Tumor Necrosis Factor (TNF)-induced Germinal Center Kinase-related (GCKR) and Stress-activated Protein Kinase (SAPK) Activation Depends upon the E2/E3 Complex Ubc13-Uev1A/TNF Receptor-associated Factor 2 (TRAF2)*

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The stress-activated protein kinases/c-Jun NH₂-terminal kinases (SAPKs/JNKs) and the p38 mitogen activated protein kinases (MAPKs) are activated by a variety of inflammatory stimuli and stresses. Many of the cytokines of the tumor necrosis factor (TNF) family, TNF in particular, potently activate the p38 MAPKs and SAPKs. Like the other MAPKs, the SAPKs are regulated as part of a three-tiered core of protein kinases. At least two MAPK/extracellular signal-regulated kinase (ERK) kinases (MEKs) lie upstream of the SAPKs, namely SAPK/ERK kinase/JNK (JNKs) and MKK7, while multiple protein kinases, including the MEK kinases (MEKK) and the mixed lineage kinases (MLK), have been implicated as proximal elements in the core SAPK pathway (reviewed in Refs. 1 and 2).

The cytoplasmic domains of the TNF receptors (TNF-R) serve as docking sites for signaling molecules that link activated receptors to downstream signaling pathways. The TNF-R uses two classes of cytoplasmic adaptor proteins, i.e. death domain (DD) molecules and TRAFs (TNF-R-associated factors) (reviewed in Ref. 3). The type 1 TNF-R (TNF-R1) recruits the death domain protein TRADD and a TRAF (TRAF2), a critical step in TNF-induced activation of nuclear factor κB (NF-κB) and SAPKs (4, 5). Genetic and biochemical studies implicate the MEKK, MEK, and GCKR, the MEK kinase-1, as an effector in TNF-induced SAPK activation (6, 7). TRAF2 and MEKK1 co-immunoprecipitate following TNF treatment, and TRAF2 activates MEKK1 in vivo. However, the mechanism by which TRAF2 activates MEKK1 remains obscure.

Several members of the germinal center kinase (GCK) family, a group of kinases homologous to the Saccharomyces cerevisiae Ste20p, a direct upstream activator of the yeast MAP3K Ste11p, are also potent and selective activators of the SAPK pathway, suggesting that they may act in an similar fashion as proximal activators of the core SAPK pathway (8). TNFα potently activates GCK and GCKR (germinal center kinase-related) and facilitates their interaction in vivo with TRAF2 (9–11). Although the activation of GCK and GCKR depends upon the RING domain of TRAF2, both required the TRAF domain to efficiently interact with TRAF2. In addition, GCK associates with MEKK1 in vivo, and purified active GCK plus TRAF2 activates MEKK1 in vitro (12). The RING domain of TRAF2 is needed for the activation of MEKK1, although the kinase domain of GCK is dispensable. GCK and, by analogy, GCKR may function by promoting the oligomerization of MEKK1, resulting in MEKK1 autophosphorylation and activation.

Additional insights into how the recruitment of TRAF molecules to TNF family receptors activates downstream signaling pathways arose from studies of the interleukin-1 receptor, TRAF6, and the IκB kinase complex (IKK), an intermediary in NF-κB activation (13, 14). TRAF6 requires the following two factors to activate IKK: (i) a dimeric ubiquitin (Ub)-conjugating enzyme composed of Ubc13 and Uev1A and (ii) the TAK1 kinase complex. Ubc13/Uev1A and TRAF6 catalyze the formation of lysine 63-linked polyubiquitin (polyUb) chains triggering the activation of the TAK1 kinase complex, which, in turn, phosphorylates and activates IKK. Ubc13, an E2 family member, forms a dimer with Uev1A, which is structurally similar to that...
of an E2 but lacks a catalytic cysteine residue. Ubc13/Uev1A along with TRAF6, which functions as an E3 ligase in this reaction, facilitate the synthesis of Lys63-linked polyUb chains (13). This contrasts with Lys48-linked polyUb chain formation catalyzed by many other E2/E3 complexes, a modification that often targets proteins for degradation (reviewed in Ref.15). The E3 ligase activity of TRAF6 requires an intact TRAF6 RING finger domain, and one of the targets of the intermolecularly-induced Lys63-linked ubiquitination is TRAF6 itself (13, 14).

