Tumor Necrosis Factor Receptor 2 Signaling Induces Selective c-IAP1-dependent ASK1 Ubiquitination and Terminates Mitogen-activated Protein Kinase Signaling*

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TRAF2 and ASK1 play essential roles in tumor necrosis factor α (TNF-α)-induced mitogen-activated protein kinase signaling. Stimulation through TNF receptor 2 (TNFR2) leads to TRAF2 ubiquitination and subsequent proteasomal degradation. Here we show that TNFR2 signaling also leads to selective ASK1 ubiquitination and degradation in proteasomes. c-IAP1 was identified as the ubiquitin protein ligase for ASK1 ubiquitination, and studies with primary B cells from c-IAP1 knock-out animals revealed that c-IAP1 is required for TNFR2-induced TRAF2 and ASK1 degradation. Moreover, in the absence of c-IAP1 TNFR2-mediated p38 and JNK activation was prolonged. Thus, the ubiquitin protein ligase activity of c-IAP1 is responsible for regulating the duration of TNF signaling in primary cells expressing TNFR2.

Tumor necrosis factor α (TNF), a key mediator of the inflammatory response, functions by increasing the expression of a variety of effector molecules such as interleukin-1, interleukin-6, and Interferon-γ (1). TNF binds to two distinct transmembrane receptors, TNF receptor 1 (TNFR1; p55) and TNF receptor 2 (TNFR2; p75). TNFR1 is ubiquitously expressed, whereas TNFR2 is found primarily on cells of the immune system and is highly regulated (2). TNFR1 is the more widely studied of this receptor pair, and its functions include signaling for apoptosis (activation of caspases) and inflammation (activation of NF-κB and inflammatory gene products). The role of TNFR2 in biological processes is less well defined. Unlike TNFR1, TNFR2 lacks an intracellular death domain, and therefore its occupancy does not cause caspase activation. Nevertheless, expression of TNFR2 in cell lines potentiates TNFR1-induced death (3, 4), and mice deficient in TNFR1 or TNFR2 are resistant to TNF-mediated toxicity and highly susceptible to infectious challenge (5, 6).

TNFR-associated factors (TRAFs) are recruited to both TNFR1 and TNFR2 signaling complexes and play critical roles in diverse biological processes (7). TRAF2 in particular is a key upstream component in the pathway leading to TNF-induced activation of the mitogen-activated protein kinases (MAPK) p38 and JNK (8). ASK1, one of the MAPK kinase kinases (MAPKKKs) upstream of p38 and JNK, associates with TRAF2 following TNF stimulation (9). In fact, the duration of p38 and JNK activation in response to TNF is impaired in ASK1-deficient murine embryonic fibroblasts (10). Other proximal MAPKKKs, such as NF-κB-inducing kinase (NIK) (11) and germinal center kinase-related (GCKR) (12), have also been implicated in the induction of MAPK signaling in response to TNF, although their role is less well described.

The cellular inhibitor of apoptosis proteins 1 and 2 (c-IAP1 and c-IAP2) are also recruited to TNFR signaling complexes through their association with TRAF2 (13). In addition to their baculovirus internal repeat-dependent caspase inhibitory effects (14), c-IAP1 and c-IAP2 have ubiquitin protein ligase (E3) activity, conferred by their C-terminal RING domains (15, 16). c-IAP1, in particular, has been found to play an important role in TNF signaling, being activated by occupancy of TNFR2 and mediating the ubiquitination and subsequent degradation of TRAF2 (17). Given that TRAF2 is degraded and TNF-induced JNK is prematurely terminated in TNFR2-expressing Jurkat cells, we asked whether upstream MAPKKKs might also be targets of ubiquitination and degradation. Here we show that ASK1, but not NIK or GCKR, is ubiquitinated in TNFR2-expressing cell lines and resting B cells in a process dependent on the E3 activity of c-IAP1. Furthermore, absence of this process in c-IAP1−/− B cells results in failure to terminate TNF-induced MAP kinase signaling.

