Activation of Stress-activated Protein Kinase/c-Jun N-terminal Kinase, but Not NF-κB, by the Tumor Necrosis Factor (TNF) Receptor 1 through a TNF Receptor-associated Factor 2- and Germinal Center Kinase Related-dependent Pathway*

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A key step by which tumor necrosis factor (TNF) signals the activation of nuclear factor-κB (NF-κB) and the stress-activated protein kinase (SAPK, also called c-Jun N-terminal kinase or JNK) is the recruitment to the TNF receptor of TNF receptor-associated factor 2 (TRAF2). However, the subsequent steps in TRAF2-induced SAPK and NF-κB activation remain unresolved. Here we report the identification of a TNF-responsive serine/threonine protein kinase termed GCK related (GCKR) that likely signals via mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase 1 (MEKK1) to activate the SAPK pathway. TNF, TRAF2, and ultraviolet (UV) light, which in part uses the TNF receptor signaling pathway, all increased GCKR activity. A TRAF2 mutant, which inhibits both TRAF2-induced NF-κB and SAPK activation, blocked TNF-induced GCKR activation. Finally, interference with GCKR expression impeded TRAF2- and TNF-induced SAPK activation but not that of NF-κB. This suggests a divergence in the TNF signaling pathway that leads to SAPK and NF-κB activation, which is located downstream of TRAF2 but upstream of GCKR.

Tumor necrosis factor (TNF) is a pleiotropic cytokine, which plays a major role in inflammation (reviewed in Ref. 1). TNF binds two distinct TNF receptors of 55 kilodaltons (TNFR1) and 75 kilodaltons (TNFR2) (reviewed in Ref. 2). TNF-induced trimerization of TNFR1 triggers an association with TNFR1-associated death domain protein (TRADD), which recruits Fas-associated death domain (FADD, also known as MORT1) protein and TRAF2 (3, 4). FADD participates in an apoptosis pathway and TRAF2 signals the activation of the NF-κB activation by both TNFR1 and the B lymphocyte co-receptor CD40 (3–5). TRAF2 contains a conserved C-terminal homology region termed the TRAF domain, which interacts with TRADD, and an N-terminal ring finger required for signaling the activation of NF-κB and SAPK (5–7).

The SAPK pathway, similar to other extracellular signal-regulated kinase (ERK) pathways, consists of a 3-tiered core of protein kinases in which a mitogen-activated protein kinase (MAPK/ERK) kinase (MEK) activates a MAPK/ERK kinase (MEK) which in turn activates SAPK (8, 9). MEKK1, which activates the SAPK pathway via a MEK termed SEK1 (10–13), has been implicated in TNF-induced SAPK activation (6). The mixed lineage kinase MLK-3 (14–16) also signals the SAPK pathway through SEK1 (17). Two other MEKKs, both of which are implicated in TNF-signaling, are NIK, which interacts with TRAF2 and signals NF-κB activation (18), and ASK1, which is TNF-inducible and activates the SAPK pathway and the related p38 pathway (19).

Above the level of the three-tiered core in the SAPK pathway are two related protein kinases, GCK (20, 21) and HPK1 (22, 23), which likely activate the SAPK pathway via MEKK1 and/or MLK3; however, they are unlikely to account for TNF-induced SAPK activation. HPK1 has a limited range of tissue expression (22, 23), inconsistent with the widespread expression of TNFR1, and although widely expressed, GCK is only marginally affected by TNF (21). However, here we report the identification of a third family member termed GCK related (GCKR) that is widely distributed, TNF-responsive, and a major mediator of TNF-induced SAPK activation.