These data suggested that TNF may trigger the Ub modification of TRAF2, thereby activating intermediaries in the NF-xB and the SAPK pathways. Here we show that TNF triggers rapid Lys63-linked ubiquitination of TRAF2. Inhibiting this modification blocks TNF-induced GCKR and SAPK activation. In contrast, it has little effect on the activation of the MAP3K, ASK1, which has also been implicated as an upstream activator in the signaling pathway leading from TRAF2 to SAPK activation (16, 17). In addition, we show that GCKR is likely a substrate for the E3 ligase activity of TRAF2, as TNF triggers Lys63-linked ubiquitination of GCKR and GCKR oligomerization. This may promote MEKK1 oligomerization and activation of the SAPK-signaling module.

EXPERIMENTAL PROCEDURES

Cell Lines, Plasmids, and Constructs—The human embryonic kidney 293 and HeLa cell line was obtained from the American Tissue Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, pMT2T-TRAF2, pcDNA3-ASK1, Ubc13 and Ubc13(C87A), and pEG GST-SEK1-KR constructs were kindly provided by Dr. U. Siebenlist (NIAID, National Institutes of Health), Dr. E. Nishida (Kyoto University), Dr. Zjiang J. Chen (University of Texas), and Dr. John Kyriakis (NIAID, National Institutes of Health), respectively. The Uev1A and Ub cDNAs were obtained (by PCR from a cDNA library created from HeLa cells. The PCR products were inserted into the pcR3.1 vector. The veracity of the collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Cell lines were used. Following cell lysis (lysis buffer contained 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1% Triton X-100, and 10% glycerol), GCKR, HA-GCKR, HA-SAPK, or TRAF2 immunoprecipitates were collected with the appropriate goat anti-Ig antibody, and 10% glycerol). GCKR, HA-GCKR, HA-SAPK, or TRAF2 im-

Fig. 1. TRAF2 undergoes TNF-induced ubiquitination that depends upon Ubc13 and Ub(K63R). a, 10-cm plates of HeLa cells were transfected with constructs that express Ubc13(C87A), 10 µg (lane 3) and 5 µg (lane 6); Ubc63R), 10 µg (lane 4) and 5 µg (lane 6); Ubc13, 5 µg (lane 5); Ub(K63R), 10 µg (lanes 1 and 2). The cells were treated with TNF for 15 min or not as indicated. TRAF2 immunoprecipitates were fractionated on SDS-PAGE and immuno-

blotted with the immunoprecipitating antibody to detect TRAF2 (top). The IgH and TRAF2 bands merge. The same blot was stripped and re-probed with a Ub-specific antibody to detect Ub containing proteins (bottom). b, HeLa cells transfected with constructs expressing Ub(K63R), 4 µg, or Ub(K48R), 4 µg, were treated with TNF or not. TRAF2 immunoprecipitates were subjected to Ub immunoblotting. In addition, the cell lysates were immunoblotted for IκB and for GCKR as a loading control. c, HeLa cells were transfected with constructs expressing TRAF2, 1 µg, or TRAF2(C34A), 1 µg, in the presence of either 4 µg of Ub, Ubc63R, or UbcK63R as indicated. Some of the cells were also transfected with constructs expressing Ubc13 or Ubc13(C87A), 4 µg, together with Uev1A, 1 µg. TRAF2 immunoprecipitates were subjected to Ub immunoblotting. In addition the cell lysates were immunoblotted for TRAF2 and HA to detect HA-tagged Ubc13 or Ubc13(C87A).

RESULTS AND DISCUSSION

To test whether TNF triggers rapid TRAF2 ubiquitination, we examined endogenous TRAF2 immunoprecipitates from HeLa cells, which had been treated or not treated with TNF for 15 min, for the presence of a ladder of molecules reactive with the TRAF2 antibody, a result consistent with Ub modification. The harvested cells were suspended in a lysis buffer, and GST-TRAF2 was isolated using glutathione-Sepharose beads (Amersham Biosciences). The beads were washed twice with the lysis buffer and three times in a similar buffer but with 500 mM NaCl. The collected GST-TRAF2 samples were boiled for 5 min in SDS-sample buffer and size fractionated by SDS-PAGE prior to immunoblotting.

