TNIK, a Novel Member of the Germinal Center Kinase Family That Activates the c-Jun N-terminal Kinase Pathway and Regulates the Cytoskeleton*

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Germlinal center kinases (GCKs) compose a subgroup of the Ste20 family of kinases. Here we describe the cloning and characterization of a novel GCK family kinase, Traf2- and Nck-interacting kinase (TNIK) that interacts with both Traf2 and Nck. TNIK encodes a polypeptide of 1360 amino acids with eight spliced isoforms. It has 90% amino acid identity to the Nck-interacting kinase in both the N-terminal kinase domain and the C-terminal germinal center kinase homology region. The homology drops to 53% in the intermediate region. TNIK specifically activates the c-Jun N-terminal kinase pathway when transfected into Phoenix-A cells (derivatives of 293 cells), similar to many GCKs. However, in contrast to other GCKs, this activation is mediated solely by the GCK homology region of TNIK. In addition, in Phoenix-A, NIH-3T3, and Hela cells, overexpression of wild type TNIK, but not the kinase mutant form of TNIK, results in the disruption of F-actin structure and the inhibition of cell spreading. Furthermore, TNIK can phosphorylate Gelsolin in vitro. This is the first time that a GCK family kinase is shown to be potentially involved in the regulation of cytoskeleton.

The Ste20 family of kinases can be divided into two structurally distinct subfamilies. The first subfamily contains a C-terminal catalytic domain and an N-terminal binding site for the small G proteins Rac1 and Cdc42 (1). The yeast serine/threonine kinase Ste20 and its mammalian homologue, p21-activated kinase 1 (PAK1), belong to this subfamily. Ste20 initiates a mitogen-activated protein kinase (MAPK) cascade that includes Ste11 (MAPK kinase kinase), Ste7 (MAPK kinase), and FUS3/KSS1 (MAPK) in response to activation of the small G protein Cdc42, as well as signals from the heterotrimeric G proteins coupled to pheromone receptors (1). Similar to Ste20, PAK1 has been demonstrated to be a Cdc42 and Rac1 effector molecule and specifically regulates the JNK pathway, one of the mammalian MAPK pathways (2, 3). The JNK pathway is activated by a variety of stress-inducing agents, including osmotic and heat shock, UV irradiation, protein inhibitors, and proinflammatory cytokines such as TNF (4). JNKs are activated through threonine and tyrosine phosphorylation by MAPK/ERK kinases 4 and 7 (MAPK kinase), which are in turn phosphorylated and activated by MAPK kinase kinases, including MEKK1, mixed lineage kinase 2, and mixed lineage kinase 3 (4). In addition to the activation of the JNK pathway, PAK1 has also been demonstrated to be a regulator of the actin cytoskeleton (5).

The second subgroup of Ste20 family of kinases is represented by the germlinal center kinase (GCK), and this family is, therefore, often referred to as GCK family of protein kinases (6). In contrast to Ste20 and PAK1, GCK family members have an N-terminal kinase domain and a C-terminal regulatory region. Many GCK family members, including GCK, GCKR, hematopoietic protein kinase 1, GCK-like kinase, HPK/GCK-like kinase, and NCK-interacting kinase (NIK), have also been demonstrated to activate the JNK pathway when overexpressed in 293 cells (7–12). Among those, GCK and GCKR have been implicated in mediating TNF-induced JNK activation through TNF receptor-associated factor 2 (Traf2) (7, 10, 13). NIK interacts with the SH2-SH3 domain containing adapter protein NCK and has been proposed to link protein tyrosine kinase signals to JNK activation (12).

Recently, Eichinger et al. (14) purified a novel GCK family kinase from Dictyostelium that can phosphorylate Severin in vitro. Severin is an F-actin fragmenting and capping enzyme that regulates Dictyostelium motility. This finding raised the intriguing possibility that the GCK family kinases may also be involved in regulating cytoskeleton function in addition to their role in regulating the JNK pathway. However, there has being no evidence suggesting the involvement of mammalian GCKs in cytoskeleton regulation.