**EXPERIMENTAL PROCEDURES**

Cell Lines, Plasmids, and Antibodies—The PC-12, COS, HeLa, Jurkat, K562, 293, and MOLT-4 cells were obtained from the American Type Culture Collection. The 293T cell line was obtained from Dr. O. Witte (UCLA) following permission from Dr. D. Baltimore (Massachusetts Institute of Technology). The CA46 and MC116 cells were obtained from Dr. I. Magrath (National Institutes of Health), and the 1108 cells were from Dr. W. Klee (National Institutes of Health). The pMT3-HA-SAPK-p46 plasmid was provided by Dr. J. Kyriakis (Harvard Medical School). The pMT2T-TRAF2 and pcDNA3-MEKK1 (K432M) plasmids were provided by Antonio Leonardi and Heidrun Ellinger (National Institutes of Health). The pCR3-TRAF2 (87–501) was created by PCR subcloning the appropriate fragment amplified from pMT2T-TRAF2 into pCR3. The pcDNA3-ASK1 (K562H) was provided by Dr. E. Nishida (Kyoto University). The anti-HA (12CA), anti-FLAG, and anti-phosphotyrosine monoclonal antibodies were purchased from Boehringer Mannheim, Eastman Kodak Co., and Upstate Biotechnology. The GCKR polyclonal antiserum was generated in rabbits by immunizing with a peptide (RKEEARDMEM) coupled to Keyhole limpet hemocyanin. The GCK polyclonal antisem was has been previously described (20).

GCKR cDNA Cloning and Construction of GCKR Expression Vectors—A human spleen cDNA library (CLONTECH) was screened with a PCR product amplified by reverse transcription PCR from human tonsil RNA using primers based on the Z52426 reported nucleotide sequence. Four overlapping cDNA clones were identified, and a com-
posite cDNA of 2746 base pairs was obtained. A GCKR clone that encompassed the open reading frame was subcloned into pcRIII (Invitrogen) in both orientations to create pCR3-GCKR and pCR3-GCKR(A/S). The nucleotide sequences of the cDNA clones and constructs were determined using an automated DNA sequencer. The inserts for plasmids pCDNA3-HA-GCKR and pFLAG-CMV2-GCKR were generated by PCR with the appropriate restriction sites incorporated into the primers and using pCR3-GCKR as a template. pCR3-GCKR-178A was created from overlapping PCR products, one of which was amplified with a mutating primer using pCR3-GCKR as a template. The two products served as a new template to generate a PCR product that spanned the coding region and contained the mutation, which was subcloned into pCR3 and pFLAG-CMV2 (Kodak). Multiple tissue Northern blots were purchased from CLONTECH and processed according to manufacturer recommendations.

In Vitro Kinase Assays, Immunoblotting, and CAT Assay—293T cells were exposed to TNF (Endogen) or UV light source (Stratagene) as indicated. GCKR and GCK immunoprecipitates were assayed for in vitro kinase activity as described (21). For the transfection studies, either a calcium phosphate method (12) or Superfect was used (Qiagen). 10-cm plates of 293T or 293 cells containing approximately 2 × 10^6 were transfected with various expression vectors or control vectors as indicated, and the total amount of DNA transfected in any one experiment was held constant among the different experimental conditions. After 48 h, cells were treated with anisomycin (10 μg/ml for 15 min), TNF, or UV light. Cell lysis, anti-HA immunoprecipitation, and SAPK immunocomplex assays were performed as described (21). The GCKR (1300 dilution), HA, FLAG, and phosphotyrosine immunoblots were performed using standard methodology with an additional amplification step using a biotinylated secondary antibody. The signals were detected by enhanced chemiluminescence (ECL, Amersham Corp.). The transfections for the NF-κB CAT assays were performed as above. Two days after the transfection, the cells were harvested and lysed, the protein concentrations were normalized, and CAT activity was measured by scintillation counting. The reporter construct contained two NF-κB sites linked to the CAT gene and was provided by Dr. Keith Brown (National Institutes of Health). The transfection efficiency was monitored by co-transfection of a β-galactosidase reporter gene whose activity varied less than 10% between samples.

