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TANK Potentiates Tumor Necrosis Factor Receptor-Associated Factor-Mediated c-Jun N-Terminal Kinase/Stress-Activated Protein Kinase Activation through the Germinal Center Kinase Pathway

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Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) are mediators of many members of the TNF receptor superfamily and can activate both the nuclear factor κB (NF-κB) and stress-activated protein kinase (SAPK; also known as c-Jun N-terminal kinase) signal transduction pathways. We previously described the involvement of a TRAF-interacting molecule, TRAF-associated NF-κB activator (TANK), in TRAF2-mediated NF-κB activation. Here we show that TANK synergized with TRAF2, TRAF5, and TRAF6 but not with TRAF3 in SAPK activation. TRAF2 and TANK individually formed weak interactions with germinal center kinase (GCK)-related kinase (GCKR). However, when coexpressed, they formed a strong complex with GCKR, thereby providing a potential mechanism for TRAF and TANK synergy in GCKR-mediated SAPK activation, which is important in TNF family receptor signaling. Our results also suggest that TANK can form potential intermolecular as well as intramolecular interactions between its amino terminus and carboxyl terminus. This study suggests that TANK is a regulatory molecule controlling the threshold of NF-κB and SAPK activities in response to activation of TNF receptors. In addition, CD40 activated endogenous GCKR in primary B cells, implicating GCK family proteins in CD40-mediated B-cell functions.

The tumor necrosis factor receptor (TNFR) family, consisting of over 20 known distinct members, plays important roles in controlling lymphocyte activation, acute-phase responses, and tumor progression (1, 24, 36, 52). Most are type I transmembrane proteins with characteristic cysteine-rich pseudorepeats in the extracellular region. They can be further classified, based on their cytoplasmic tails, into three groups. The death domain-containing receptor group, which includes TNFRI and Fas, contains a homologous region of about 80 amino acids (aa) called the death domain (21, 55). The members of the non-death domain-containing group, which includes TNFRII, CD40, and CD30, share little homology in their cytoplasmic tails. Members of the third group of this family, including TRID and osteoprotegerin, do not contain functional cytoplasmic tails and are believed to function as inhibitors by competing for ligands with the other two groups of the TNFR family (38, 49, 51). Most of the TNFR family proteins are able to send signals activating two distinct signal transduction pathways, nuclear factor κB (NF-κB) and the stress-activated protein kinase (SAPK; also known as c-Jun N-terminal kinase [JNK]), which lead to TNF-mediated transcriptional regulation for cell survival, cell proliferation and inflammatory responses.

Significant progress has recently been made in the study of TNF-mediated NF-κB and SAPK activation, with identification of the TNFR-associated factors (TRAF) (45). TRAF proteins either directly bind to the cytoplasmic tails of non-death domain-containing TNFRs or indirectly bind to death domain-containing TNFRs through intermediate molecules such as TRADD and RIP (4, 6, 16, 17, 32, 40, 45). In addition, TRAF proteins are involved in signaling through non-TNFR family proteins, including Epstein-Barr virus latent membrane protein 1, which plays an essential role in virus-mediated cell transformation, and the Toll family proteins, which belong to a family of receptors responding to various inflammatory stimuli such as interleukin-1 and lipopolysaccharide (32, 58). To date, six distinct TRAF proteins, TRAF1 through TRAF6, have been isolated. TRAF2, TRAF3, and TRAF6 are ubiquitously expressed, whereas TRAF1 and TRAF5 are preferentially expressed in the spleen, thymus, and lung, and TRAF4 expresses at low levels in normal tissues but is abundant in breast cancer cells (4, 6, 18, 20, 33, 45, 48). All TRAF family proteins have related TRAF-N and TRAF-C domains in their carboxyl-terminal regions (6). In addition, all TRAF family members except TRAF1 have two amino-terminal zinc-binding domains, including a ring finger and five zinc fingers (6). Overexpression of TRAF2, TRAF5, and TRAF6, but not TRAF3, in human embryonic kidney 293 cells activates both the NF-κB and SAPK pathways (2). With the recent identification of NF-κB-inducing kinase (NIK) and the I-κB kinases (IKK), a cascade of signals in the TNF-α-mediated NF-κB activation pathway, from TNF to TNFRs, TRAFs, NIK, IKKs and then to IκB and NF-κB/Rel family transcription factors, has been proposed (12, 28, 29, 41, 56, 60). In JNK activation, TNF-α is thought to signal through a three-tiered mitogen-activated protein kinase

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(MAPK) pathway, from the MAPK kinase kinase MEKK1, to the dual specificity MAPK kinase MKK/SEK/JNKK proteins, to phosphorylation of JNK, leading to the activation of the AP-1 transcription factor (11, 27, 30, 34, 47). Less clear, however, are the direct links between TNFR and MEKK1. Furthermore, the regulation of the NF-κB and SAPK pathways in response to the activation of various TRAF-associated receptors remains to be elucidated.

We previously isolated a TRAF-associated NF-κB activator (TANK) by yeast two-hybrid screening, using TRAF3 as a bait. TANK binds all known TRAF proteins except the predominantly nuclear TRAF4 and synergistically activates TRAF2-mediated NF-κB activation in a biphasic manner with increasing levels of TANK (5, 8). Here we report that TANK is also a mediator of TRAF-induced SAPK activation, which synergistically activated SAPK with TRAF2, TRAF5, and TRAF6 but not with TRAF3. This robust SAPK activation was potentially due to synergistic association of TANK and TRAF proteins with germinal center kinase (GCK) family kinases. A dominant-negative form of GCK-related kinase (GCKR) abrogated CD40 activation of SAPK in vivo, while endogenous GCKR was activated by the B-lymphocyte coreceptor, CD40. In addition, we suggest that TANK, which may form both inter- and intramolecular associations, can act as a regulator by controlling the activities of both the TRAF-mediated NF-κB and SAPK pathways.