Here, we report a novel mammalian GCK family kinase identified in our yeast two-hybrid screening. It interacts with both Traf2 and NCK, and was therefore designated Traf2- and NCK-interacting kinase (TNIK). It shares highest homology to NIK. We demonstrate in this report that TNIK, like many other GCK family members, is able to specifically activate the JNK pathway when overexpressed in Phoenix-A cells. In addition, overexpression of TNIK results in the disruption of F-actin structure in Phoenix-A, NIH-3T3, and Hela cells, thereby providing for the first time evidence that a mammalian GCK family kinase may regulate the cytoskeleton.

EXPERIMENTAL PROCEDURES

Antibodies and Cytokines—Antibodies used in this report include anti-HA mAb (Babco) and pAb (Santa Cruz Biotechnology), anti-FLAG...
mAb (Sigma) and pAb (Santa Cruz), anti-Myc mAb (Babco), anti-Traf2 pAb (Santa Cruz), anti-NCK mAb (Transduction Laboratories), and anti-β-actin mAb (Sigma). TNFα was purchased from Calbiochem.

Cloning of Full-length TNIK and Northern Blotting—Using yeast two-hybrid screening, overlapping cDNA fragments were identified that interacted with Traf2 and NCK. The sequences of the fragments were contained in a partial cDNA clone, KIAA0551 (GenBankTM accession number AB011123). Antisense oligos TGCGCTTATATTCCAGAGTAGAGCT and CTGTCTCTGCTCCTCCTA were designed according to the 5′ end sequence of KIAA0551, and the full-length TNIK cDNA was cloned from reverse-transcribed human brain mRNA by rapid amplification of cDNA ends-PCR. Northern blotting was performed on human multitissue Northern blot according to the manufacturer’s recommendations (CLONTECH). A PCR product amplified from nt 1264 to 2427 of TNIK coding region was used as a probe.

Plasmid Construction—Full-length human TNIK was cloned into pCI (Promega)-derived expression vector pYCI under the control of the cytomegalovirus tag with an HA epitope tag (AYPYDVPDYA) inserted on the N terminus by PCR. A kinase mutant form of TNIK, designated as TNIK (KM) was constructed using the QuikChange mutagenesis kit (Stratagene) with oligos AGCTTGCAGCCATCAGGTTATGGATGTCAC and GTGACATCCATAACCTTGATGGCTGCAAGCT to change the highly conserved lysine 54 in the kinase domain to arginine. Full-length human Traf2 was cloned into pYCI with a FLAG epitope tag (DYKDDDDK) inserted on the N terminus by PCR. Full-length human NCK was similarly cloned into pYCI with a FLAG epitope tag at the N terminus. Myc-JNK2 and Myc-ERK1 were constructed in the pCR3.1 vector with a Myc epitope tag (ASMEQKLI-SEEDLN) inserted on the N terminus of JNK2 and ERK1, respectively. All of the truncation mutants were constructed by PCR. All constructs were verified by DNA sequencing.

Cell Culture, Transfection of Phoenix-A Cells, and Immunoprecipitation—Phoenix-A cells (derivatives of 293 cells) (15) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfection of Phoenix-A cells was performed using the standard calcium phosphate method (15). Eight 10^6 cells were seeded 16 h before transfection. 3 μg of DNA was used in the transfection for each well of a six-well plate, and 10 μg DNA was used for each 100-mm dish. Medium was changed 8 h after transfection. Cells were
lysed in lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl) with protease inhibitors (Roche Molecular Biochemicals) and analyzed 24 h after transfection. Cell lysates were cleared by centrifugation (14,000 rpm for 10 min). For immunoprecipitation studies, cell lysates (2 × 10^6 cells/lane) were rotated with 2–3 μg of desired antibodies and 20 μl of a 50% slurry of protein A-Sepharose (Amersham Pharmacia Biotech) for 1.5 h. Immune complexes were precipitated, and the pellets were washed three times with lysis buffer. Washed precipitates were subjected to SDS-PAGE analysis and Western blotting. Supersignal and Supersignal West Duro substrates (Pierce) were used as detection systems for the Western blotting.

