker or other caspase target linkers leads to a significant loss of the FRET signal. The flow cytometric optical configuration with the FACS DiVa (BD Biosciences) for measuring CFP, YFP, and FRET signals and the method for analyzing FRET-related caspase activity were described previously (23). Similarly, this method was also used to measure caspase 3-, 8-, and 9-related activ-
ity in viable cells by using CFP-DEVD-YFP, CFP-IETD-YFP, and CFP-LEHD-YFP probes, respectively. Briefly, HeLa cells were co-transfected with the CFP-LEVD-YFP probe or other caspase-sensitive indicators and RFP-hTRAF6, RFP-hTRAF6c70a, His-hTRAF6, or His-hTRAF6c70a. After overnight culture, transfected cells were analyzed for CFP, YFP, FRET, and RFP signals by flow cytometry. Cells gated for CFP, YFP, and RFP signals in a plot of RFP versus YFP were analyzed for the percentage of FRET-diminished cells to all transfected cells in a plot of CFP versus FRET. An increase of percentage of cells with diminished FRET indicates caspase activation.

When caspase activity was measured by using the fluorogenic caspase substrate D2R (36), which will emit green fluorescence upon caspase activation, the green signals were collected in FL1 on the FACSCalibur (BD Biosciences). When caspase activity of caspase 3 was measured by using FLICA-DEVDase and flow cytometry, a method described previously was employed (23).

**FRET Measurement by a λ-Mode Using Confocal Microscopy**—A previously described confocal microscopic λ-mode FRET technique was used to document direct protein-protein interaction (37–39). Briefly, the 405-nm line emitted from a violet laser was used to excite only CFP molecules in CFP/YFP complexes, and then the emission spectrum was documented continuously in a 12-nm interval from 440 to 600 nm. CFP/YFP FRET will result in the occurrence of a peak at 524 nm. To compare the FRET signal throughout all samples quantitatively, a ratio of emissions at 524 nm (FRET) to 470 nm (CFP) was calculated with sufficient sample size to achieve statistical significance.

**Immunofluorescence, Annexin V Staining, and Flow Cytometry**—The method for TRAF6 immunostaining for flow cytometric analysis was adapted from one used for heat shock protein immunostaining, which was described previously (40). Briefly, HeLa cells in suspension were fixed in 70% ice-cold methanol for at least 20 min and then exposed to three cycles of pelleting/washing, blocked for nonspecific binding using...
5% bovine serum albumin, and then sequentially incubated with a mAb against hTRAF6 (Active Motif, Canada) for 1 h at 37 °C and with a mouse IgG-specific antibody conjugated with FITC for an additional hour at 37 °C. Finally the hTRAF6-stained cells were analyzed in FL1 with the FACSCalibur (BD Biosciences). The FITC-conjugated annexin V binding assay was carried out to distinguish apoptotic cells using the protocol provided by Pharmingen. The cells with positive and negative annexin V-FITC staining were measured using a FACSVantage DiVa (BD Biosciences) and then sorted for analysis by TRAF6 immunoblotting. Similarly APC-conjugated annexin V binding assay was carried out in cells transfected with YFP-hTRAF6, YFP-mTRAF6, or YFP alone. The percentage of YFP/APC double-positive cells was measured using a FACSCalibur (BD Biosciences).

**RESULTS**

**The RING Domain Mediates hTRAF6 Degradation**—To visualize the cellular distribution of hTRAF6 and facilitate its detection by Western blotting, a construct was prepared in which full-length hTRAF6 was fused to YFP and then was transfected into HeLa cells. GFP and TRAF6 Western blots were initially carried out to validate the expression of the fusion proteins. It has been reported that the RING domain of mTRAF6 is required for its E3 ligase activity that can catalyze the addition of ubiquitin molecules to a variety of proteins, including TRAF6 itself (18). The rapid degradation of hTRAF6 suggested that the RING domain of hTRAF6 might initiate this ubiquitin-mediated autodegradation. To test this
possibility, a mutant hTRAF6 with a RING domain mutation (Cys → Ala at amino acid 70, C70A) was prepared. As anticipated, this RING domain mutation prevented modification and degradation of hTRAF6 (Fig. 1B). Stabilization did not depend on the YFP tag as it was also observed with His-tagged proteins in both HeLa and 293 cells (Fig. 1C). Notably, endogenous hTRAF6 was also stabilized by addition of proteasome inhibitors, MG132 (Fig. 1D, left) or lactacystin (Fig. 1D, right). Notably, in HeLa cells TRAF6 is maintained as a distinct band with the molecular mass of unmodified TRAF6, whereas in Jurkat cells, it is maintained as a family of higher molecular weight, presumably modified proteins. Importantly, hTRAF6 was clearly shown to be ubiquitylated by immunoprecipitating expressed YFP-hTRAF6 with antibody to GFP followed by ubiquitin immunoblotting (Fig. 1E). These results strongly indicate that endogenous hTRAF6 is degraded through a RING domain-mediated ubiquitylation pathway. To determine the portions of the hTRAF6 molecule involved in degradation, a series of deletion constructs was prepared (Fig. 1F). Although the intact RING domain was necessary for modification, the RING domain itself was not modified. At a minimum, the RING domain and the zinc fingers were required for modification and preferably a portion of the TRAF domain as well. Finally, this modification was not observed when other TRAFs (TRAF1–3 and -5) were expressed as YFP or CFP fusion proteins in HeLa cells (see Ref. 20 and data not shown).

