Tumor Necrosis Factor (TNF) Receptor-associated Factor 7 Is Required for TNFα-induced Jun NH2-terminal Kinase Activation and Promotes Cell Death by Regulating Polyubiquitination and Lysosomal Degradation of c-FLIP Protein*

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The pro-inflammatory cytokine tumor necrosis factor (TNF) α signals both cell survival and death. The biological outcome of TNFα treatment is determined by the balance between survival factors and Jun NH2-terminal kinase (JNK) signaling, which promotes cell death. Here, we show that TRAF7, the most recently identified member of the TNF receptor-associated factors (TRAFs) family of proteins, is essential for activation of JNK following TNFα stimulation. We also show that TRAF6 and TRAF7 promote unconventional polyubiquitination of the anti-apoptotic protein c-FLIP, and demonstrate that degradation of c-FLIP also occurs through a lysosomal pathway. RNA interference-mediated depletion of TRAF7 correlates with increased c-FLIP expression level, which, in turn, results in resistance to TNFα cytotoxicity. Collectively, our results indicate an important role for TRAF7 in the activation of JNK following TNFα stimulation and clearly point to an involvement of this protein in regulating the turnover of c-FLIP and, consequently, cell death.

Death receptors are cell surface receptors that belong to the tumor necrosis factor (TNF) receptor superfamily, the most well known members of which are tumor necrosis factor receptor 1 (TNFR1), the CD95/Fas receptor, and TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4/TRAIL-R1 and DR5/TRAIL-R2 (1). Upon ligand binding, activated death receptors oligomerize, thereby inducing the formation of the death-inducing signaling complex (DISC) on their intracellular parts (2). The DISC consists of oligomerized death receptor, the adaptor molecule FADD, two isoforms of procaspase-8, procaspase-10, and c-FLIP proteins (2, 3). Upon binding to the DISC, procaspase-8 undergoes oligomerization, which results in processing of the zymogen (3–5). In responsive cells, activated caspase-8 is then able to activate effector caspsases, thereby initiating apoptosis (3–5).

Cellular FLICE-inhibitory protein (c-FLIP), also known as Casper, iFLICE, FLAME-1, CASJ, CLARP, MRIT, or usurpin, is known as a crucial inhibitor of death receptor-mediated apoptosis by interfering with caspase-8 activation at the DISC signaling (6). c-FLIP exists as three splice variants, which give rise to a long form of c-FLIP (c-FLIPL) polypeptide of 55 kDa (7, 8), the short form (c-FLIPS) polypeptide of 26 kDa (7, 8), and a third 23-kDa form, called c-FLIPs (9). As a characteristic feature, c-FLIP proteins contain tandem death effector domains, and all three isoforms of c-FLIP can be recruited to the DISC through an interaction of their tandem death effector domains with the adaptor protein FADD. Due to its structural similarity to caspase-8 and caspase-10, c-FLIP can therefore remain bound to FADD and inhibit complete caspase-8 processing and activation (8).

Consistent with its role of crucial negative regulator of the apoptotic pathway, c-FLIP has been found overexpressed in different types of cancer. Increased levels of c-FLIP have been observed in a large number of tumor cell lines of various type, including carcinoma, gastric adenocarcinoma, pancreatic carcinoma, melanoma, ovarian carcinoma, and prostate carcinoma (10, 11). In primary tumors, an elevated level of c-FLIP has been observed in B-cell chronic lymphocytic leukemia (12, 13), bladder urothelial carcinoma (14), lung adenocarcinoma (15), gallbladder carcinoma (16), and hepatocellular carcinoma (17). Generally, a high level of c-FLIP expression correlates with...
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...a more aggressive form of the tumor and with a poor prognosis (18). For this, c-FLIP represents a promising target for cancer therapy, and a large amount of work is currently underway to validate c-FLIP as a therapeutic target to restore and/or increase an apoptotic response in cancer cells (10, 11).