To test whether TNF triggers rapid TRAF2 ubiquitination, we examined endogenous TRAF2 immunoprecipitates from HeLa cells, which had been treated or not treated with TNF for 15 min, for the presence of a ladder of molecules reactive with the TRAF2 antibody, a result consistent with Ub modification. Following TNF treatment, such a ladder of molecules appeared that ranged from ~60 to 180 kDa (Fig. 1a). Because TRAF2 shares a similar molecular mass as the immunoglobulin heavy chain (IgH) used to immunoprecipitate it, unmodified TRAF2 merged with the IgH band. To verify that Ub accounts for the higher molecular weight TRAF2 molecules that we had detected, we stripped and re-probed the above blot with a Ub-specific antibody. A similar ladder of molecules resulted, although it extended to a higher molecular mass than that observed with the TRAF2 antibody, perhaps because the multiple Ub molecules needed to achieve the higher molecular masses can be more readily detected with the Ub antibody than with the TRAF2 antibody (Fig. 1a, bottom panel).
Although this result indicates that TRAF2 undergoes Ub modification, it did not address the type of Ub modification. Therefore, to determine whether Ubc13 and Ub Lys63 participated in the rapid ubiquitination of TRAF2 observed following TNF treatment, we used constructs expressing either Ubc13(C87A) or Ub(K63R). These constructs express proteins that antagonize the activities of endogenous Ubc13 and Ub, respectively. Ubc13(C87A) no longer functions as an E2 but acts to interfere with wild type Ub13 activity. The expression of Ubc13(C87A) blocked TRAF2-, TRAF6-, and TNF-induced NF-kB activation as assessed by a reporter gene assay without affecting the activation of NF-kB by NF-kB-inducing kinase (NIK) or Tax (10). Ub(K63R) cannot be used to create Lys63-linked polyUb and thereby competes with wild type Ub for the generation of Lys63-linked polyUb chains (13). We found that the expression of high amounts of either Ubc13(C87A) or Ub(K63R) significantly blocked both the appearance of the slower mobility TRAF2 molecules as well as the appearance of Ub in the TRAF2 immunoprecipitates (Fig. 1e). The origin of the ~100 kDa band detected with the TRAF2 antibody and not with the Ub antibody is unknown, although its mobility is that of a TRAF2 dimer (Fig. 1a, lane 3). Surprisingly, the simple overexpression of Ubc13 and Ub also caused TRAF2 polyubiquitination. In contrast, the overexpression of Ubc13(C87A) and Ub(K63R) did not (Fig. 1a). As a further specificity control, we compared the effects of expressing Ub(K63R) to that of Ub(K48R), which should inhibit Lys48-linked polyUb chain formation. As before, Ub(K63R) blocked, whereas Ub(K48R) enhanced the detection of polyUb-modified endogenous TRAF2 (Fig. 1b). Interestingly, both mutants interfered with TNF-induced IκB degradation. Presumably, the expression of Ub(K63R) inhibited TRAF2 signaling, whereas Ub(K48R) interfered with the targeting of IκB for proteasomal destruction. Ub(K63R), but not Ub or Ub(K48R) also interfered with the TRAF2 polyubiquitination that occurred following overexpression of Ubc13 (Fig. 1c). Underscoring the importance of the E3 ligase activity of TRAF2, a mutation in TRAF2 at a site predicted to cripple its E3 ligase activity (14) resulted in a TRAF2 protein that failed to undergo Ub modification following Ubc13 overexpression.