Both hTRAF6 RING and TRAF Domains Are Required for Programmed Cell Death—The cellular fate of YFP-hTRAF6 was examined by confocal microscopy (Fig. 2). It was notable that hTRAF6-transfected HeLa cells or 293 cells (data not shown) quickly loosened from the culture dish, rounded up, and finally progressed to apoptosis that was visualized by DNA fragmentation after nuclear staining with DAPI and staining with annexin V, as well as by the induction of PARP cleavage detected by immunoblotting (Fig. 2). Analysis of the activity of a family of hTRAF6 deletion mutants indicated that an intact RING domain as
well as the five zinc fingers and parts of the TRAF domain were minimally required for induction of apoptosis assessed either by annexin V binding or PARP cleavage (Fig. 2C).

To delineate the activity of hTRAF6 in greater detail, series of chimeric proteins were constructed (Fig. 3A). Expression of the constructs was analyzed by GFP immunoblotting in HeLa cells either 2 or 16 h after transfection (Fig. 3A). The induction of apoptosis in cells expressing the indicated constructs was assessed by annexin V binding and PARP cleavage (data not shown). Moreover, when the RING domain of hTRAF6 was incorporated into hTRAF2, no modification of the chimeric protein and no induction of apoptosis were noted. These results confirm that aspects of the hTRAF6 protein other than the required RING domain are necessary for the induction of apoptosis.

Human TRAF6 induced apoptosis and also was rapidly degraded. Both effects apparently required the E3 ligase activity of the RING domain. To examine this in greater detail, apoptosis of Jurkat cells was induced by anti-Fas antibody or the chemical inducer of apoptosis, staurosporine (STS). In both circumstances, increased degradation of hTRAF6 was induced (Fig. 3B). Human TRAF6, but not human TRAF2, induced apoptosis, although hTRAF2 and hTRAF6 share a structurally similar RING domain (data not shown). Moreover, when the RING domain of hTRAF6 was incorporated into hTRAF2, no modification of the chimeric protein and no induction of apoptosis were noted. These results confirm that aspects of the hTRAF6 protein other than the required RING domain are necessary for the induction of apoptosis.

hTRAF6 Induces Apoptosis via a Caspase-dependent Pathway—Apoptosis can be mediated through a caspase-dependent pathway and a mitochondria-dependent pathway (21, 22). To examine the mechanism of apoptosis induced by hTRAF6, experiments were carried out with a pan-caspase inhibitor, Z-VAD. Addition of Z-VAD largely suppressed the programmed cell death induced by hTRAF6 (Fig. 4A). This protection from apoptosis by Z-VAD was also demonstrated by a decrease of PARP cleavage (data not shown). Notably, further degradation of ubiquitylated hTRAF6 molecules required the activity of functional caspases, as this was inhibited by Z-VAD-fmk (Fig. 4B).

To confirm hTRAF6-induced caspase activation, a FRET probe, consisting of CFP and YFP separated by a caspase cleavage site (CFP-LEVD-YFP) that has been demonstrated to be cleaved directly by caspases 6, 8, and 4 (23), was co-transfected with RFP-hTRAF6 or its mutant RFP-hTRAF6C70A into HeLa cells. Appearance of cells with diminished FRET indicates a high degree of caspase activity. As can be seen in Fig. 5A, only slightly increased caspase activity was found in cells (R3) co-expressing the caspase-sensitive probe and RFP-hTRAF6 compared with those co-expressing the probe and RFP-hTRAF6C70A at 2 h after transfection (55.2 versus 42.1%). In contrast, nearly all hTRAF6-transfected cells (98.1%; Fig. 5A, R3), but not those expressing the RING domain mutant hTRAF6C70A (44.2%), underwent caspase-mediated cleavage of the probe after overnight expression (16 h), indicating that there had been up-regulation of caspases that subsequently cleaved the FRET probe. These results indicate that caspase activity could be induced by hTRAF6 in a manner that required the intact RING domain.