EXPERIMENTAL PROCEDURES

Reagents, Plasmids, and Cell Lines—Antibodies used for immunoblotting include anti-β-actin and anti-FLAG (Sigma), anti-ASK1, anti-ubiquitin, anti-HA, anti-Myc, anti-TRAF2, anti-NIK, and anti-GCKR (Santa Cruz Biotechnology), anti-Thr-180/Tyr-182-phosphorylated p38, anti-p38, and anti-Thr-183/Tyr-185-phosphorylated JNK (Cell Signaling), and anti-JNK1/JNK2 (BD Biosciences). Secondary antibodies used for immunoblotting are anti-rabbit (or anti-mouse) IgG horseradish peroxidase-linked whole antibody (Amersham Biosciences) and Alex Fluor 680 anti-rabbit (or anti-mouse) IgG (Molecular
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Probes. The following antibodies, cytokines, and chemicals were used for cell culture: human TNF-α (R&D systems), MG-132 (Sigma, used at 10 μM), lactacystin (Sigma, used at 25 μM), and agonic anti-TNFRI and anti-TNFRII antibodies (Abcam). The plasmids used for transfection were: pcDNA3 encoding human HA-ASK1 (18), pCMV2 encoding human FLAG-TRAF2 (Dr. Zheng-Gang Liu, National Institutes of Health), pcDNA3 encoding human HA-GCKR (19), and pcDNA3 encoding human Myc-NIK (Dr. Srinivasa Srinivasula, National Institutes of Health). Expression vectors pcDNA3 encoding human Myc-c-IAP1 and human Myc-c-IAP1-H588A (E3-dead; c-IAP1-mut) were generated in our laboratory (15). Jurkat and 293 cells were obtained from American Type Culture Collection. Jurkat cells stably transfected with TNFR2 (4E3 cells) were a gift from Dr. Michael Lenardo (4). 293 cells stably transfected with TNFR2 have been described (20). HeLa cells stably transfected with TNFR2 were kindly provided by Dr. Harald Wajant (21).

Cell Transfection—293 cells were seeded on a 10-cm plate and transfected with the indicated expression vectors using Lipofectamine 2000 (Invitrogen). After 24 h, the cells were harvested and lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) with EDTA-free protease inhibitor mixture (Roche Applied Science).

In Vitro Protein Interaction and Ubiquitination Assays—In vitro protein pulldown assays have been described (20). Briefly, GST or the GST-c-IAP1 fusion protein were coated onto glutathione-Sepharose 4B beads and incubated with in vitro translated (IVT) [35S]Met-labeled ASK1 at 4 °C for 2 h. After extensive washing, the beads were eluted by boiling in SDS sample buffer and analyzed by SDS-PAGE followed by autoradiography. In vitro ubiquitination assays were performed by adding 1 μl of IVT [35S]Met-labeled ASK1 in 25 μl of reaction buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM dithiothreitol, 2 mM ATP, and 2 mM MgCl2) in the presence of IVT [35S]Met-labeled c-IAP1 or IVT [35S]Met-labeled c-IAP1-mut, 200 nM ubiquitin-activating enzyme, 1 μM Ubch45b, and 10 μg of ubiquitin (Boston Biochem). After incubation at 30 °C for 1 h, the reactions were terminated with SDS sample buffer and analyzed by SDS-PAGE followed by autoradiography.

Mouse B Cell Isolation—Small resting lymphocytes from wild type or c-IAP1-deficient mice (22) were isolated by centrifugation through a discontinuous Percoll gradient (the layer at the 60–70% Percoll interface) (23). B cells were purified by negative selection using the Mouse B Cell Recovery Column kit (Cedarlane Laboratories Ltd). After experimental manipulation, B cells were lysed in 2× sample buffer (100 mM Tris, 25% glycerol, 2% SDS, 0.01% bromphenol blue, pH 6.8, and 10% 2-Me).