Having established that TRAF2 likely undergoes Lys63-linked polyubiquitination, we tested whether this modification contributes to the ability of TRAF2 to activate the SAPK pathway and GCKR. We transfected constructs that express HA-SAPK, FLAG-GCKR, and TRAF2 into HEK 293 cells, a highly transfectable cell line that requires GCKR for TNF-induced SAPK activation (9). To assess GCKR and SAPK kinase activities, we performed in vitro kinase assays with immunoprecipitated GCKR or SAPK using MBP and c-Jun(1–79) as substrates, respectively (Fig. 2a). By intention, we expressed relatively low amounts of FLAG-GCKR to avoid significant SAPK activation by GCKR alone. As expected, the addition of TRAF2 enhanced both GCKR and SAPK activation. When we co-expressed low amounts of Ubc13(C87A) and Ub(K63R) together or antisense Ubc13 along with Ub(K63R), we blocked the in vitro kinase activities of GCKR and SAPK (Fig. 2a). Whereas the expression of low amounts of Ub(K63R) partially blocked GCKR and SAPK activation, the addition of either the antisense Ubc13 or Ub(C87A) further enhanced the inhibition. In contrast, the expression of the inhibitors had little effect on GCKR-mediated SAPK activation even when we expressed higher amounts of GCKR (data not shown). We also showed that, although the expression of Ub(K63R) blocked TRAF2-induced GCKR and SAPK activation, the expression of Ub(K48R) did not (Fig. 2b).

Having relied on a transfection system, we checked the importance of Ubc13 in TNF-induced activation of endogenous GCKR and SAPK. We treated HEK 293 cells with TNF or not, immunoprecipitated endogenous GCKR, checked its activity by an in vitro kinase assay, and immunoblotted for pSAPK. As expected, the treatment of HEK 293 cells with TNF increased the activity of endogenous GCKR and elevated the levels of pSAPK. The expression of either Ubc13(C87A) and Ub(K63R) or antisense Ubc13 along with Ub(K63R) nearly abolished it (Fig. 2c). Both isoforms of pSAPK were resolved in panel c, whereas a non-gradient gel failed to resolve the two isoforms in panel b. Finally, just as the expression of the RING finger domain deleted a version of TRAF2 (9), the expression of the E3 ligase-crippled TRAF2, TRAF2(C87A), also failed to activate GCKR (Fig. 2d).

![FIG. 2. Ubc13 and Ub(K63R) are needed for TRAF2- and TNF-induced GCKR and SAPK activation.](Image)
expression of Ubc13(C87A). HEK 293 cells were transfected with expression of Ubc13(C87A).

GST alone. TNF treatment enhanced the recruitment of endog-

pSAPK in HEK 293 cells as compared with those expressing

GST-TRAF2 became ubiquitinated. The expression of GST-

Of note, the amount of GST-TRAF2 detected in each of the

Ub in the GST-TRAF2 pull downs, but not the GST pull downs.

resulted in a dramatic dose-dependent increase in the amount of

UB in the GST-TRAF2 pull downs, but not the GST pull downs.

Next, we examined the effect of Ubc13(C87A) on TRAF2- and

TNF-mediated ASK1 activation. ASK1 has also been impli-
cated in linking TRAF2 to the SAPK pathway in TNF signaling
(16, 17). We transfected constructs expressing ASK1 and

TRAF2 in the presence or absence of the Ubc13(C87A) con-

struct into HEK 293 cells or treated the cells with TNF rather
than transfecting the TRAF2 construct. Afterward, we per-
formed an ASK1 in vitro kinase assay using ASK1 immunopre-
icipitates with the substrate GST-SEK1-KR. Although the

Ubc13(C87A) construct again blocked TRAF2-mediated GCKR

activation, it had a very minor effect on TRAF2- or TNF-

induced ASK1 activation (Fig. 3). We repeated the experiments

but substituted MBP, also an ASK1 substrate, for GST-

TRAF2 pull downs prepared from HEK 293 cells transfected

with GST-TRAF2. The precipitates were subjected to immu-

noblotting using antibodies specific for GST, TRAF2, GCKR, and Ub.

longevity proteins as well.