To identify the specific caspases activated by hTRAF6, we constructed three additional CFP-YFP FRET probes with distinct linkers containing a caspase 3 target sequence DEVD (DEVDase), a caspase 8 target sequence IETD (IETDase), and a caspase 9 target sequence LEHD (LEHDase). Each of these three probes was co-transfected along with wild type hTRAF6 or the RING mutant hTRAF6C70A into HeLa cells. As shown in Fig. 5B, the downstream effector caspase 3 (DEVDase) was significantly activated by hTRAF6 but not the hTRAF6 RING domain C70A mutant (22 versus 6%). Similarly, expression of hTRAF6 induced significantly enhanced cleavage of the caspase 8-sensitive probe, CFP-IETD-YFP (27%), whereas the TRAF6 RING domain C70A mutant induced minimal activity (6%). In contrast, hTRAF6 did not induce cleavage of the caspase 9-sensitive inhibitor, CFP-LEHD-YFP, and no significant difference in the cleavage of this probe was found between hTRAF6 and the RING domain C70A mutant. These results strongly indicate that hTRAF6-induced apoptosis is predominantly mediated by the caspase pathway initiated by caspase 8 and not the mitochondrial pathway, involving caspase 9.

Direct Interaction of Pro-caspase 8 with hTRAF6—To examine in greater detail the putative caspase 8-dependent pathway of apoptosis triggered by hTRAF6, HeLa cells were co-transfected with YFP-hTRAF6 and a mitochondrial indicator, CFP-Mito. YFP-hTRAF6 did not co-localize with CFP-Mito (Fig. 6A). In contrast, immunostaining indicated that caspase 8, but not caspase 2 or 3, strongly co-localized with YFP-hTRAF6 (Fig. 6B). The co-localization of caspase 8 and hTRAF6 was further validated in the cells co-transfected with CFP-pro-caspase 8C360S and YFP-hTRAF6 (Fig. 6C). Because transfection of pro-caspase 8 alone strongly and rapidly induced apoptosis, the caspase-inactive mutant, C360S, was employed in the co-localization study. Cells transfected with CFP-caspase 8C360S alone
FIGURE 5. Activation of caspases in cells transfected with hTRAF6. A, the hTRAF6-mediated cleavage of a FRET probe, CFP-LEVD-YFP, that was determined previously to be sensitive to caspase 6, 8, and 4 (23). HeLa cells were co-transfected with CFP-LEVD-YFP and dsRFP-hTRAF6 or the RING domain mutant dsRFP-hTRAF6C70A, and 2 h after overnight incubation (16 h), the CFP, YFP, RFP, and FRET signals were determined using the FACS DiVa. The cells expressing both the dsRFP-TRAF6 construct and the CFP-LEVD-YFP probe were gated (R1) and analyzed for FRET. The expression of the intact CFP-LEVD-YFP protein resulted in intense FRET (R2), whereas the cleavage of the linker, LEVD, between CFP and YFP resulted in diminished FRET (R3). The cells expressing all three distinct fluorescent signals (CFP, YFP, and RFP) were gated (shown in a plot of YFP versus dsRFP), and the percentage of FRET-positive (R2) and FRET-negative cells (R3) was determined in a plot of CFP versus FRET and expressed as a fraction of all transfected cells (R2/H11001 R3). Expression of hTRAF6 but not the hTRAF6 RING domain C70A mutant resulted in a significant increase in the FRET-negative caspase-expressing population. Data from one of three independent experiments with similar results are shown.