Transcriptionally, c-FLIP expression is regulated by several transcription factors, including NF-κB (19, 20) and p53 (21). However, in addition to transcriptional regulation, c-FLIP expression level is also greatly regulated by post-transcriptional mechanisms. In fact, c-FLIP expression is regulated by heat stress (22), by the JNK-mediated phosphorylation and activation of the E3 ubiquitin ligase Itch (23), and by the ubiquitin/proteasomal pathway (24). In addition, JNK-independent degradative mechanisms of c-FLIP also have been described (25–27).

TRAF7 is the most recently identified member of the TVF receptor-associated factor (TRAF) proteins, a family of cytoplasmic regulatory molecules that functions as signal transducers for receptors involved in both innate and adaptive humoral immune responses (28–30). Functionally, TRAF7 potentiates MEKK3-mediated signaling and regulates activation of NF-κB transcription factor by promoting K29-linked polyubiquitination of NEMO and p65 (28–31).

Here, we show that TRAF7 is essential for activation of JNK following TNFα stimulation. We also show that TRAF7 promotes cell death by modulating the expression level of the anti-apoptotic protein c-FLIP.L.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Antibodies—HEK293 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS. Jurkat cells were cultured in RPMI medium supplemented with 10% FCS. HEK293 cells were transfected by calcium phosphate precipitation. TRAF2/−/− embryonic fibroblasts (EFs) were provided by Drs. T. W. Mak and W. C. Yeh (32), JNK DK0 EFs were provided by Dr. R. Davis (33), and p65/−/− EFs were provided by Dr. G. Franzoso (34). Cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Lentiviral vectors expressing shTRAF7 RNAs were obtained from Sigma and used according to the manufacturer’s instructions. Plasmids encoding mutant ubiquitins were a kind gift of Dr. C. Sasakawa, University of Tokyo.

Sources of antisera and monoclonal antibodies were the following: anti-c-FLIP and anti-Fas from Alexis; anti-FLAG and anti-β-actin from Sigma; anti-phosho-Jun, anti-phosho-p38, anti-p38, and anti-JNK1/3 (anti-JNK) from Cell Signaling; and anti-ubiquitin (P4D1), anti-phosho-JNK, and anti-HA from Santa Cruz Biotechnology. TNFα, MG132, leupeptin, and cycloheximide were from Sigma.

Immunoblot Analysis and Coprecipitation—Cell lysates were made in lysis buffer (150 mM NaCl, 20 mM Hepes, pH 7.4, 1% Triton X-100, 10% glycerol, and a mixture of protease inhibitors). Proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Blots were developed using the ECL system (Amersham Biosciences). For coimmunoprecipitation experiments, cells were lysed in lysis buffer, and immunocomplexes were bound to protein A/G (Roche Applied Science), resolved by SDS-PAGE, and analyzed by immunoblot assay.

Luciferase Assay—To assess AP1 activation, HEK293 cells were transfected with the indicated plasmidic DNAs together with pAP-1-luc (Clontech) in 6-well plates. 24 h after transfection, luciferase activity was determined with the Luciferase Assay System (Promega). Plasmids expressing RSV-β-galactosidase or Tk- Renilla were used in transfection mixtures to normalize for efficiency of transfection.

In Vitro Kinase Assay—In vitro kinase assay was performed as reported by Herr et al. (35). Briefly, following stimulation, cell lysates were first immunoprecipitated with rabbit polyclonal anti-JNK1/3 (Santa Cruz Biotechnology) conjugated with protein A-Sepharose beads. Then, samples were spun down and washed to remove nonspecifically bound proteins. The kinase reaction was carried out in the presence of nonradioactive ATP and GST-c-Jun 1–79 fusion protein for 25 min at 30 °C. The reaction was terminated by adding Laemmli buffer, and samples were resolved by 12% SDS-PAGE. After blotting onto nitrocellulose membrane, phosphorylated c-Jun protein was detected using a rabbit polyclonal antibody specifically raised against phosphorylated Ser-73. Signals were developed by ECL.