RESULTS

Using the nucleotide sequence of a PCR product, Z25426, that had been amplified from a gene clearly related to GCK, we isolated a cDNA that encompassed an open reading frame predicted to encode an 846 amino acid protein kinase with a molecular mass of 95 kilodaltons, which we termed GCKR (Fig. 1A). GCKR possesses an amino-terminal catalytic domain 73% identical with that of GCK and related to those of the yeast protein kinases SPS1 and STE20 (24, 25), the human and mouse PAK protein kinases (26), and HPK1 (22, 23). The catalytic domain of HPK1 shares approximately 66% amino acid identity with GCK and GCKR, but overall HPK1 shares less identity with GCKR than does GCK (40 versus 54%, Fig. 1A). GCKR and GCK also contain three conserved regions in their putative regulatory domains of approximately 80 amino acids each (GCKR amino acids 485–568, 604–689, and 762–842 share 66%, 61%, and 72% identity with GCK amino acids 459–543, 579–665, and 735–815, respectively), whereas over similar regions, GCKR and human HPK1 share 47%, 51%, and 34% identity. While HPK1 has four potential SH3 binding sites (P1–P4) (23), GCKR shares only the P2 site, and it lacks the P3 and P4 sites, which bind the SH3 domain of MLK-3. To determine the range of GCKR mRNA expression, we analyzed GCKR mRNA transcripts in a variety of tissues. We found a 4.4-kilobase GCKR mRNA transcript in all tissues examined (Fig. 1B). In addition, we generated a rabbit anti-GCKR antiserum that immunoblotted a 97-kDa band in all cell lysates (Fig. 1C) examined although we detected relatively low levels in the B cell lines GA46, MC116, NALM-6, and RAMOS (bands detected on a longer exposure).

Similar to GCK and HPK1, GCKR activated the SAPK pathway. 293T cells co-expressing a hemagglutinin (HA)-tagged SAPK and either GCKR, GCK, or both showed a 3.5-, 5.5-, or 4.5-fold increase in HA-SAPK activity, respectively (Fig. 2A). These levels compared favorably with those achieved by anisomycin, an established SAPK activator. The increase in SAPK activity depended upon the catalytic activity of GCKR since a GCKR kinase-deficient mutant, GCKR (T178A), contains a threonine to alanine substitution at amino acid position 178 and did not activate the pathway (Fig. 2B). Like GCK, GCKR phosphorylated the prolinc-rich myelin basic protein (MBP), while GCKR (T178A) did not (Fig. 2C). Also similar to previous studies with GCK (21), GCKR did not efficiently activate either an epitope-tagged MAPK or an epitope-tagged p38 kinase, and a catalytically inactive form of SEK1 inhibited GCKR-mediated SAPK activation (data not shown).

To determine whether GCKR may be involved in TNF-induced SAPK activation, we exposed 293T cells to 50 or 100 ng of TNF for varying duration times, immunoprecipitated GCKR, and performed GCKR immune complex kinase assays. Consistent with TNF-induced GCKR activation, GCKR immune complexes from TNF-treated cells contained a marked elevation in in vitro protein kinase activity (Fig. 3A). UV light, which in part utilizes TNF receptor signaling in its induction of SAPK (27), also increased GCKR activity. Exposing 293T cells to increasing amounts of UV light resulted in a dose-dependent activation of GCKR (Fig. 3B). In contrast, UV light did not significantly activate endogenous GCK (Fig. 3C). Phosphotyrosine immunoblots of the GCKR immunoprecipitates that had been subjected to an in vitro kinase assay revealed a 97-kDa UV light-inducible band consistent with UV light-induced GCKR tyrosine phosphorylation. Autoradiography of the same immunoblot revealed phosphorylation of a 97-kDa band (Fig. 3D). Since UV light utilizes other cell surface receptors besides the TNF receptor in activating SAPK in HeLa cells (27), we examined the effects of optimal concentrations of interleukin-1 (IL-1), epidermal growth factor (EGF), and TNF on GCKR in those cells. EGF and IL-1 had minimal effects on GCKR activity; however, their combination with TNF resulted in a marked increase in activity, exceeding that achieved with UV light (Fig. 3E).