MATERIALS AND METHODS

Tissue culture and transfection. For transfection studies, 293T human embryonic kidney cells were maintained in Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, Calif.) and 1% penicillin and streptomycin at 37°C in a 5% CO_2 atmosphere in a tissue culture incubator. Cells were transfected in 10-cm-diameter tissue culture dishes at densities of approximately 40% confluency, using a calcium phosphate method. Briefly, 435 μl of sterile water containing plasmid DNA was mixed with 65 μl of 2 M $CaCl_2$ and 500 μl of 2× HEPES-buffered saline solution (50 mM HEPES [pH 7.05], 10 mM KCl, 12 mM dextrose, 280 mM NaCl, 1.5 mM Na_2HPO_4). After thorough mixing, the DNA solution was added to the culture dish. After 12 h, the medium was changed, and the cells were incubated for another 24 h before harvesting. HS-Sultan and Ramos cells were maintained in RPMI medium (Mediatech) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C in a 5% CO_2 atmosphere.

Plasmid constructs. The plasmid constructs used in the experiments were as follows. Hemagglutinin epitope (HA)-tagged TRAF2, TRAF3, TRAF5, and TRAF6 were all subcloned in pEBB vectors and contained full-length murine cDNA of these molecules; glutathione *S*-transferase (GST)-tagged pEBG-TANK and Flag-tagged pCMV TANK contained full-length murine TANK cDNA; pEBB HA- and GST-tagged pEBG-TANK-C contained the carboxyl-terminal portion of TANK (aa 190 to 413); GST-tagged pEBG- and Flag-tagged pCMV TANK-N contained the amino-terminal portion of TANK (aa 1 to 168); full-length human CD40 and murine CD40L were both subcloned in pBABE vectors; pEBB HA-tagged TRAF2-C contained the C-terminal portion of murine TRAF2 (aa 94 to 501), with deletion of the zinc ring finger; the kinase-deficient mutant GST-tagged pEBG-DN-MEKK1 contained an amino acid change of lysine to arginine at position 447; GST-tagged pEBG-DN-SEK1 contained a mutation from lysine to arginine at position 129 and was thus kinase inactive; pCMV2-Flag-GCKR contained Flag-tagged wild-type GCKR; pCMV2-Flag-DN-GCKR contained the dominant-negative form of GCKR, which has a lysine-to-aspartic acid mutation at amino acid position 178 (50); pCR3.1-Flag-GLK contained Flag-tagged wild-type GCK-like kinase (GLK); and pCR3.1-Flag-DN-GLK contained the dominant-negative form of GLK, which has a lysine-to-glutamic acid mutation at position 35 (13). The Fyn and Btk expression constructs were previously described (7).

Cell lysis. At 36 to 48 h after calcium phosphate transfection, the cells were washed once in ice-cold phosphate-buffered saline and lysed with 800 μl of radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA, 5 mM NaF, 1 μg each of aprotinin, leupeptin, and pepstatin per ml, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride) for 30 min. The cell lysate was transferred to a microcentrifuge tube and then centrifuged at 15,000 × *g* for 10 min at 4°C to remove the cell pellet.

In vitro kinase assays. Equal amounts of supernatants from various transfections were incubated at 4°C on a rotating mixer, first with 1 μg of monoclonal anti-HA antibody or 1 μg of polyclonal anti-JNK1 antibody C-17 (Santa Cruz Biotechnology, Inc.) for 1 h and then with protein A/G-agarose (Calbiochem, San Diego, Calif.) for 1 h. Solutions for the JNK assay were made as previously described (15). The agarose beads with bound JNK molecules were washed twice in radioimmunoprecipitation assay buffer and twice in HEPES binding buffer. Remaining supernatant above the agarose beads was carefully removed, and the kinase reaction was performed in 30 μl of kinase buffer containing 1.0 μg of purified GST-Jun fusion protein containing the amino-terminal 79 aa [GST-Jun(1-79)] of c-Jun (GST-Jun expression plasmid kindly provided by Dennis Templeton, Case Western Reserve University) and 2 μCi of [γ-^{32}P]ATP (NEN Life Science Products Inc., Boston, Mass.) per sample. The kinase reaction was carried out at 30°C for 30 min. and then terminated by addition into the reaction mixture of equal volumes of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heating in a boiling water bath for 5 min. After SDS-PAGE on an 11% gel, the gel was dried and exposed to a Kodak X-ray film. Fold activation quantitated was with an the Alpha Imager 2000 densitometer (Alpha Innotech Corporation). Human tonsil B and T cells were prepared from tonsillar mononuclear cells by rosetting with aminoethylisothiouronium bromide-treated sheep erythrocytes as described previously (22). The B- and T-cell populations were greater than 97% pure, as assessed by flow cytometry with CD19 and CD3 monoclonal antibodies, respectively. B cells were stimulated with an antibody to either CD40, G28.5 (ATCC, Rockville, Md.), or a soluble CD40 ligand fusion protein, CD8-gp39 (gift from Marilyn Kehry, Boehringer Ingelheim Pharmaceuticals Inc.). Myelin basic protein (MBP) in vitro kinase assays were performed as previously described (22, 39).

Coimmunoprecipitation. Coimmunoprecipitation was performed from cell extracts prepared 36 to 48 h after transient transfection of 293T cells by calcium phosphate precipitation. Cell extracts were incubated with either 3 μg of anti-HA monoclonal antibody, 3 μg of anti-Flag monoclonal antibody, or 2 μl of anti-GST polyclonal antibody for 3 to 4 h, followed by incubation with 20 μl of protein A/G beads (Oncogene Sciences) for 1 h. Beads were extensively washed in buffer (1% NP-40, 150 mM $NaCl_2$, 50 mM Tris, 1 mM EDTA, protease inhibitors) before being boiled in sample buffer, and bound proteins were fractionated by SDS-PAGE and immunoblotted with the respective antibodies.