In Vitro Kinase Assays—For the JNK in vitro kinase assay, Myc-JNK2 was co-transfected into Phoenix-A cells with TNIK mutants, Traf2, or MEKK1 as described above. 24 h after transfection, cells were lysed with lysis buffer supplemented with 20 mM β-glycerophosphate, 1 mM NaF, 1 mM Na3VO4, and protease inhibitors. Myc-JNK2 was precipitated from clarified cell lysates with an anti-Myc mAb, and the pellets were washed three times with lysis buffer and two times with kinase buffer (20 mM HEPES, pH 7.4, 10 mM MnCl2, 10 mM MgCl2, 20 mM β-glycerophosphate, 1 mM NaF, 1 mM Na3VO4, 0.5 mM dithiothreitol). For the kinase reactions, immunoprecipitates were incubated with 1 μg of glutathione S-transferase (GST) c-Jun-(1–79) (Santa Cruz Biotechnology) in 20 μl of kinase buffer supplemented with 1 μM PKI peptide (Sigma), 10 μM ATP, 5 μCi of [γ-32P]ATP for 20 min at 30 °C. Kinase reactions were stopped by addition of 20 μl of 2× SDS sample buffer (Norvex), heated at 95 °C for 5 min, and then loaded onto SDS-PAGE. ERK and p38 in vitro kinase assays were conducted in a similar fashion. For ERK kinase assays, an anti-Myc mAb was used to immunoprecipitate Myc-ERK1, and myelin basic protein (Sigma) was used as an exogenous substrate. For p38 kinase assays, an anti-FLAG mAb was used to immunoprecipitate FLAG-p38, and GST-ATF2 (Santa Cruz) was used as an exogenous substrate. For in vitro kinase assays on TNIK, 3 μg of wild type HA-TNIK or 3 μg of kinase mutant form of HA-TNIK was expressed in Phoenix-A cells and immunoprecipitated with an anti-HA antibody. Immune complexes were subjected to kinase assays as described above in the absence or presence of 0.5 μg of Gelsolin as an exogenous substrate.

Fluorescence Microscopy—Phoenix-A cells seeded in six-well plates were co-transfected with GFP and TNIK constructs as described above. 24 h after transfection, cells were observed using a Nikon Eclipse TE 300 fluorescent microscope. For detection of apoptosis, Hoechst 33258 (Sigma) was added to transfected Phoenix-A cells (final concentration, 2 μg/ml), and the cells were incubated for 30 min at 37 °C before microscopic observation.

Determination of Actin Distribution—4 × 10^5 Phoenix-A cells per well in a six-well plate were transfected with 3 μg of control vector, HA-TNIK(WT) or HA-TNIK(KM). 24 h after transfection, culture media were carefully removed. Cells were lysed directly on the plate using 250 μl of Triton X-100 lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4) with protease inhibitors. Cell lysates were centrifuged at 14,000 rpm for 10 min. Supernatants represented the Triton X-100-soluble fraction. Pellets were washed once with 500 μl of Triton X-100 lysis buffer and dissolved in 500 μl of 1× SDS sample buffer. DNA was sheared by sonication. This represented the Triton X-100-insoluble fraction. Triton X-100-soluble and -insoluble fractions derived from the same number of cells were resolved on SDS-PAGE and blotted with an anti-β-actin mAb to determine the content of F- and G-actin.

**RESULTS**

Molecular Cloning of TNIK—Using a human brain cDNA library and a T/B cell library in our yeast two-hybrid pathway mapping effort, we identified a novel germinal center kinase family member that interacted with both Traf2 and NCK. A GenBank search revealed that this kinase is identical to a partial cDNA clone with unknown function, KIAA0551 (GenBank accession number AB011123). The 5′ end sequence of KIAA0551 was cloned from cDNAs prepared from human brain mRNA by rapid amplification of cDNA ends-PCR, and full-length cDNA clones of KIAA0551 were obtained by RT-PCR. We designated this protein TNIK, for Traf2 and NCK Interacting Kinase (Fig. 1A).