It has been suggested that oligomerization of TRAF2 or

TRAF6 through their carboxy-terminal TRAF domains is

needed to activate IKK and the SAPK pathway by proinflam-
matory cytokines (6). To determine whether TRAF2 ubiquiti-
nation enhances TRAF2-TRAF2 and TRAF2-GCKR interac-
tions, we constructed a GST-TRAF2 expression vector. When

we transfected HEK 293 cells with this construct, the GST-

TRAF2 pull downs contained low amounts of Ub, whereas

similar pull downs prepared from HEK 293 cells transfected

with GST alone lacked Ub (Fig. 4a). The GST-TRAF2 pull
downs also contained low amounts of endogenous TRAF2 and

GCKR. Consistent with our initial results, TNF treatment re-
sulted in a dramatic dose-dependent increase in the amount of

Ub in the GST-TRAF2 pull downs, but not the GST pull downs.

Of note, the amount of GST-TRAF2 detected in each of the
lanes appeared similar, indicating that only a minority of the

GST-TRAF2 became ubiquitinated. The expression of GST-

TRAF2 also potently enhanced the TNF-induced appearance of

pSAPK in HEK 293 cells as compared with those expressing

GST alone, TNF treatment enhanced the recruitment of endog-

enous TRAF2 and GCKR to GST-TRAF2, and blocking Ubc13

activity strikingly reduced it. Expression of Ubc13(C87A) also
decreased the appearance of polyUb-modified proteins in the

GST-TRAF2 pull downs and the presence of pSAPK in the

TNF-treated HEK 293 cells (Fig. 4a). Because the GST-TRAF2

pull downs included endogenous TRAF2 and GCKR, the

polyUb-modified proteins detected in the Ub immunoblot may
not only include GST-TRAF2 but also modified endogenous

proteins as well.

To test whether TRAF2 ubiquitination also led to GCKR

oligomerization, we co-transfected constructs expressing HA-

GCKR and FLAG-GCKR along with TRAF2 or treated the cells
with TNF. We found significantly higher amounts of FLAG-GCKR in the HA-GCKR immunoprecipitates following either expression of TRAF2 or TNF treatment, which Ubc13(C87A) expression blocked (Fig. 4b). Thus, the assemblies of stable higher order TRAF2 and GCKR complexes and the optimal recruitment of GCKR to TRAF2 following TNF treatment depends, at least in part, upon Ubc13 and the E3 ligase activity of TRAF2.

The interaction of GCKR with TRAF2 suggested that GCKR might serve as a TRAF2 substrate. To test that possibility, we transfected HeLa cells with constructs that expressed either HA-GCKR or FLAG-GCKR in the presence or absence of constructs expressing TRAF2, Ubc13, and Ub. We extensively washed HA immunoprecipitates to remove any co-immunoprecipitating proteins and immunoblotted for the presence of Ub. From those cells transfected with the construct expressing HA-GCKR, but not FLAG-GCKR, we observed a smear of molecules between 110 and 200 kDa that reacted with the Ub antibody (Fig. 5a). A similar although less intense smear appeared when we re-blotted with the HA antibody. These results indicate that GCKR can undergo Ub modification. To test the involvement of Ubc13 and Ub(K63R), we transfected HeLa cells with constructs expressing TRAF2 and HA-GCKR in the presence of constructs expressing either Ubc13 and Ub, Ubc13(C87A) and Ub, Ubc13 and Ub(K48R), or Ubc13 and Ub(K63R). We found that the expression of Ubc13(C87A) or Ub(K63R) inhibited the detection of a polyUb ladder in the HA-GCKR immunoprecipitates, whereas Ub(K48R) enhanced it (Fig. 5b). Similarly, the expression of Ubc13(C87A), Ub(K63R), or TRAF2(87–501) inhibited the appearance of a polyUb ladder in the HA-GCKR immunoprecipitates following 15 min of TNF treatment. As expected, TNF enhanced the presence of pSAPK, and each of the three inhibitors reduced its induction. Besides these data, a Ub immunoblot of endogenous GCKR immunoprecipitated from TNF-treated cells also contains a smear of molecules consistent with Ub-modified GCKR; however, the GCKR antibody has not been useful for re-blotted GCKR. Finally, we tested whether the impairment of the E3 ligase activity of TRAF2 would interfere with the appearance of the polyUb ladder detected in the HA-GCKR immunoprecipitates. In contrast to the overexpression of TRAF2, the expression of a similar amount of TRAF2(C3A) failed to induce GCKR polyUb or SAPK activation. Furthermore, the same construct inhibited TNF-induced SAPK activation and GCKR polyUb.