Immunoprecipitation—4E3 cells were lysed in a buffer containing 50 mM Tris HCl, pH 8.0, 137 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 0.1% phenylmethylsulfonyl fluoride, and fresh protease inhibitors (lysis buffer). To disrupt noncovalent intermolecular interactions, lysates were heated in the presence of 1% SDS and 5 mM dithiothreitol at 95 °C for 5 min before immunoprecipitation. The heated lysates were pre-cleared with protein A-Sepharose beads for 1 h at 4 °C and centrifuged. Lysates were incubated with the immunoprecipitating antibody for 1 h on ice, protein A-Sepharose beads were added, and the tubes were rotated at 4 °C overnight. The beads were washed five times with lysis buffer and heated at 95 °C for 5 min in sample buffer.

Immunoblotting—Cell lysates and immunoprecipitates were resolved on 8 or 12% SDS-PAGE. The proteins were detected by either enhanced chemiluminescence (Pierce) or infrared fluorescence (Odyssey Infrared Imaging System) following the manufacturer’s instructions.

RESULTS

TNF Induces TNFR2-dependent ASK1, but Not NIK or GCKR, Ubiquitination and Degradation—We have previously shown that c-IAP1 mediates TNFR2-dependent TRAF2 ubiquitination and there is premature termination of TNF-induced JNK activation in Jurkat cells expressing TNFR2 (17). ASK1, NIK, and GCKR are TRAF2-interacting MAPKKs upstream of p38 and JNK (9, 11, 12). Considering that TRAF2 is degraded in cells expressing TNFR2, we asked whether ASK1, NIK, or GCKR are also subject to degradation upon TNFR2 occupancy. Jurkat T cells that express TNFR1 and TNFR2 (4E3 cells) (4) were treated with TNF for 4 h, at which time TRAF2, ASK1, NIK, and GCKR levels were determined (Fig. 1A). As previously shown, stimulation with TNF resulted in a substantial decrease in TRAF2 expression. Notably, expression of ASK1, but not NIK or GCKR, also
decreased in response to TNF. To assess whether TNFR2 signaling is required for this decrease, ASK1 levels were determined in Jurkat T cells (TNFR1+/TNFR2−) and 4E3 cells stimulated with TNF (Fig. 1B). Unlike the TNFR2+ 4E3 cells, the level of ASK1 was unaffected by TNF treatment in Jurkat cells. Furthermore, the loss of ASK1 in TNF-treated 4E3 cells was prevented by MG-132, indicating that it was undergoing proteasome-mediated degradation (Fig. 1C). Similar results were obtained with non-lymphoid epithelial cells expressing TNFR2 (Fig. 1, D and E). Therefore, as with TRAF2, signals downstream of TNFR2 induce proteasome-mediated ASK1 degradation.

Proteasome-mediated degradation of ASK1 implies that it is a target for TNFR2 signaling-induced polyubiquitination. To determine whether this is the case, 4E3 cells were stimulated with TNF in the presence of MG-132. After lysis of the cells, protein-protein interactions were disrupted by heating to prevent co-immunoprecipitation of putative ASK1-interacting and ubiquitinated molecules. The lysates were then diluted in lysis buffer without SDS, and ASK1 was immunoprecipitated and then subjected to immunoblotting with anti-ubiquitin. As shown in Fig. 2, high molecular weight bands representing extensively polyubiquitinated ASK1 were markedly elevated 20 min after TNF stimulation. Together, these data demonstrate that ASK1 is a target for TNF-induced polyubiquitination and subsequent proteasomal degradation.