B, the specific cleavage of a caspase 8-sensitive FRET probe, CFP-IETD-YFP, and a caspase 3-sensitive FRET probe, CFP-DEVD-YFP, but not of a caspase 9-sensitive FRET probe, CFP-LEHD-YFP, in cells transfected with wild type hTRAF6 but not in those transfected with the RING domain C70A mutant. Similar to A, the percentages of cells with an intense FRET signal and diminished FRET were determined in cells co-transfected with either wild type hTRAF6 or its RING domain C70A mutant, along with each individual caspase-sensitive FRET probe. The expression of wild type hTRAF6 but not the RING domain C70A mutant resulted in a significant increase in the fraction of cells with diminished FRET (R3) among those co-expressing a caspase 3-sensitive FRET probe (DEVDase) and a caspase 8-sensitive FRET probe (IETDase), but not a caspase 9-sensitive FRET probe (LEHDase).
displayed a diffuse uniform distribution throughout the cytoplasm, whereas those transfected with YFP-hTRAF6 RING domain C70A mutant or wild type hTRAF6 exhibited concentrated focal and a faint diffuse distribution in the cytoplasm (Fig. 6C). Importantly, the co-localization between caspase 8 and hTRAF6 was documented in cells co-transfected with CFP-caspase 8C360S and YFP-hTRAF6 (Fig. 6C). Most strikingly, this co-transfection triggered the focal aggregation of caspase 8 in a majority of cells. In contrast, caspase 8 remained in a uniform distribution in the cytoplasm in cells co-transfected with the hTRAF6 RING domain C70A mutant and CFP-pro-caspase 8C360S, which is catalytically inactive. Cells expressing CFP-pro-caspase 8C360S alone displayed uniform distribution in the cytoplasm (1st row), whereas those expressing YFP-hTRAF6C70A or YFP-hTRAF6 exhibited mostly punctate expression along with a diffuse distribution in the cytoplasm (2nd and 3rd rows). Both partial (I, 84% of cells) and complete co-localization (II 16% of cells) was observed in cells co-transfected with wild type YFP-hTRAF6 and the CFP-pro-caspase 8C360S mutant. The vast majority (82%) of cells transfected with YFP-hTRAF6 RING domain C70A mutant and CFP-pro-caspase 8C360S displayed faint co-localization in the cytoplasm only, whereas only 18% of cells manifested co-localization in a punctate distribution. Results are representative of those obtained from four individual experiments.

The association of hTRAF6 and caspase 8 was validated using a confocal FRET technique and a flow cytometric FRET approach at an early time point, 2 h after transfection in the presence of the pan-caspase inhibitor, Z-VAD-fmk (Fig. 7, A and B). Cells were transfected with CFP-caspase 8C360S and YFP-hTRAF6 and a 405 nm line was used in ZeissMeta mode to excite only CFP, but not YFP molecules (Fig. 7A, rows 1 and 2, respectively). By confocal microscopy, the occurrence of a sensitized YFP emission peak detected at 524 nm results from the direct transfer of energy from excited CFP to YFP moieties, indicating close proximity between CFP- and YFP-tagged molecules. Emission detected at 470 nm is from CFP and diminishes when there is transfer of energy from CFP to YFP. Cells transfected with a FRET-positive control, CFP-YFP, display an emission peak at 524 nm with the ratio of emission detected at 524/470 nm being 1.66 (Fig. 7A, row 6), which is significantly higher than that in cells expressing a FRET-negative control (0.76, Fig. 7A, row 5). Positive FRET (a peak at 524 nm) was documented in cells co-expressing caspase 8-CFPC360S and YFP-hTRAF6 (524/470 nm emission = 1.45) or the RING domain C70A mutant (524/470 nm emission = 1.26, p < 0.05 when compared with wild type hTRAF6) (Fig. 7A, rows 3 and 4). A close interaction between caspase 8 and hTRAF6 was also confirmed using a flow cytometric FRET technique (Fig. 7B). Experiments using a series of hTRAF6 and caspase 8 deletion mutants indicated that the TRAF domain is required for the interaction of hTRAF6 with pro-caspase 8 and that the death-effector-domains (DEDs), most specifically the DED-2 of pro-caspase 8 is essential for the caspase 8 interaction with hTRAF6 (Fig. 7C). Notably, there was no co-localization between hTRAF6 and pro-caspase 8C360S (data not shown). Importantly, the interaction between pro-caspase 8 and hTRAF6 was confirmed by immunoprecipitation in HeLa cells after