Western blot analysis. Equal amounts of proteins in cell extracts or immunoprecipitated proteins were separated by SDS-PAGE (9% gel) and blotted to nitrocellulose. Filters were incubated with the appropriate antibodies, either anti-HA monoclonal antibody 12CA5 (BAbCo, Richmond, Calif.), anti-Flag monoclonal antibody M2 (BAbCo), or an anti-GST polyclonal antibody, followed by horseradish peroxidase-labeled anti-mouse or anti-rabbit immunoglobulin G (Southern Biotechnology Associates, Inc.). The proteins were detected by enhanced chemiluminescence (Amersham). Endogenous GCKR was identified by using rabbit polyclonal antiserum as previously described (50), while endogenous JNK1 was identified with anti-JNK1 antibody C-17 (Santa Cruz Biotechnology).

RESULTS

TANK synergistically activated SAPK with TRAF2, TRAF5, and TRAF6 but not with TRAF3. TANK (or I-TRAF) was originally isolated in a yeast two-hybrid screen as TRAF2- and TRAF3-associated proteins (5, 46). It binds to all known TRAF proteins except TRAF4, which is mostly insoluble in lysis buffer (5, 8, 46). When increasing amounts of TANK were coexpressed with a constant amount of TRAF2, a biphasic pattern of NF-κB activation was obtained with an initial dose-dependent enhancement, while a further increase in TANK leads to inhibition of TRAF2-mediated NF-κB activation (5). To determine the effect of TANK in TRAF-mediated SAPK activation, pEBB HA-tagged plasmids carrying TRAF2, TRAF3, TRAF5, and TRAF6 were cotransfected with an expression plasmid for JNK1 at constant levels, with increasing amounts of TANK-expressing constructs, into 293T cells. To detect SAPK activity, immunoprecipitated JNK1 was subjected to an in vitro kinase assay, using c-Jun(1-79) as the substrate. To control for the effect of TANK expressed alone in SAPK activation, 0, 1, 3, and 10 μg of pEBG-TANK was transfected into 293T cells with an expression plasmid for JNK1 at constant levels. No activation of SAPK was observed with increasing amounts of TANK (Fig. 1A). Furthermore, no increase in SAPK activation was detected when 3 μg of pEBB-TRAF3 was transfected with 1, 3, or 10 μg of pEBG-TANK into 293T cells (Fig. 1B), whereas overexpression of either TRAF2, TRAF5, or TRAF6 led to strong activation (Fig. 1B and C). However, the dose-dependent synergistic activation of SAPK varied among individual TRAF proteins. Strong synergistic SAPK

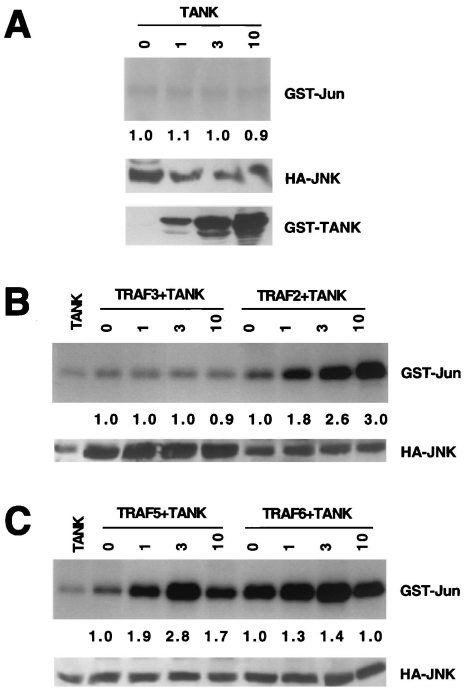

FIG. 1. Synergy between TANK and TRAF proteins in activating SAPK. (A) TANK alone has no effect on SAPK activity. Human kidney 293T cells were transiently transfected with 0, 1, 3, or 10 μg of pEBG-TANK. Each plate received 2 μg of HA-tagged JNK1 expression plasmid, while total DNA amount was maintained with empty vector. Cell lysate preparations and in vitro SAPK assays were performed as described in Materials and Methods. The gel bands represent GST-Jun proteins phosphorylated by SAPK in the cell lysates (top). Fold activation of SAPK is shown below the phosphorylated bands and represents fold activation versus 0 μg of transfected TANK. Similar expression of HA-JNK is shown as a control (middle). Increasing expression of TANK is shown by immunoblotting of lysates (bottom). (B) TRAF2-induced SAPK activity is enhanced by coexpression of TANK. Human kidney 293T cells were transiently transfected with 3 μg of pEBG-TANK and 3 μg of pEBB-TRAF3 or pEBB-TRAF2 with 0, 1, 3, or 10 μg of pEBG-TANK. The gel bands represent GST-Jun proteins phosphorylated by SAPK in the cell lysates (top). Fold activation is shown below the phosphorylated bands and represents fold activation of a specific TRAF with increasing amounts of TANK versus the activation of the specific TRAF alone, which is set at 1. Similar expression of HA-JNK is shown as a control (bottom). TRAFs are consistently expressed at similar levels for each particular TRAF (data not shown). (C) TRAF5- and TRAF6-induced SAPK activity is enhanced by coexpression of TANK. In vitro SAPK assays were performed as described for panel B, except that 1 μg of pEBB-TRAF5 or pEBB-TRAF6 was used with 0, 1, 3, or 10 μg of pEBG-TANK expression plasmids.

activation was observed with TRAF2 and TANK in a dose-dependent manner, with higher SAPK activities obtained when more TANK was transfected. The synergy between TANK and TRAF5 or TRAF6 in activating SAPK displayed a biphasic pattern, with an initial increase at low dosages of TANK followed by decreased activation with larger dosages of TANK (Fig. 1C). These results indicate the differences in the signaling capacities of various TRAF molecules and their abilities to cooperate with TANK.