Because TNF-induced SAPK activation requires TRAF2 (6, 7), we examined the effect of TRAF2 on GCKR activity by co-transfecting TRAF2 along with HA-GCKR into 293T cells. In these experiments, we used only 1 μg of HA-GCKR expression vector to reduce its basal level of activity. The presence of TRAF2 increased HA-GCKR activity by 5-fold (Fig. 3F). In addition, co-transfection of HA-GCKR along with the mutant TRAF2 (87–501), which blocks TNF-induced SAPK activation (7), blocked TNF-induced GCKR activation (Fig. 3F). These results indicated that GCKR is a downstream target of TRAF2 in TNF receptor signaling; however, they do not establish its importance in TNF-induced SAPK activation.

To examine whether GCKR might mediate TNF-induced SAPK activation required inhibiting endogenous GCKR. We used two approaches, one to reduce endogenous levels by using a GCKR antisense plasmid and the second to block downstream GCKR signaling by using the catalytically inactive form. The GCKR antisense plasmid markedly inhibited HA-GCKR-induced SAPK activation and reduced HA-GCKR protein levels (data not shown). Furthermore transient transfection of the GCKR antisense construct reduced endogenous GCKR protein levels by approximately 50%, suggesting a significant reduction in GCKR levels in the transfected cells (Fig. 4A). Next, we introduced the GCKR antisense construct along with HA-SAPK into 293T cells. The antisense construct, but

2 The Genbank accession number is pending.
not a control vector, reduced UV light-induced HA-SAPK activity by 75% in cells exposed to 80 joules of energy and by 35% in cells exposed to 160 joules (Fig. 4B, lanes 1–5). Consistently, the antisense construct reduced UV-induced SAPK activation more effectively at lower energy levels. The GCKR antisense construct also inhibited TRAF2-induced SAPK activation. Co-transfection of the construct along with TRAF2 and HA-SAPK into 293T cells reduced SAPK activity 50–70% in the HA immunoprecipitates, as compared with a control construct (Fig. 4B, lanes 6–9). In contrast, it had no effect on anisomycin-induced SAPK activation (Fig. 4B, lanes 10–12). In addition, co-transfection of the GCKR antisense construct, 0.5, 1.0, or 2.0 \( \mu \)g, along with HA-SAPK into 293T cells reduced the TNF-induced SAPK activity in HA immunoprecipitates by 34, 72,

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**Fig. 1. GCKR amino acid sequence and its expression in various cell lines.** A, the GCKR predicted amino acid sequence aligned to GCK and HPK1 amino acid sequence. All three proteins have an N-terminal catalytic domain: amino acids 21–278 in GCKR, 15–272 in GCK, and 18–275 in HPK1. Identical amino acids are indicated. The potential SH3 binding sites are underlined. B, Northern blot analysis of multiple tissues. A multiple tissue Northern blot was sequentially hybridized with a full-length GCKR cDNA and a \( \beta \)-actin cDNA. The GCKR blot was exposed overnight. C, immunoblot analysis of GCKR in various cell lines.
and 78%, respectively (Fig. 4C). Finally, we determined whether the catalytically inactive form of GCKR impaired TRAF2-induced SAPK activation. Co-transfection of 293 cells with HA-SAPK and TRAF2 in the presence of increasing concentrations of GCKR(T178A) markedly impaired TRAF2-induced SAPK activation (Fig. 4D). These results indicate that in 293 cells, UV light and TNF signal SAPK activation by activating GCKR.