FIGURE 6. Documentation of co-localization of hTRAF6 with caspase 8. A, lack of localization of hTRAF6 to mitochondria. HeLa cells co-transfected with YFP-hTRAF6 and a mitochondrial indicator, CFP-Mito, were incubated for 3 h and fixed in 4% paraformaldehyde in PBS, and then their CFP and YFP images were examined using confocal microscopy. B, YFP-hTRAF6 co-localizes with endogenous caspase 8, but not caspase 3 or caspase 2. Cellular distribution of endogenous caspase 2, 3, and 8 in YFP-hTRAF6-transfected HeLa cells 3 h after transfection was visualized by immunostaining analysis using specific antibodies followed by rhodamine-conjugated secondary antibodies. Only caspase 8 co-localizes with YFP-hTRAF6. C, co-localization between hTRAF6 and caspase 8 in HeLa cells 3 h post-co-transfection with YFP-hTRAF6 or the RING domain C70A mutant and CFP-pro-caspase 8C360S, which is catalytically inactive. Cells expressing CFP-pro-caspase 8C360S alone displayed uniform distribution in the cytoplasm (1st row), whereas those expressing YFP-hTRAF6C70A or YFP-hTRAF6 exhibited mostly punctate expression along with a diffuse distribution in the cytoplasm (2nd and 3rd rows). Both partial (I, 84% of cells) and complete co-localization (II 16% of cells) was observed in cells co-transfected with wild type YFP-hTRAF6 and the CFP-pro-caspase 8C360S mutant. The vast majority (82%) of cells transfected with YFP-hTRAF6 RING domain C70A mutant and CFP-pro-caspase 8C360S displayed faint co-localization in the cytoplasm only, whereas only 18% of cells manifested co-localization in a punctate distribution. Results are representative of those obtained from four individual experiments.
transfection (Fig. 8A, I) as well as in A20 mouse lymphoma cells, in which the interaction of native proteins was examined (Fig. 8A, II).

To examine the interaction of caspase 8 and hTRAF6 in greater detail, their ability to interfere with each other’s function was assessed. Co-expression of the inactive caspase 8C360S mutant partially suppressed human TRAF6-induced caspase 3 activation (DEVDase) (Fig. 8B, rows 1 and 2). Similarly, co-expression of the inactive hTRAF6 RING domain C70A mutant also inhibited wild type caspase 8-induced caspase 3 activation (Fig. 8B, rows 3 and 4). Taken together, these data demonstrate that hTRAF6 induces apoptosis by a mechanism that involves the RING domain and other unique elements of the hTRAF6 molecule and that hTRAF6-induced apoptosis is achieved through its direct interaction with and activation of caspase 8. The TRAF domain of human TRAF6 mediates interaction with pro-caspase 8, whereas the RING domain is necessary for aggregation and activation of caspase 8.

To explore whether caspase 8 is specifically required for hTRAF6 to induce apoptosis, we transfected the wild type hTRAF6-YFP fusion protein into the caspase 8-deficient Jurkat I9.2 cell line and control Jurkat-neo cells (Fig. 8C). The deficiency of caspase 8 significantly inhibited hTRAF6-induced apoptosis to a level similar to that induced by the hTRAF6 RING domain mutant. It is notable that Z-VAD further suppressed the extent of apoptosis induced by
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hTRAF6, as well as that induced by hTRAF6 RING domain mutant hTRAF6C70A, in both wild type and caspase 8-deficient Jurkat cells (Fig. 8C). Notably, hTRAF6 appeared to be more stable in Jurkat cells compared with HeLa cells (Fig. 8C, inset), shown by GFP Western blotting. Despite this relative stability, the deficiency of caspase 8 partially blocked hTRAF6 cleavage in Jurkat cells. These data suggest that caspase 8 plays an important role in hTRAF6-mediated apoptosis and hTRAF6 cleavage but that other caspase members are also involved.

Involvement of Endogenous hTRAF6 in Spontaneous or MG132-induced Apoptosis in HeLa Cells—The data demonstrate clearly that the overexpression of hTRAF6 induces apoptosis. To determine whether endogenous hTRAF6 also regulates apoptosis, we carried out three different experiments. In the first experiment, the level of endogenous...
hTRAF6 protein was visualized on a single cell basis using TRAF6 immunostaining and flow cytometry (Fig. 9A). Spontaneously apoptotic cells were distinguished by a sub-diploid DNA content after propidium iodide staining and displayed higher hTRAF6 staining than cycling cells. In the second experiments, spontaneously apoptotic HeLa cells that bound annexin V were sorted, as were living cells that did not bind annexin V, and both were analyzed for hTRAF6 expression by immunoblotting (Fig. 9B). The apoptotic cells expressed high levels of TRAF6, whereas the living cells that did not bind annexin V expressed undetectable levels of hTRAF6.