The carboxyl-terminal portion of TANK strongly inhibited TRAF- and TANK-mediated SAPK activation. We previously mapped a 21-aa fragment in the middle of TANK as the TRAF family member-interacting motif in TANK (TIMtk) and defined the portion amino-terminal to TIMtk as TANK-N(1-168) and the portion carboxyl-terminal to TIMtk as TANK-C(190-413) (5). Synergistic NF-κB activation was obtained by coexpression of TANK-N and TRAF2, whereas TANK-C strongly inhibited CD40- and TRAF2-mediated NF-κB activation. To understand the role of TRAF2 and TANK in SAPK activation, we determined the effects by in vitro kinase assays of TANK-C and the dominant-negative form of TRAF2, which lacks the amino-terminal zinc ring domain of TRAF2, TRAF2-C, in CD40-mediated SAPK activation after cotransfection into 293T cells. Neither TANK-C nor TRAF2-C alone could activate SAPK (Fig. 2, lanes 8 and 9). However, TANK-C was able to completely inhibit SAPK activation induced by CD40 plus CD40L, TRAF2, or TRAF2 plus TANK (lanes 3, 6, and 13). TRAF2-C could fully abrogate SAPK activation by CD40, but its blockage of SAPK activation induced by TRAF2 or TRAF2 plus TANK was not as complete as that of TANK-C (lanes 4, 7, and 12). When TANK-N was coexpressed in 293T cells with TRAF2, it enhanced TRAF2-mediated SAPK activation (data not shown). These results suggest that TANK-C is a potent inhibitor of both the NF-κB and SAPK pathways, mediated through TNFRs, and that TANK-N can synergistically activate TRAF-mediated NF-κB and SAPK activation.

TANK may self-associate through both inter- and intramolecular interactions. When coexpressed in 293T cells, full-length Flag-tagged TANK and GST-tagged TANK were associated with each other, suggesting that TANK can exist as a dimer (Fig. 3, lane 1). To further understand the nature of this interaction and the activating and inhibiting properties of the amino- and carboxyl-terminal regions of TANK, we performed a series of coimmunoprecipitations, using a dual-epitope tag strategy between various domains of TANK to explore the binding interactions. Antibodies to one epitope tag were used to immunoprecipitate protein complexes, while antibodies to a second epitope tag were used to detect the presence of an interacting protein by immunoblotting. TANK-N was found to strongly associate with full-length TANK and itself (Fig. 3, lanes 2 and 3), while TANK-C interacted very weakly with full-length TANK but did not associate with itself (lanes 5 and 6). In addition, coimmunoprecipitation between TANK-N and TANK-C was observed (lane 4). Thus, the dimerization of TANK can be explained in one of two ways, either through an intermolecular association of amino-termini or through the amino terminus of one TANK polypeptide binding to the carboxyl terminus of another TANK polypeptide. Based on the strength of the coimmunoprecipitation of the TANK amino termini and the relative weakness of the interaction between the carboxy terminus of TANK and the full-length protein, a TANK dimer is predicted to be formed through association

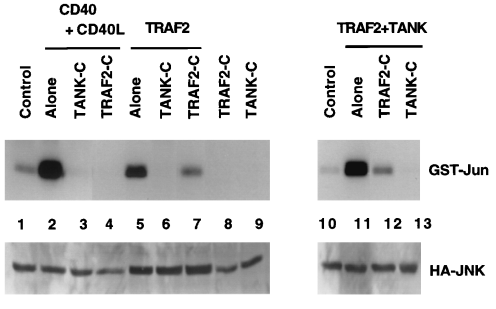

FIG. 2. Inhibition of SAPK activation by TANK-C. TANK-C and TRAF2-C inhibit SAPK activation by CD40 plus CD40L, TRAF2, and TRAF2 plus TANK. Human kidney 293T cells were transfected with 3 μg of pBABE-CD40 plus 3 μg of pBABE-CD40L, 6 μg pEBB-TRAF2, or 3 μg of pEBB-TRAF2 plus 3 μg of pEBG-TANK, together with 5 μg of either pEBG vector, pEBG-TANK-C, or pEBB-TRAF2-C in the presence of 2 μg of HA-tagged JNK1 expression plasmid. Cell lysate preparations and in vitro SAPK assays were performed as described in Materials and Methods. The gel bands represent GST-Jun proteins phosphorylated by SAPK in the cell lysates (top); similar expression of HA-JNK is shown as a control (bottom).

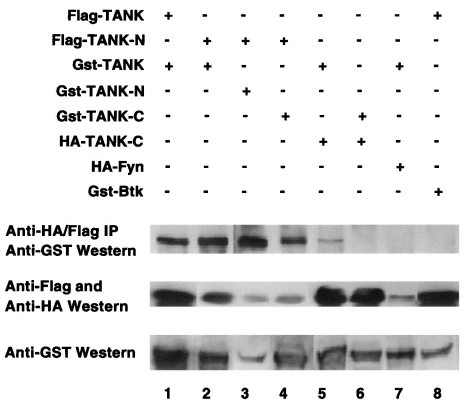

FIG. 3. Self-association and potential intramolecular interaction of TANK. TANK can associate with itself, with interactions observed between the amino termini and between the amino and carboxyl termini. Various plasmids expressing Flag-TANK, Flag-TANK-N, GST-TANK, GST-TANK-N, GST-TANK-C, HA-TANK-C, HA-Fyn, and GST-Btk were transfected in combination, as indicated, into 293T cells. Total DNA amounts were maintained at 10 μg, using empty vector. Cell lysates were immunoprecipitated (IP) with an anti-Flag (lanes 1 to 4 and 8) or anti-HA (lanes 5 to 7) monoclonal antibody. Coprecipitated proteins were detected by immunoblotting with an anti-GST polyclonal antibody. Cutouts of the interacting bands are shown (top); aliquots of cell extracts were immunoblotted with the appropriate antibodies to confirm expression of proteins (middle and bottom). Coexpression of HA-Fyn and GST-TANK and of Flag-TANK and GST-Btk served as negative controls.