ATPLite Assay—A total of 8 × 10³ cells per well was cultured in a flat-bottom 96-well plate in quadruplicates in 10% FBS/DMEM medium. Cell viability was determined using an ATPLite™ (PerkinElmer Life Sciences) kit according to the manufacturer’s instructions.

Statistical Analysis—Results are expressed as mean ± S.D. from a number of samples as indicated in the corresponding figure legends. Student’s t test was used to determine statistical significance of two-group comparisons. Multiple comparisons statistical significance was verified by analysis of variance with a one-tailed F-test, the posthoc Tukey honestly significant difference test, and Bonferroni and Dunnett corrections.

RESULTS

We and others have previously reported that ectopic expression of TRAF7 induces activation of AP1 transcription factor (28–31). To verify whether TRAF7 plays a physiological role in TNFR1 signaling, we determined the effect of RNA interference-mediated depletion of TRAF7 on TNFα-induced AP1 activation, as assessed by an AP1-luciferase reporter assay. For this, HEK293 cells, transfected with a vector encoding for a short hairpin RNA (shRNA) designed to target human TRAF7 (30) or a scramble shRNA, were exposed to TNFα for 6 h, and luciferase activity was determined. As shown in Fig. 1A, depletion of TRAF7 reduces AP1 transcriptional activity induced by TNFα exposure. This effect was specific for the TNFR1 signaling, because AP1 activity induced by a constitutively active mutant of MEKK7 was not altered by TRAF7 depletion in the same cells (Fig. 1A). Next, we determined whether induction of AP1 activity due to TRAF7 expression occurs through activation of JNK. To this end, HEK293 cells were transfected with an expression vector empty or encoding for TRAF7, and the activation state of JNK was monitored by in vitro kinase assay. As
shown in Fig. 1, TRAF7 expression induces activation of JNK, independently of TNFα stimulation. Activation of JNK following TRAF7 expression was confirmed by immunoblot assays probed with anti phospho-JNK antibodies (Fig. 1C). Finally, we determined whether TRAF7 function is required for JNK activation following TNFα stimulation. Thus, HEK293 cells depleted of TRAF7 were exposed to TNFα. Cell lysates were then immunoprecipitated with anti-JNK antibody, and the immunoprecipitates were subjected to in vitro kinase assays. The result of the experiment, shown in Fig. 1D, indicates that depletion of TRAF7 reduces activation of JNK following TNFα stimulation. Overall, from these experiments we concluded that TRAF7 expression is sufficient for JNK activation, and its function is required for complete activation of JNK following TNFα stimulation.

It is known that TNFα-mediated JNK activation accelerates turnover of c-FLIP, via induction of the E3 ubiquitin ligase Itch, which specifically polyubiquitinates c-FLIP, thereby inducing its proteasomal degradation (23). In addition, we have recently shown that TRAF7 mediates polyubiquitination events that play a key role in regulating cellular functions (31). Taking into account that TRAF7 expression induces cell death (29, 31), we examined whether TRAF7 influences the ubiquitination state of c-FLIP. For this, we transfected HEK293 cells with TRAF7 and assessed the ubiquitination state of endogenous c-FLIP by immunoblot experiments. As shown in Fig. 2A, TRAF7 expression promotes polyubiquitination of endogenous c-FLIP, and correlates with a reduction in the expression level of c-FLIP, (Fig. 2B). Because ubiquitin possesses seven lysines (Lys-6, Lys-11, Lys-27, Lys-33, Lys-48 and Lys-63), and the fate of ubiquitinated proteins depends on the K-type of ubiquitin linkage, we used a series of ubiquitin mutants possessing single lysine residues to investigate the nature of TRAF7-mediated polyubiquitination of c-FLIP. In the presence of TRAF7, c-FLIP was polyubiquitinated by Lys-29-, Lys-33-, Lys-63-, and, to a lesser extent, Lys-48-linked ubiquitin mutants (Fig. 2C). Given this result, we examined whether also TRAF6, another member of the TRAF family that promotes polyubiquitination (36), was capable of inducing polyubiquitination of c-FLIP. In fact, as shown in Fig. 2D, TRAF6 induced polyubiquitination of c-FLIP, although with a pattern different than that used by TRAF7. Indeed, TRAF6 triggered Lys-27-, Lys-29-, and, to a lesser extent, Lys-48-linked ubiquitin mutants (Fig. 2C). Interestingly, TRAF6, but not TRAF7, also promoted a weak polyubiquitination of JNK (Fig. 2E).