In the third experiment, three distinct shRNA constructs were generated. HeLa cells were co-transfected with three hTRAF6-specific shRNA constructs to deplete endogenous hTRAF6 or were transfected with control shRNA vectors. The shRNA constructs successfully deleted the cells of hTRAF6 (Fig. 9C, III). MG132, a proteasome inhibitor, was used to induce apoptosis (24, 25) that was assessed by measuring caspase 3 activity by flow cytometry (Fig. 9C, I and II). Deletion of hTRAF6 significantly inhibited the induction of caspase 3 activation by MG132. Moreover, a modest but significant decrease in caspase 3 activity was also observed after deletion of TRAF6 in the absence of MG132 stimulation. Protein levels of both caspase 3 and caspase 8 did not change significantly from cells transfected with TRAF6-specific shRNA to those transfected with the control shRNA plasmid (Fig. 9C).

Murine TRAF6 Displays More Stable Expression, Higher NF-κB Activation, and Less Capacity to Induce Apoptosis than hTRAF6—Because of the significant sequence difference in the connecting region between the zinc finger 5 and the TRAF domain from murine to human TRAF6, we were interested in determining whether the molecules were functionally similar. It has been reported that mTRAF6 can activate NF-κB in a RING domain-dependent manner (18, 19), but its capacity to induce apoptosis is unknown.

As shown in Fig. 10A, hTRAF6 induced apoptosis as assessed by the induction of annexin V binding to a markedly greater extent than mTRAF6. Similarly, hTRAF6 was more effective at inducing caspase 3 activity than was mTRAF6 (Fig. 10B). Furthermore, expression of mTRAF6 was much more stable than was hTRAF6 in HeLa cells (Fig. 10C).
Similar to hTRAF6, the mTRAF6-induced apoptosis was mediated via a mechanism involving a direct interaction with pro-caspase 8 (Fig. 10D). However, the association of mTRAF6 with pro-caspase 8 was much less tight than the interaction of hTRAF6 with pro-caspase 8 (FRET emission ratio at 524/470 nm, 1.07 versus 1.45). Notably, mTRAF6 triggered NF-κB much more effectively than hTRAF6 (Fig. 10E). These data indicate there are important functional differences between murine and human TRAF6. To explore the structural basis for these differences, the capacity of the various hTRAF6 deletion mutants to activate NF-κB was examined. Although the RING domain was essential for the activation of NF-κB, alone it was insufficient to activate this transcriptional activity (Fig. 10F). The RING domain, all five zinc fingers, and likely a portion of the TRAF region are necessary for hTRAF6 to activate NF-κB maximally. Of note, when the C-terminal portion of the TRAF-C domain was deleted, NF-κB activation was more effective than with the wild type molecule. The context of the hTRAF6 molecule is important in the activation of NF-κB as documented by the finding that a construct composed of the hTRAF6 RING domain inserted into hTRAF2 failed to activate NF-κB as effectively as intact hTRAF6.

Functional Discrepancy between Human and Murine TRAF6 Results from a Difference in the Connecting Region between the Zinc Fingers and the TRAF Domain—As noted previously, the major sequence difference between human and murine TRAF6 resides in the connecting region between the fifth zinc finger and the TRAF domain (Fig. 11A). To determine whether this region contributes to the functional discrepancy between human and murine TRAF6, we generated two chimeras that exchanged these regions, designated as hTRAF6-m20 (2) and mTRAF6-h12 (4), respectively (Fig. 11A, right panel). Although hTRAF6 is heavily modified on expression (Fig. 11B, lane 1), the substitution of the murine connecting region into hTRAF6 prevented modification and stabilized
expression (Fig. 11B, lane 2). Murine TRAF6 is not significantly modified, and insertion of the human connecting region did not change this significantly. Remarkably, however, insertion of the murine connecting region into hTRAF6 inhibited the induction of apoptosis (Fig. 11C) and increased NF-κB activation (Fig. 11D), whereas insertion of the human connecting region into mTRAF6 did the reverse. The data strongly indicate that the differences in functional impact of human and murine TRAF6 are governed by the connecting region between the zinc fingers and the TRAF region.

**DISCUSSION**

In this study we define a novel function of hTRAF6 in mediating programmed cell death in addition to its established roles as an E3 ligase and an adaptor in mediating downstream activation of NF-κB and survival signals. Although the RING domain of hTRAF6 was essential in both the activation of NF-κB and the induction of apoptosis, we could distinguish major functional discrepancies between human and murine TRAF6 that can be accounted for by amino acids in the connecting region between the zinc fingers and the TRAF region. Importantly, the disruption of endogenous TRAF6 by expressing TRAF6-specific shRNAs in HeLa cells suppressed spontaneous and induced apoptosis, indicating that the results were not merely related to overexpression. Finally, apoptosis induced by hTRAF6 is mediated by a direct interaction with pro-caspase 8 and the activation of caspase 8 governed by the TRAF domain and the RING domain, respectively.