between the amino termini. Furthermore, the weaker association between TANK-N and TANK-C may be significant in the formation of an intramolecular interaction between the amino terminus of TANK folding back to interact with its own carboxyl terminus. Supporting this prediction, the dimerization of full-length TANK is weaker by coimmunoprecipitation than dimerization of TANK-N with itself, although TANK-N expression is severalfold lower, suggesting that the potential intramolecular interaction between TANK-N and TANK-C may be inhibitory to TANK dimerization through the amino termini. Full-length TANK did not interact with the proteins Fyn and Btk, demonstrating binding specificity (Fig. 3, lanes 7 and 8). Thus, TANK may form both intermolecular and intramolecular interactions through either the TANK-N and TANK-N association or the TANK-N and TANK-C association.

The dominant-negative form of either GCKR, MEKK1, or SEK1, but not that of Rac1 or cdc42, strongly inhibited TRAF2- and TANK-mediated SAPK activation. The upstream links where the stimulatory signals enter the SAPK pathway are complex and often depend on the nature of the stimuli and the cell type involved. Stimulation from growth factors, cytokines, and environmental stress may employ different entry points that connect to SEK1 (MKK4), which is an immediate upstream activator of SAPK. MEKK1 has been shown to be a common upstream regulator of SEK1 activity, which may be responsible for transmitting SAPK activation signals from various small GTPases such as Ras, Rac, cdc42, and Rho. On the other hand, some stimuli may enter the SAPK kinase cascade directly at the link of SEK1, as is the case with anisomycin (19), or through multiple pathways which cannot be effectively blocked by the MEKK1 dominant-negative mutant, as is the case with UV and hyperosmolarity (43).

To determine whether MEKK1, SEK1, and various small GTPases are in the path of signal transmission of SAPK activation induced by TRAF2 plus TANK stimulation, kinase-inactive dominant-negative mutants of MEKK1 and SEK1 were coexpressed with TRAF2 and TANK in 293T cells, and the lysates were assayed for SAPK activation by an in vitro kinase assay. As indicated in Fig. 4B, the dominant-negative forms of MEKK1 and SEK1 strongly blocked activation of SAPK activity induced by TRAF2 plus TANK (lanes 3 and 4), as well as by CD40 and TRAF2 alone (data not shown). On the other hand, overexpression of the dominant-negative forms of cdc42 and Rac1 did not affect TRAF2-plus-TANK-mediated SAPK activation (lanes 5 and 6). This finding suggests that TRAF2 and TANK activation of SAPK is mediated by MEKK1 and SEK1 but not by cdc42 and Rac1.

It has been previously reported that a kinase-inactive dominant-negative form of GCKR inhibits SAPK activation mediated by TNF-α and TRAF2 (50). To determine whether GCKR is downstream of SAPK activation by TANK synergy with TRAF2 or CD40, dominant-negative GCKR was coexpressed with TRAF2 plus TANK and CD40 plus CD40L in 293T cells and assayed by in vitro kinase activity. As a positive control, we showed that the dominant-negative form of GCKR inhibited activation of SAPK by TNF-α and TRAF2 (Fig. 4A, lanes 3 to 6). Furthermore, we found that the same dominant-negative form of GCKR also strongly inhibited SAPK activation induced by TRAF2 plus TANK and CD40 plus CD40L (lanes 7 to 10). According to sequence homology, GCKR is a