It is known that, although Lys-48 polyubiquitination targets proteins for proteasomal degradation, unconventional Lys-29 polyubiquitination promotes lysosomal protein degradation (37). Thus, we tried to determine whether, in addition to proteasomal degradation, also lysosomal mechanisms regulate the
FIGURE 2. TRAF7 promotes polyubiquitination of c-FLIP. A, HEK293 cells were transfected with an expression vector encoding for HA-ubiquitin along with a vector empty or expressing a FLAG-tagged version of TRAF7. 24 h later, cell lysates were immunoprecipitated with anti-c-FLIP antibody, separated by SDS-PAGE, and transferred onto membranes subsequently probed with anti-HA. B, HEK293 cells transfected with an expression vector encoding for TRAF7 were monitored for c-FLIP expression by immunoblot assay. c-FLIP expression levels were quantitated by ImageJ (lower panel). C and D, HEK293 cells were cotransfected with HA-tagged ubiquitin mutants and FLAG-tagged TRAF7 (C) or TRAF6 (D). The numbers indicate the only lysine residue remaining in the ubiquitin molecule. Immunoprecipitates with anti-c-FLIP antibody were resolved by SDS-PAGE and blotted onto a membrane subsequently probed with anti-HA. E, HEK293 cells were transfected as indicated, and the ubiquitination state of JNK was monitored by immunoblot assay probed with an anti-ubiquitin antibody (P4D1).
expression level of c-FLIPL. To do this, we used a very sensitive cellular experimental system, the EFs from TRAF2/H11002 mice. In fact, these cells are particularly sensitive to the cytotoxic activity of TNF/H9251, because they express very low levels of c-FLIPL due to a rapid degradation of this protein (38). Thus, we monitored the expression levels of c-FLIPL in TRAF2/H11002 EFs untreated or treated with lysosomal (leupeptin/NH4Cl) or proteasomal (MG132) inhibitors. As shown in Fig. 3A, TRAF2/H11002 EFs express almost undetectable levels of c-FLIPL. Treatment with MG132 restores c-FLIPL expression, confirming the existence of a proteasomal-mediated degradation of the protein (24, 36). Interestingly, c-FLIPL expression was also restored in cells exposed to lysosomal inhibitors. Significantly, also treatment with cycloheximide, which inhibits the lysosomal proteases cathepsins, restores c-FLIPL expression in TRAF2/H11002 EFs. Collectively, these results show, for the first time, that degradation of c-FLIPL also occurs through a lysosomal pathway, which, together with the proteasomal pathway, regulates the expression level of this protein. The recovery of c-FLIPL due to proteasomal and lysosomal inhibitors is functionally relevant, because TRAF2/H11002 EFs treated with these inhibitors are considerably resistant to TNFα cytotoxicity (Fig. 3B) and show a lower level of caspase-8 activation following TNFα stimulation (Fig. 3C). To verify that the protective effect exerted by lysosomal inhibitors was not limited to the TRAF2/H11002 murine EFs, we depleted TRAF2 from the human cell line HEK293 via infection with a retroviral vector encoding a shRNA targeting human TRAF2. As shown in Fig. 3D, also in this experimental system lysosomal inhibitors confer resistance to TNFα cytotoxicity. Lysosomal inhibitors treatment induces accumulation of c-FLIPL also in the mouse embryonic fibroblast cell line NIH-3T3 (Fig. 3E).