**Fig. 2.** GCKR activates the SAPK pathway while GCKR-178A does not. A, 293T cells were transfected with pCR3-GCK (5 µg), pCR3-GCKR (5 µg), pCR3-GCK and pCR3-GCKR together (2.5 µg each), or a control vector (5 µg) along with HA-SAPK (1 µg). After 48 h, HA-SAPK immunoprecipitates were assayed for protein kinase activity using GST-Jun(79) as a substrate. Anisomycin-treated cells (10 µg/ml) were exposed for 15 min before lysis. HA immunoblotting was performed by ECL. B, a GCKR mutant (pFLAG-CMV2-GCKR-178A, 5 µg), pFLAG-CMV2-GCKR (5 µg), or control vectors along with pMT3-HA-SAPK (1 µg) were transfected into 293T cells. SAPK assays were performed as above. C, pFLAG-CMV2-GCKR (5 µg), pFLAG-CMV2-GCKR-178A (5 µg), or a control vector was transfected into 293T cells. After 48 h, FLAG-GCKR immunoprecipitates were assayed for kinase activity using MBP as a substrate. Following autoradiography, the amount of \([^{32}P]ATP\) incorporated into MBP was measured by excising the bands and liquid scintillation counting. All experiments were performed in duplicate, and at least three times with similar results.

**Fig. 3.** GCKR is potently induced by TNF, UV light, and TRAF2. A, activation of endogenous GCKR activity by TNF. 293T cells (2 × 10^6) were exposed to 50 ng/ml of TNF for 15 or 30 min or to 50 ng/ml or 100 ng/ml for 20 min. The cells were lysed and GCKR was immunoprecipitated. The immunoprecipitates were assayed in an in vitro protein kinase assay using myelin basic protein as a substrate. Immunoblotting verified equivalent levels of GCKR in the immunoprecipitates (not shown). B, activation of endogenous GCKR activity by UV light. 293T cells were exposed to varying amounts of UV light (joules indicated above). GCKR immunoprecipitates were assayed as above. C, UV light does not induce GCK activity. 293T cells were exposed to varying amounts of UV light (joules), and protein kinase activity was assayed in GCK immune complexes. D, UV light triggers GCKR tyrosine phosphorylation. 293T cells were exposed to increasing amounts of UV light (joules), and the amount of protein kinase activity was assayed as above (MBP). The GCKR immunoprecipitates from the in vitro kinase assay were also analyzed by immunoblotting with an anti-phosphotyrosine antibody (second panel), and the immunoblot was exposed via autoradiography (third panel). Equivalent levels of GCKR were detected by immunoblotting. E, GCKR activation by IL-1, TNF, and EGF. HeLa cells were exposed for 15 min to IL-1 (100 ng/ml), TNF (100 ng/ml), or EGF (1 µg/ml), the combination of the three, or 100 µg/ml of UV light (joules). GCKR immunoprecipitates were subjected to an in vitro kinase assay as above. GCKR levels were assayed by immunoblotting (second panel). F, TRAF2 induces GCKR activity, and a TRAF2 mutant blocks TNF-induced GCKR activation. 293T cells were transfected with pMT2T-TRAF2 (2 µg) or control vector (2 µg) along with pcDNA3-HA-GCKR (1 µg). HA immunoprecipitates were assayed in an in vitro kinase assay as above (lanes 1 and 2). 293T cells were also transfected with pcDNA3-HA-GCKR (1 µg) and exposed to TNF (50 ng/ml) for 15 min, and HA-GCKR immunoprecipitates were assayed for activity (lanes 3–6). HA immunoblot is shown below. All the experiments were repeated a minimum of three times with similar results with the exception of the UV-GCK experiment, which was performed twice.
Based on the previous observation that a catalytically inactive form of MEKK1 inhibited TNF-induced SAPK activation (6), we examined whether a similar construct inhibited GCKR-induced SAPK activation. We co-transfected 293T cells with HA-SAPK, GCKR, and either ASK1 (K709M) (19) or MEKK1 (K432M). The catalytically inactive form of MEKK1 significantly impaired GCKR-induced SAPK activation, whereas the catalytically inactive form of ASK1, which inhibits TNF-induced apoptosis (19), had no effect (Fig. 4E). We also observed that MEKK1 (K432M) impaired TNF-induced SAPK activation but ASK1 (K709M) did not.3

Finally, since both MEKK1 and TRAF2 have been reported to activate NF-κB (6, 7, 28), we tested whether GCKR does. Cell lysates from 293T cells transfected with GCKR and an NF-κB reporter construct were assayed for reporter gene activity (Fig. 5). GCKR did not induce NF-κB reporter gene activity although TRAF2 and TNF did. Also, the GCKR antisense construct did not block TNF-induced activation of the NF-κB reporter construct. The reduction of TNF-induced reporter gene expression by GCKR, but not GCKR (T178A), was consistently observed although of unknown significance.