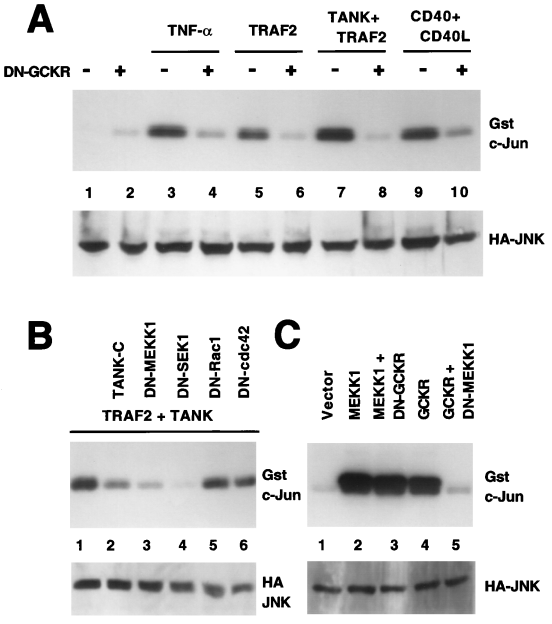

FIG. 4. Inhibition of SAPK activation with dominant-negative forms of GCKR, MEKK1, and SEK1 (DN-GCKR, DN-MEKK1, and DN-SEK1). (A) Dominant-negative GCKR inhibits TRAF2-mediated SAPK activation. Human 293T cells were transiently transfected with 6 μg of pEBB, 6 μg of pEBB-TRAF2, 3 μg of pEBB-TRAF2 plus 3 μg of pEBG-TANK, or 3 μg of pBABE-CD40 plus 3 μg of pBABE-CD40L in the presence of 5 μg of either pcDNA3 or pcDNA3-DN-GCKR. The cells in lanes 3 and 4 were treated with 10 ng of human TNF-α per ml for 10 min before lysing. Cell lysate preparations and in vitro SAPK assays were performed as described in Materials and Methods. The gel bands represent GST-Jun proteins phosphorylated by SAPK in the cell lysates (top rows in all panels); similar expression of HA-JNK is shown as a control (bottom rows in all panels). (B) Dominant-negative MEKK1 and SEK1 but not dominant-negative Rac1 and cdc42 inhibit TRAF2- and TANK-mediated SAPK activation. For transient transfections, 3 μg of pEBB-TRAF2 and 3 μg of pEBG-TANK, together with 3 μg of pEBG vector, pEBG-TANK-C, pEBG-DN-MEKK1, pEBG-SEK1, pEBG-DN-Rac1, or pEBG-DN-cdc42 were used. (C) MEKK1 is downstream of GCKR. For transient transfections, 6 μg of pEBG, 3 μg of pEBG-MEKK1, 3 μg of pEBG-MEKK1 plus 3 μg of pcDNA3-DN-GCKR, 3 μg of pcDNA3-GCKR, or 3 μg of pEBG-DN-MEKK1 plus 3 μg of pcDNA3-GCKR were used.

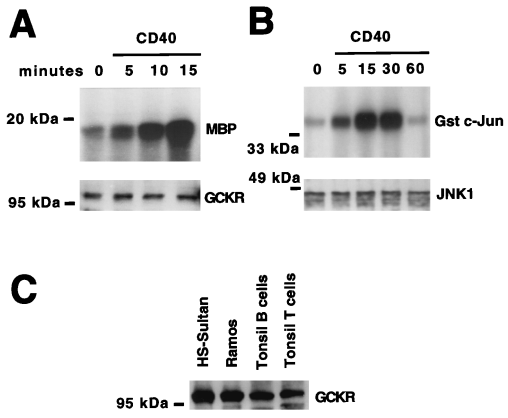

FIG. 5. Activation of endogenous GCKR in human tonsil B cells. (A) GCKR activity is enhanced by stimulation of CD40 in tonsil B cells. Lysates from human tonsil B cells were immunoprecipitated with an antiserum to GCKR and assayed for the ability to phosphorylate MBP at 0, 5, 10, and 15 min after CD40 stimulation with anti-CD40 monoclonal antibody G28.5 (top); expression of endogenous GCKR is shown by immunoblotting with antiserum to GCKR (bottom). (B) Kinetics of JNK1 activation are similar to those of GCKR activation by CD40 stimulation in Ramos B cells. Activation of JNK1 by CD40 in Ramos B cells was performed at 0, 5, 15, 30, and 60 min after stimulation of cells by soluble CD8-gp39. Lysates were immunoprecipitated with a polyclonal anti-JNK1 antibody and assayed for the ability to phosphorylate GST c-Jun(1-79) (top); expression of endogenous GCKR is shown by immunoblotting with a polyclonal anti-JNK antibody (bottom). (C) Expression of endogenous GCKR was examined in the indicated cell types. GCKR was detected by immunoblotting with antiserum to GCKR and migrates at about 95 kDa.

member of the MAP4K family, whereas MEKK1 belongs to the MAP3K family. To determine the functional relationship between GCKR and MEKK1, we found that the dominant-negative GCKR did not affect MEKK1-mediated SAPK activity, whereas the dominant-negative MEKK1 abolished GCKR-mediated SAPK activity, indicating that GCKR is upstream of MEKK1 (Fig. 4C).

CD40 stimulation of primary tonsil B cells activated endogenous GCKR. To further confirm the role of GCKR in the CD40 signaling pathway in more physiological conditions, we examined the activation of GCKR by CD40 in primary human tonsil B cells. Lysates from CD40-activated tonsil B cells were immunoprecipitated with a polyclonal antiserum to GCKR, and kinase activity was assayed by an in vitro kinase assay, using MBP as the substrate. The kinase activity of GCKR was upregulated within 5 min of CD40 stimulation and progressively increased through the 15-min time course (Fig. 5A). These kinetics correlated with the kinase activity of endogenous JNK1 (Fig. 5B). Expression of GCKR was also examined in primary cells and cell lines. Migrating as a 95-kDa protein, endogenous GCKR was similarly found to be expressed in HS-Sultan human plasmacytoma cells, Ramos human B cells, and human tonsil B and T cells (Fig. 5C). Thus, GCKR is a downstream target of CD40 and is inducible by CD40 in primary B cells.

TRAF2 and TANK synergistically interacted with GCKR. To understand the mechanism of TRAF2 and TANK synergy in GCKR-mediated SAPK activation, we tested the possible interactions among TRAF2, TANK, and GCKR in a series of coimmunoprecipitation assays. When coexpressed in 293T cells, Flag-tagged GCKR was found in the complex coprecipitated with HA-tagged TRAF2 (Fig. 6A, lane 4). Interestingly, overexpression of TANK strongly enhanced the association between TRAF2 and GCKR (Fig. 6A, lane 6). Similarly, a weak association between Gst-tagged TANK and Flag-tagged GCKR was obtained in coimmunoprecipitation assays (Fig. 6B, lane 5), but its association becomes much stronger when TRAF2 is coexpressed (Fig. 6B, lane 6). As shown previously, TRAF2 and TANK form a strong interaction (Figure 6C, lane 6). These results suggest that TRAF2 and TANK individually interact with GCKR but together synergistically interact with GCKR to form a TRAF2-TANK-GCKR complex.