Next, we examined whether TRAF7 was involved in the degradation of c-FLIP in TRAF2/H11002 EFs. To do this, we abrogated TRAF7 expression in TRAF2/H11002 EFs via infection with a retroviral vector encoding two different shRNA designed to target murine TRAF7. As shown in Fig. 4A, depletion of TRAF7 in TRAF2/H11002 EFs correlated with an increase in the level of
**DISCUSSION**

There are several aspects that make particularly interesting the finding we report herein. The first one is the demonstration that TRAF7 is required for activation of JNK following TNFα stimulation. It is well established that, following TNFα stimulation, the balance between NF-κB and JNK activities determines the outcome of TNFR1 signaling, such that NF-κB promotes cell survival, whereas JNK activation enhances TNFα-induced death (40). Therefore, the discovery here that TRAF7 has an essential role in activation of JNK following TNFα stimulation is, *per se*, valuable information that adds a further element in the map of knowledge we have about TNFR1 signaling.

At least in part, JNK activity controls TNFα-induced death through the proteasomal processing of c-FLIP, via activation of the ubiquitin ligase Itch (23). This brings us to the second important finding contained in this report: *i.e.*, the evidence for a lysosomal pathway that controls c-FLIP turnover. This discovery is significant and potentially of great consequence, because many *in vitro* studies have demonstrated the importance of the role of c-FLIP in resistance to apoptosis induced by death receptors and to conventional chemotherapy (10, 11). Elevated expression of c-FLIP is often identified in malignant cancers, and it strongly correlates with a poor prognosis (10, 11).

Finally, we examined whether the observation obtained using cell lines derived from knockout mice was extensible to other cell types. For this, we used two well known cellular systems, namely Jurkat cells and HeLa cells. As shown in Fig. 6A, Jurkat cells depleted of TRAF7 were significantly more resistant to the cytotoxic activity of both anti-Fas and TNFα. In addition, TRAF7-depleted Jurkat cells showed a remarkable accumulation of c-FLIP, following TNFα stimulation (Fig. 6B). Similarly, TRAF7-depleted HeLa cells showed an increased expression level of both c-FLIP and c-FLIP following TNFα stimulation (Fig. 6C).
Additionally, preclinical data clearly indicate that selective inhibitors of c-FLIP, in combination with conventional chemotherapy, could represent an effective antitumor therapy (41–43). Therefore, the finding here, that a lysosomal degradation pathway regulates cellular turnover of c-FLIPL, offers additional tools for anticancer strategies based on down-regulation of c-FLIP.

The last, but not less important aspect emphasized in this report, is that TRAF7 is involved in regulating the expression level of c-FLIPL. First, TRAF7 could regulate degradation of c-FLIPL via activation of JNK and, thus, through the pathway JNK/Itch/proteasome described by Chang et al. (23). The evidence that TRAF7 promotes Lys-48-linked polyubiquitination of c-FLIP (Fig. 2B) is consistent with this possibility. However, it should definitely be noted that TRAF7 promotes Lys-29-linked polyubiquitination of c-FLIPL, and this type of polyubiquitination has been associated with lysosomal degradation of proteins (37). Also striking is the evidence that lysosomal degradation of proteins by Lys-29-linked polyubiquitination occurs through the E3 ubiquitin ligase Itch (37).

In addition, because TRAF6 promotes polyubiquitination of c-FLIPL, it would be interesting to see if it regulates the expression level of c-FLIPL as well. However, this analysis is compli-
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cated by the fact that TRAF6 is a potent activator of NF-κB, which, in turn, positively regulates transcription of c-FLIP (19, 20). Also intriguing is the experiment conducted on the JNK−/− DKO E6s, which would suggest that the positive effect exerted by TRAF7 depletion on c-FLIP turnover is independent of JNK function.

Clearly, our work opens many interesting questions concerning TRAF7 function that require further investigation. In this context, the generation of animal models genetically modified in the locus encoding for TRAF7 will certainly be of enormous value to finally define the physiological role of this protein.

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