Recent studies (18, 19) have shown that mTRAF6 functions as an E3 ligase by forming a complex with the ubiquitin-conjugating enzymes, Ubc13 and the Ubc-like protein Uev1A, that in turn synthesizes polyubiquitin chains linked through lysine 63 of ubiquitin to their substrates, which include mTRAF6 itself. This complex activates IκB kinase by means of a complex involving TAK1, TAB1, and TAB2, resulting in the induction of NF-κB transcriptional activity. A RING mutation of mTRAF6 abolished its E3 ligase function and its ability to initiate this pathway of NF-κB activation. In these studies ubiquitylation of mTRAF6 initiated NF-κB activation but not degradation of mTRAF6. In contrast, another member of the TRAF family, hTRAF2 was reported to be degraded via a ubiquitin- and proteasome-mediated pathway initiated by the E3 ligase activity of the IAP-1 (inhibitor of apoptosis) protein associated with TNF receptors (26). Finally, mTRAF2 has been shown to mediate the proteolysis of mTRAF3 in a manner that is dependent on the RING finger of TRAF2 as well as its own ubiquitylation (27). These results indicate that the RING domain of the TRAF
family of molecules can function as an E3 ligase and that TRAFs themselves can be subjected to ubiquitin-mediated proteolysis. Indeed, we found that endogenous hTRAF6 can mediate its own ubiquitylation and degradation as one pathway of cellular metabolism. However, the full spectrum of the processes mediated by these mechanisms has not been delineated.

We focused on TRAF6 because its activity is central to the signaling capability of a variety of TNF-R family members as well as members of the TLR family and the IL-1R. Moreover, we were intrigued that hTRAF6 was biologically active, but its expression was difficult to detect (28). By using a series of fusion proteins, we found rapid ubiquitin modification of hTRAF6 followed by degradation. This was distinctly different from the behavior of mTRAF6 that undergoes some ubiquitylation but is not degraded to an appreciable degree. Importantly, a point mutation (Cys → Ala) at amino acid 70 within the RING domain of hTRAF6 stabilized expression of the protein. Furthermore, degradation of endogenous unubiquitylated hTRAF6 was prevented by a proteasome inhibitor, clearly indicating that degradation resulted from auto-ubiquitylation mediated by the RING domain of hTRAF6. It is notable that the RING domain was required for ubiquitylation but was not the site of ubiquitin modification. Analysis of the various deletion mutants indicated that the RING domain-mediated ubiquitylation of hTRAF6 was targeted to zinc fingers 4 and 5 and the TRAF domain. This is similar to the site of ubiquitylation of mTRAF6 (18, 19). Of interest, in addition to the ubiquitylation-mediated degradation pathway, caspases are also involved in human ubiquitin-modified TRAF6 degradation (Fig. 4). These two coupled degradation pathways likely make human TRAF6 tightly regulated in vivo.

Not only was hTRAF6 auto-ubiquitylated and degraded, but it also exhibited unique RING domain-mediated functional properties, including the capacity to induce apoptosis. Even though the induction of apoptosis required the RING domain of hTRAF6, this segment of the molecule was insufficient to induce programmed cell death; the TRAF domain was also required. Moreover, the RING domain of hTRAF6 was not specifically required as the RING domain of mTRAF6 could substitute (data not shown). This is consistent with the previous finding suggesting that the RING domain of TRAF2 is an E3 ligase capable of ubiquitylating itself and TRAF3 (26, 27). Moreover, the amino acid sequences of the hTRAF2 and hTRAF6 RING domain are similar, supporting the conclusion that they might function in an interchangeable manner. It is notable, however, that the hTRAF6 RING domain embedded into the hTRAF2 molecule did not induce apoptosis, strongly implying that the unique ability of hTRAF6 to induce apoptosis resulted from specific aspects of the protein besides its capacity to function as an E3 ligase. Part of this relates to the capacity of the TRAF domain of hTRAF6 to bind to the upstream caspase, caspase 8. This was documented by co-localization studies, examination of the induction of specific caspase activity, and examination of protein-protein interaction by FRET and immunoprecipitation.