GLK is another GCK family protein involved in TRAF- and TANK-mediated SAPK activation. A member of the GCK protein family, GLK, possesses 61% amino acid identity to GCKR. To explore the role of GLK in CD40-mediated activation of SAPK, we determined the effect of the kinase-inactive dominant-negative form of GLK in CD40-induced SAPK activation. As indicated in Fig. 7, dominant-negative GLK strongly inhibited SAPK activation induced by CD40 plus CD40L or TRAF2 plus TANK, as shown by an in vitro kinase assay after coexpression in 293T cells (lanes 5 to 8). Similar to observations for GCKR, GLK-induced SAPK activation could be inhibited by dominant-negative forms of MEKK1 and SEK1, whereas MEKK1-induced SAPK could not be inhibited by the dominant-negative form of GLK (lanes 2 to 4, 9, and 10).

DISCUSSION

We previously reported that point mutations in the amino-terminal zinc ring finger domain of TRAF5 abolished its ability to mediate NF-κB activation but did not affect its ability to activate SAPK. In addition, we found that TRAF2A, an alternative spliced form of TRAF2 with a 7-aa insertion in the zinc ring finger domain of TRAF2, activates the SAPK pathway but not the NF-κB pathway (10). We therefore suggested that

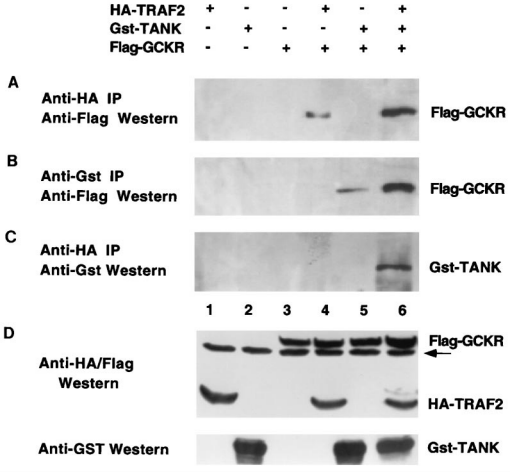

FIG. 6. Synergistic interaction of TRAF2 and TANK with GCKR. Individual TRAF2 and TANK interactions with GCKR are enhanced by coexpression of TRAF2 and TANK. Human 293T cells were transfected with pEBB-HA-TRAF2, pEBB-GST-TANK, or pcDNA3-Flag-GCKR, either alone or in combination, as indicated. (A) Total DNA amounts were maintained at 10 μg, using empty vector. Cell lysates were immunoprecipitated (IP) with an anti-HA antibody and the coimmunoprecipitated complexes were analyzed by immunoblotting with an anti-Flag antibody to demonstrate TRAF2 and GCKR interactions (B). Lysates were precipitated with an anti-GST antibody, and the coimmunoprecipitated complexes were immunoblotted with an anti-Flag antibody to show TANK and GCKR interactions (C). TRAF2 and TANK interactions are shown by immunoprecipitation of lysates with an anti-HA antibody and subsequent immunoblotting with an anti-GST antibody (D). Expression levels of input proteins were obtained by immunoblotting lysates with appropriate antibodies. The arrow indicates a nonspecific protein found in 293T cells that cross-reacts with the anti-HA monoclonal antibody.

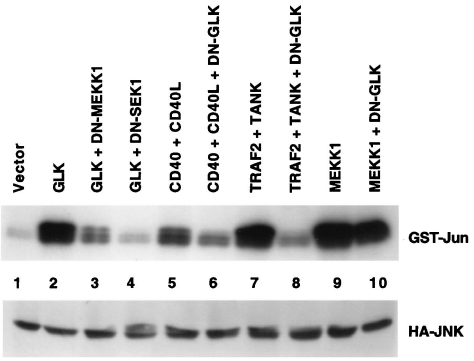

FIG. 7. Involvement of GLK in TRAF- and TANK-mediated SAPK activation. GLK-induced SAPK activity is inhibited by dominant-negative MEKK1 and SEK1 (DN-MEKK1 and DN-SEK1), while dominant-negative GLK (DN-GLK) inhibits SAPK activation by CD40 and TRAF2 plus TANK but not by MEKK1. Human 293T cells were transfected with 2 μg of plasmid expressing HA-JNK1 and 3 μg of pEBB vector (lane 1), pcDNA3-GLK (lane 2), pcDNA3-GLK plus pEBB-DN-MEKK1 (lane 3), pcDNA3-GLK plus pEBB-DN-SEK1 (lane 4), pBABE-CD40 plus pBABE-CD40L (lane 5), pBABE-CD40 and pBABE-CD40L plus pcDNA3-DN-GLK (lane 6), pEBB-TRAF2 plus pEBG-TANK (lane 7), pEBB-TRAF2 and pEBG-TANK plus pcDNA3-DN-GLK (lane 8), DN-MEKK1 (lane 9), or DN-MEKK1 plus pcDNA3-DN-GLK (lane 10). Total DNA amounts were kept at 9 μg, with empty vector. Cell lysate preparations and in vitro SAPK assays were performed as described in Materials and Methods. The gel bands represent GST-Jun proteins phosphorylated by SAPK in the cell lysates (top); similar expression of HA-JNK is shown as a control (bottom).

TRAF proteins are the last common molecules between the NF-κB and SAPK pathways and may branch these two distinct pathways through different TRAF complexes. TRAF proteins have been reported to associate with many intracellular proteins, including TRADD, RIP, IRAK, NIK, TANK, TRIP, Peg3/Pw1, c-IAP1, c-IAP2, and A20 (4, 5, 16, 17, 26, 28, 42, 44, 54). These proteins potentially form large TRAF-associated complexes important in TRAF-mediated cell signaling. Nevertheless, the roles and the mechanisms of these molecules in TRAF-mediated NF-κB and SAPK activation are not known.