c-IAP1 Mediates ASK1, but Not NIK or GCKR, Ubiquitination and Degradation—c-IAP1 is a TNFR2-associated ubiquitin protein ligase that ubiquitinates TRAF2 upon stimulation with TNF (17). To determine whether c-IAP1 was responsible for ASK1 ubiquitination, 293 cells were transfected with expression plasmids encoding HA-ASK1 and either Myc-c-IAP1 or an E3-inactive c-IAP1 that has single amino acid substitution in the RING domain (c-IAP1-mut). After 24 h the cells were lysed and the levels of the transfected gene products were determined by immunoblotting. The levels of TRAF2 were determined with an anti-FLAG antibody. C, 293 cells were transfected with expression plasmids encoding HA-ASK1, FLAG-TRAF2, Myc-NIK, FLAG-GCKR, or Myc-c-IAP1 in the indicated combinations. After 24 h ASK1 and c-IAP1 levels were detected by immunoblotting. The levels of TRAF2 were determined with an anti-FLAG antibody. D, 293-TNFR2 cells were transfected with an expression plasmid encoding HA-ASK1. After 16 h the cells were incubated with or without MG-132 for 3 h and then treated with TNF. ASK1 and TRAF2 levels were determined by immunoblotting.
c-IAP1 reduced the level of TRAF2 and ASK1, but not NIK or GCKR. Thus, unlike TRAF2 and ASK1, NIK and GCKR are unlikely to be c-IAP1 substrates in vivo.

To determine the rate of ASK1 and TRAF2 down-regulation, HA-ASK1-transfected 293-TNFFR2 cells were treated with TNF and ASK1 and TRAF2 levels were monitored over time. The levels of both proteins were reduced as early as 5 min after treatment and were substantially decreased by 30 min (Fig. 3D). The decreases were largely prevented by MG-132, indicating that they were due to proteasome-mediated degradation.

**c-IAP1 Interacts with ASK1 and Induces Its Ubiquitination in Vitro**—To test whether c-IAP1 can directly bind and ubiquitinate ASK1, beads coated with GST or GST-c-IAP1 were incubated with [35S]Met-labeled ASK1, and the pulled down material was detected by autoradiography. Beads coated with GST-c-IAP1, but not with GST alone, pulled down ASK1 (Fig. 4A). Furthermore, IVT [35S]Met-labeled ASK1 was ubiquitinated by c-IAP1 in vitro in an E2-dependent fashion, as indicated by a decrease in the amount of input unmodified protein (Fig. 4B). A RING mutant of c-IAP1 that lacks E3 activity had no effect on ASK1 levels. Taken together, these results show that c-IAP1 can bind and ubiquitinate ASK1.

**Maintenance of ASK1 and TRAF2 Levels in TNFR2-stimulated c-IAP1−/− Resting B Cells**—The preceding results were obtained using recombinant proteins and cultured cell lines. To determine the physiologic relevance of these findings, cells from mice with targeted disruption of the c-iap1 locus were studied. The major manifestation of this genetic manipulation is a marked up-regulation of c-IAP2 levels, which is a consequence of the failure of c-IAP1 to ubiquitinate c-IAP2 and cause its degradation (22). Because c-IAP1, but not c-IAP2, has been shown to ubiquitinate TRAF2 (17), cells from c-IAP1−/− animals should provide a suitable environment for testing the hypothesis that c-IAP1 is the major effector of TRAF2 and ASK1 ubiquitination downstream of TNFR2 signaling. B cells constitutively express TNFR2, and the levels expressed on wild type and c-IAP1−/− B cells are similar (25).3 Purified B cells from wild type and c-IAP1-deficient mice were treated with agonistic anti-TNFR2 antibodies for 6 h, at which time the amounts of TRAF2 and ASK1 were determined (Fig. 5A). Signaling through TNFR2 induced a decrease in TRAF2 that was abrogated by the proteasome inhibitor lactacystin. Importantly, and in agreement with results obtained by overexpressing the dominant negative c-IAP1-mut in 4E3 cells (17), TRAF2 expression was not affected by TNFR2 signaling in the absence of c-IAP1. Analysis of ASK1 protein levels in TNFR2-stimu-