Specifically, the TRAF domain of TRAF6 bound the DED of pro-caspase 8. In contrast, hTRAF2 did not bind pro-caspase 8, explaining the inability of hTRAF2 to induce apoptosis. The interaction of hTRAF6 with pro-caspase 8 was further documented by the capacity of either an enzyme-inactive construct of caspase 8 or the E3 ligase-deficient hTRAF6 to function as a dominant negative and inhibit apoptosis induced by TRAF6 or pro-caspase 8, respectively. It is notable that the interaction between the TRAF domain of hTRAF6 and the DED of caspase 8 appeared to alter the threshold for apoptosis induction, as cells expressing dominant negative TRAF6 (C70A) still remained viable, even though NF-κB activity was significantly diminished. Although interaction with pro-caspase 8 did not require the E3 ligase activity of the RING domain of hTRAF6, activation of caspase 8 was dependent of the presence of an intact RING domain. Whether this involves ubiquitylation of hTRAF6, caspase 8 or an intermediary molecule is unknown. In this regard, the recently cloned CARPs (caspase 8 and 10 associated RING proteins) were found to bind and induce ubiquitin-mediated proteolysis of pro-caspase 8 and 10 in a RING domain-dependent manner (29). Similarly, the X-linked inhibitor of apoptosis degraded the active form but not the pro-form of caspase 3 by ubiquitylating the protein by means of its RING finger domain (30). These data are consistent with a role for RING domain-mediated ubiquitylation and degradation, but whether ubiquitylation of pro-caspases plays a role in their activation is unknown. In this regard, we have not observed ubiquitylation of pro-caspase 8 after expression of hTRAF6 (data not shown). On the other hand, TRAF6-induced activation of NF-κB requires ubiquitylation of TRAF6, but not TAK1, TAB1, or TAB2 (19), whereas activation of caspase 8 can occur with aggregation of the protein that could be fostered by the ubiquitin-modified hTRAF6. It is notable that oligomerization of mTRAF6 is known to be associated with its ubiquitylation (19), providing a mechanism for aggregation and activation of bound pro-caspase. It is also noteworthy that ubiquitylation of hTRAF6 appears to trigger a negative regulatory loop in that the pan-caspase inhibitor, Z-VAD, partially blocks degradation of hTRAF6, but not hTRAF6 ubiquitylation (data not shown). This suggests that the interaction with and activation of caspase 8 may serve to limit the signaling capability of hTRAF6. Differences in the ability to activate caspase 8 may contribute to the relative stability of murine compared with human TRAF6.

Even though caspase 8 plays a central role in hTRAF6-induced apoptosis, caspases other than caspase 8 may also be involved. Clearly one of these is caspase 3. Whether hTRAF6 can directly activate caspase 3 or whether other upstream caspases are involved remains to be explored.

Of interest, mTRAF6 exhibited a reduced capacity to induce apoptosis when compared with hTRAF6. Despite this, TRAF6-deficient mice exhibited a substantial reduction of programmed cell death or apoptosis within the ventral diencephalon and mesencephalon (31), suggesting a role for TRAF6 in regulating apoptosis in vivo. A role for a c-Jun N-terminal kinase-dependent pathway was suggested to explain the TRAF6-mediated pathway of apoptosis triggered by lipopolysaccharide (32), whereas the current results suggest an alternative mechanism, namely the direct binding and activation of pro-caspase 8.

Significant functional differences were noted between mTRAF6 and hTRAF6, with the former being more stable, as well as inducing more NF-κB activity and less apoptosis. This difference could be accounted for by differences in the amino acid sequence between the zinc finger 5 and the TRAF-N domain. Within this region, mTRAF6 has eight additional amino acids. Protein modeling of this region suggests that the mouse protein is generally similar but may have an additional domain that potentially could function to foster different interactions with proteins than hTRAF6. This possibility is supported by the observation that mTRAF6 binds more weakly to pro-caspase 8 than hTRAF6, possibly explaining the diminished capacity of mTRAF6 to induce apoptosis. It is also possible that differential interactions with the TAK1-TAB1-TAB2 complex could explain the enhanced capacity of mTRAF6 to activate NF-κB. It is clear, however, that in both mouse and human TRAF6 plays a pivotal role in cell fate decisions with hTRAF6 favoring programmed cell death and mTRAF6 biased toward the program of survival and host-response genes regulated by NF-κB. The clear difference in these outcomes mandates caution when inferring outcomes in one species from those in another.
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