**DISCUSSION**

GCKR is a potent activator of the SAPK pathway and is responsive to UV light, TNF-α, and TRAF2. We propose that

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3 C. Shi, unpublished observation.
activators (20). The limited tissue distribution of HPK1 suggests that it may be linked to a similarly distributed receptor (22, 23).

While TNF-induced GCKR activation required TRAF2, we have not determined the mechanism that couples TRAF2 to GCKR activation. Since a serine/threonine protein kinase and a phosphoprotein of the molecular mass of GCKR immunoprecipitates with TNFR1 following TNF signaling (29), we examined whether GCKR might be assembled into the TNFR1 signaling complex via an interaction with TRAF2. However, in preliminary experiments, we have been unable to co-immunoprecipitate TRAF2 along with GCKR using 293T cells transfected with expression vectors that encode for epitope-tagged versions of TRAF2 and GCKR. Further experiments examining this issue are in progress.

The downstream kinases involved in GCKR-induced SAPK remain to be delineated. ASK1 is an interesting candidate as it is also TNF-inducible; however, it is involved in TNF-induced apoptosis and TRAF2-induced SAPK activation proceeds through a non-cytotoxic mechanism (19). Also arguing against ASK1, a catalytically inactive form of ASK1 did not block GCKR-induced SAPK activation, nor did it block TNF-induced SAPK activation.3 Another candidate is MEKK1, particularly since a catalytically inactive form of MEKK1 inhibited TNF-induced SAPK activation (6). However, the studies with MEKK1 (K432M) have been contradictory, leading to diametrically opposed conclusions concerning the role of MEKK1 in NF-κB activation (6, 28). While MEKK1 (K432M) inhibited GCKR-induced SAPK activation, additional studies will be needed to substantiate that GCKR signals via MEKK1. Since MLK-3 is a downstream target of both GCK and HPK1 (23), we are also interested to determine whether GCKR may also use MLK-3 to signal the SAPK pathway. MEKK2 and MEKK3 seem less likely to link GCKR to the SAPK pathway as they activate both the SAPK and MAPK pathways, whereas GCKR specifically activates the SAPK pathway (30–31). Finally, a newly identified MEKK, MEKK4, which specifically activates the SAPK pathway, is another potential target of GCKR. However, it appears to be a downstream mediator of Cdc42 and Rac since a dominant negative form of MEKK4 blocked Cdc42 and Rac induced SAPK activation (32).

While it is clear that GCKR is a mediator of TNF-induced SAPK activation, we have no evidence that GCKR is involved in TNF-induced NF-κB activation. GCKR failed to activate NF-κB as assessed by the use of an NF-κB reporter construct, and the antisense GCKR, which inhibited TNF-induced SAPK activation, failed to inhibit TNF- or TRAF2-induced NF-κB activation. Furthermore, GCKR’s activation of the SAPK pathway is also inhibited by MEKK1 (K432M) also failed to activate NF-κB in co-transfection assays. Thus, either the TNF receptor signaling pathway leading to SAPK and NF-κB diverges above the level of MEKK1 or perhaps MEKK1 can be independently activated or segregated into autonomous signaling modules such that it differentially activates the IκB kinase complex and SEK1.

In the course of these studies an identical kinase to GCKR was independently isolated by two groups and termed KHS and GLK. Similar to our results, KHS and GLK were shown to activate the SAPK pathway (33, 34). However, our studies significantly extend those observations to show that GCKR is a major mediator of TNF- and UV-induced SAPK activation in some cell types. Our current studies are aimed at determining how TRAF2 activates GCKR and clarifying whether MEKK1 is the major downstream target of GCKR.

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