The ability of TANK to modulate TRAF2-mediated NF-κB activation suggested that TANK could potentially function as either an activator or an inhibitor of NF-κB activation induced by TNFR family proteins. In this report, we showed that TANK synergistically activated SAPK with TRAF proteins. TANK-mediated synergistic SAPK activation varies with individual TRAF proteins. It strongly enhanced TRAF2-mediated SAPK activation in a dose-dependent manner but did not synergize with TRAF3 in SAPK activation. As with TRAF2-mediated NF-κB activation, the effects of TANK upon TRAF5- and TRAF6-mediated SAPK activation were biphasic, with enhancement by lower levels of TANK and decreased enhancement by higher levels. These results suggest that TANK might function as a regulatory molecule controlling the threshold of TRAF-mediated NF-κB and SAPK activation.

This report also describes a potential mechanism of TANK function during TRAF-mediated NF-κB and SAPK activation. We previously defined a 21-aa peptide in the middle of TANK as the TRAF family member-interacting motif in TANK (TIMtk), and showed that the TIMtk can compete with the TRAF binding motif in the CD40 cytoplasmic tail for binding to the TRAF-C domain of TRAF proteins (5). In the work reported here, we predict that TANK forms a homodimer through an interaction in the TANK-N region, as well as forming an intramolecular association through the interaction between TANK-N and TANK-C, which may inhibit TANK dimerization. All these studies are consistent with our previously proposed model, suggesting that TANK in its ground state is autoinhibited by an intramolecular interaction of its carboxyl and amino termini. The binding of TRAF2 to the TIMtk motif in the middle of TANK may change TANK's conformation, favoring the intermolecular TANK-N and TANK-N interaction to form a TANK dimer. Because TRAF binds TANK through the TIMtk, dimerization of TANK would lead to aggregation of TRAF2, which may trigger a signal for NF-κB and SAPK activation. Consistent with this model, we found that overexpression of TANK-C strongly inhibited NF-κB and SAPK activation induced by CD40, TRAF2 or TRAF2 plus TANK, presumably by competing away TANK dimerization sites at the amino terminus of endogenous TANK. Conversely, TANK-N overexpression synergistically activated TRAF-mediated NF-κB and SAPK activation, possibly by titrating away the autoinhibitory carboxyl-terminal portion of endogenous TANK.

Multiple pathways may be involved in SAPK activation. The SAPK activation initiated from many stress stimuli requires activation of a signaling cascade from the Rho group of small G proteins (such as Rac1 or cdc42) to mixed lineage kinases (such as MLK-2 and MLK-3), to MEKK1, to SEK1 or SEK2, to JNK family proteins, to c-Jun (3, 9, 11, 27, 30, 31, 37, 47, 57). Other stress inducers may activate JNK through a family of germinal center kinases (25). Recent studies suggested two potential signal transduction pathways for TRAF-induced SAPK activation (35, 50, 59). One is mediated by ASK1 (or MAPKKK5), which is a serine/threonine kinase at the level of the MAPK kinase kinases (35). Studies showed that ASK1 coprecipitates with TRAF2 upon TNF-α stimulation. The dominant-negative ASK1, which contains a lysine-to-alanine mutation at the ATP binding site, however, only partially inhibits TNF-α- or TRAF-induced SAPK activation (8, 35). Other studies suggest the involvement of GCK and GCKR in TRAF-induced SAPK activation (50, 59). In this report, we showed that although TRAF2 or TANK alone bound to GCKR weakly, together they formed a strong complex with GCKR. This synergistic binding of TRAF2 and TANK to GCKR may result in synergistic activation of SAPK by TRAF2 and TANK. Consistent with the role of GCKR in TRAF2- and TANK-mediated SAPK activation, we showed that the dominant-negative GCKR but not the dominant-negative Rac1 or cdc42 strongly inhibited SAPK activation induced by TRAF2 plus TANK. The GCK family proteins are kinases at the MAP4K level. Although their physiological targeting molecules in the kinase cascade are not yet confirmed, activation of some GCK family proteins is likely to lead to activation of MEKK1. We showed that the dominant-negative MEKK1 strongly inhibited SAPK activation induced by GCKR or GLK, whereas the dominant-negative GCKR or GLK did not affect MEKK-1-induced SAPK activation. Furthermore, coprecipitation between GCK and MEKK1 was recently reported (59). The dominant-negative SEK1 abolished SAPK activation induced by upstream activators such as CD40, TRAF2, and TRAF2 plus TANK, suggesting that SEK1 may be a potential kinase involved in the TRAF-mediated SAPK activation pathway (8). In addition to SAPK activation, TRAF proteins have also been reported to activate the p38 pathway, a stress-activated pathway parallel to the SAPK pathway (2). Further studies are necessary to determine the contributions of ASK1 and GCK family proteins in TRAF- and TANK-mediated SAPK and p38 activation.

We also reported the endogenous activation of GCKR by CD40 in primary B cells. Dominant-negative TRAF2 and GCKR completely abrogated CD40-mediated SAPK activation in vivo. Together, these data suggest that GCKR is a

critical mediator in CD40 activation of SAPK. Interestingly, the kinase known as GCK derived its name by virtue of its high expression in the follicular germinal center, with putative roles in B-cell differentiation and selection (22, 39). In B cells, CD40 has been implicated in a variety of functions, including germinal center formation, immunoglobulin isotype switching, antibody affinity maturation, and generation and maintenance of memory B cells (14, 23). However, how GCKR-mediated SAPK activation contributes to CD40-mediated B-cell functions is a critical and open question which should be addressed further. In addition to GCKR, our and other studies suggest that GLK, another GCK family protein, and GCK may also be involved in TNFR-mediated and in TRAF- and TANK-mediated SAPK activation. With 11 known members of the GCK family, it will be of interest to determine whether additional members are involved in signal transduction mediated by CD40 as well as other TNFRs. Specificity of GCK members to distinct TNFRs in vivo will be important in delineating the function of GCK family-mediated signaling.

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A. Chin, J. Shu, and C. Shi contributed equally to this work.

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