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responsible for activation of MAPKs in Caenorhabditis elegans (Neuronal symmetry 1 (Nsy1)) (27), Drosophila melanogaster (DASAK1) (28), and mammalian cells (18, 29). In mammalian cells, ASK1 participates in the JNK and p38 MAP signaling cascades by phosphorylating MKK4/MKK7 and MKK3/MKK6, respectively (9). ASK1 is activated in response to various extracellular and intracellular stimuli, such as lipopolysaccharide, reactive oxygen species, endoplasmic reticulum stress, and signaling through such as Fas and TNF and TNFR (30). Overexpression of TRAF2 strongly activates ASK1, and in the case of TNF an interaction between ASK1 and TRAF2 is essential for activation of the MAPK cascade (9). Binding of TRAF2 to ASK1 requires the N-terminal RING domain of the former and the C-terminal coiled-coil domain of the latter (9). Notably, both truncated (RING-deleted) TRAF2 and catalytically inactive TRAF2 activation, and although ASK1−/− cells have no obvious developmental abnormalities, ASK1−/− mouse embryonic fibroblasts have sustained activation of JNK and p38 in response to TNF and are resistant to TNF-induced cell death (10). Therefore, TNF-induced p38 and JNK activation requires and is downstream of ASK1.

This report demonstrates that c-IAP1 is responsible for the ubiquitination and subsequent degradation of TRAF2 and ASK1. It is noteworthy that we have previously shown that c-IAP1 is capable of ubiquitinating TRAF2 and that in 4E3 cells dominant negative c-IAP1 prevented TNF-induced TRAF2 down-regulation (17). Another study proposed that the ubiquititin protein ligase Siah2 is a key regulator of TRAF2 expression and cell viability in cells treated with TNF (31). Subsequent analysis, however, failed to find a defect in TNF-induced signaling or apoptosis in primary siah2−/− murine embryonic fibroblasts and CD8+ T cells (32). Our observation that TNFR2-sigaledged degradation of TRAF2 is ablated in primary c-IAP1-deficient B cells shows that this receptor-associated E3 is in fact a physiologic regulator of TRAF2 expression. Because c-IAP1 binds TRAF2 (13) and TRAF2 and ASK1 are known to inducibly associate (9), it might be thought that TRAF2 simply acts as a bridge between c-IAP1 and ASK1, providing a mechanism for the regulation of this ubiquitination. However, NIK and GCKR also associate with TRAF2 in the TNF "signalosome" yet their expression was not altered by c-IAP1 overexpression or by TNFR2-initated signals. This suggests that merely being present in a complex containing TRAF2 and c-IAP1 is insufficient to cause ubiquitination and that c-IAP1 has an inherent specificity for ASK1. Precedent for this exists in the observation that although c-IAP1 binds both TRAF1 and TRAF2 in vitro it only ubiquinitates TRAF2 (17). Moreover, c-IAP2 binds TRAF2 in vitro but does not ubiquitiniate it. The highly selective substrate specificity of the c-IAPs is further supported by the finding that although c-IAP2 expression is highly elevated in c-IAP1−/− cells (22) it could not substitute for c-IAP1 for the TNFR2-induced ubiquitination of TRAF2 and ASK1.

The controlled termination of signaling after ligand-induced activation is a key requirement for the orderly regulation of cellular processes. Cellular signals can be terminated by various mechanisms, such as protein oligomerization, translocation, phosphorylation/dephosphorylation, cleavage, or degradation. Many cell surface receptors (for example G protein-coupled receptors, receptor tyrosine kinases, cytokine and antigen receptors) inactivate and down-regulate their ligand-induced signals by internalization and lysosomal degradation (33). The ubiquitin-proteasome system, the major non-lysosomal cellular protein degradation pathway, contributes to signaling attenuation as well. In the ubiquitin-proteasome system, substrate specificity is determined by E3s, and a variety of such enzymes target critical molecules in the EGF, TGF-β, and bone morphogenetic protein signaling pathways for degradation (34–36). The present study adds the RING-containing E3 c-IAP1 as a physiologic regulator of MAPK signaling downstream of TNFR2. Its activation results in TRAF2 and ASK1 (and perhaps other as yet unidentified substrates) ubiquitination and subsequent degradation, providing an example of proteasome-regulated termination of MAP kinase signaling in the TNF pathway. Furthermore, the results suggest that TNFR2 up-regulation, which occurs in many cell types under various inflammatory conditions (37–39), may be a feedback mechanism to dampen signals generated by TNFR1 occupancy.

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