How TRAF2 activates GCKR remains unknown, although it does depend upon the E3 ligase activity of TRAF2, which we show promotes or perhaps stabilizes the oligomerization of TRAF2 and GCKR. This, in turn, likely facilitates GCKR transautophosphorylation, which may alter the conformation of GCKR, thereby facilitating MEKK1 oligomerization and its activation of subsequent steps in the SAPK pathway as has been recently proposed for GCK (12). Alternatively, TRAF2-mediated ubiquitination of GCKR may have a direct role in kinase activation, resulting in a similar scenario. The identification of the specific lysines in TRAF2 that undergo a Lys63-linked chain modification should assist in discriminating the relative importance of TRAF2 self-ubiquitination versus its modification of other substrates such as GCKR for TNF-mediated GCKR and SAPK activation. Besides functioning as a target and catalyst for Lys63-linked Ub chains, TRAF2 also undergoes tagging with Lys48-linked chains. Signaling through the TNF-RII, where the anti-apoptotic molecules c-IAP1 and c-IAP2 function as E3 ligases, generates polyUb-modified TRAF2 (20). In addition, TRAF2 may also use a classical E2 for self-ubiquitination and proteasomal dependent degradation (21). Thus, TRAF2 may undergo either Lys48- or Lys63-linked Ub modification, each with a far different functional consequence. Supporting that concept, the overexpression c-IAP1 significantly inhibited TRAF2-mediated GCKR activation.2

In this report we demonstrate a requirement for Ubc13 in TNF-mediated activation of GCKR and SAPK and for the optimal recruitment of GCKR to TRAF2. Ubc13/UevA1 along with TRAF2 catalyze the synthesis of Lys63 polyUb chains that modify TRAF2 and likely GCKR. In the absence of these modifications, TNF treatment fails to activate the SAPK pathway due to a failure to recruit and/or activate upstream kinases that lead to pathway activation. Polyubiquitination through

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2 C.-S. Chi, unpublished observation.

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**Fig. 5.** GCKR undergoes Ubc13-dependent ubiquitination. a, HeLa cells were transfected with constructs expressing HA-GCKR (0.5 μg, lanes 1 and 2) or FLAG-GCKR (0.5 μg, lanes 3 and 4) plus constructs expressing Ubc13 (0.1 μg) and Uev1A (0.1 μg) and Ub, 1 μg, as indicated. HA-immunoprecipitates were subjected to immunoblotting with Ub-specific antibody, which reacts with both HA- and FLAG-GCKR. b, HeLa cells were transfected with constructs expressing HA-GCKR (0.5 μg), TRAF2 (1 μg, lanes 2–5) was expressed, or the cell was treated with TNF (150 ng/ml, lanes 7–10). Constructs expressing Ubc13 and Uev1A, Ubc13(C87A), Ub, UbcK48R, TRAF2(87–501), or Ub(K63R) were transfectected as indicated. TRAF2(87–501) (TRAF2**) has a mobility on SDS-PAGE that merges with that of β-actin. HA-GCKR immunoprecipitates were subjected to immunoblotting with a Ub-specific antibody. Levels of HA-GCKR, TRAF2, and β-actin in the cell lysates are shown. The induction of pSAPK in cell lysates verified that the TNF treatment activated the SAPK pathway. c, HeLa cells were transfected with constructs expressing HA-GCKR (0.5 μg), Constructs expressing Ubc13, Ub, TRAF2, and TRAF2(C3A) (designated as TRAF2**) were transfected as indicated. Cells used for lanes 4 and 5 were treated with TNF (150 ng/ml) for 15 min prior to lysis. HA-GCKR immunoprecipitates were subjected to immunoblotting with an Ub-specific antibody. Levels of HA-GCKR, TRAF2, β-actin, and pSAPK in the cell lysates are shown.
Lys^63-linked ubiquitin has emerged as a general modification used to activate upstream kinases in the SAPK and NFκB pathways.

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