The longest TNIK clone was encoded by a polypeptide of 1360 amino acids. It had an N-terminal kinase domain, an intermediate domain and a C-terminal germinal center kinase homology (GCKH) region. It shared about 90% amino acid identity with a previously cloned GCK family member, NIK, in both the kinase domain and the GCKH domain (12). However, TNIK was only 53% identical to NIK in the intermediate region (Fig. 1,A and C). Two shorter clones of TNIK were also obtained: one lacked nt 1338–1424 (amino acids 447–475) and nt 2383–2406 (amino acids 795–802), and the other lacked those two regions plus nt 1609–1773 (amino acids 537–591) (Fig. 1C). These clones suggested that TNIK may have multiple spliced isoforms. Primers encompassing these three alternatively spliced regions were designed and used for PCR from spleen, heart, and brain cDNAs. The relative amounts of the different isoforms, seen as multiple bands amplified from both spleen and brain, varied among the different tissues (Fig. 1B). Amplified DNA fragments were cloned into a TA cloning vector and the inserts sequenced. All eight combinations from the alternative splicing of these three regions were identified. These eight spliced isoforms of TNIK were designated as TNIK_1 to TNIK_8...
The expression pattern of the TNIK message was examined by human multi-tissue Northern blot. A strong phosphorylated band at 150 kDa was detected in the TNIK(WT) expressed lane, but not in the TNIK(KM) expressed lane (Fig. 1D, lanes 1 and 2). Immunoblotting with an anti-HA antibody showed equal levels of expression of both TNIK(WT) and TNIK(KM) at 150 kDa (Fig. 1D, lanes 3 and 4). Therefore, the phosphorylated band in the in vitro kinase assay represented autophosphorylated TNIK, and the TNIK(KM) mutant was deficient in protein kinase activity.

**Tissue Distribution of TNIK**—The expression pattern of the TNIK message was examined by human multi-tissue Northern blot. Because TNIK shared homology with NIK, a probe corresponding to nt 1264–2427 of TNIK was used to rule out any potential cross-hybridization. This region shared only 40% amino acid identity with NIK. Three major bands of sizes 6.5, 7.5, and 9.5 kilobases were detected (Fig. 2). Alternative splicing in the coding region described above is unlikely to account for the size differences among the three messages, because the largest isoform is only 273 base pairs bigger than the smallest isoform. Alternative splicing in the untranslated region or alternative usage of poly(A) sites could be possible explanations. This phenomenon is not unique to TNIK. NIK and HPK/GCK-like kinase also have multiple message sizes. TNIK is ubiquitously expressed, with higher levels of message detected in heart, brain, and skeletal muscle. Interestingly, heart and skeletal muscle predominantly expressed the 6.5-kilobase form; placenta, kidney, and pancreas predominantly expressed the 7.5-kilobase form; brain, lung, and liver expressed all three forms at a similar level. It is currently unknown whether these messages have different functional roles.

**Interaction of TNIK with Traf2 and NCK**—To confirm the interaction of TNIK with Traf2, N-terminal HA-tagged TNIK was transiently expressed in Phoenix-A cells, and HA-TNIK was immunoprecipitated by an anti-HA antibody. The immune complexes were resolved on SDS-PAGE and immunoblotted with an anti-Traf2 antibody. Endogenous Traf2 specifically co-immunoprecipitated with HA-TNIK (Fig. 3A, top panel). To map the interaction domain on TNIK that mediated its interaction with Traf2, we constructed several truncated forms of HA-tagged TNIK (Fig. 3B) and co-expressed them with FLAG-tagged Traf2. Anti-HA immunoprecipitates were then blotted with an anti-FLAG antibody to detect the co-immunoprecipitated FLAG-Traf2. TNIK(WT), TNIK(N2), TNIK(C1), and TNIK(KM) all co-immunoprecipitated with FLAG-Traf2, suggesting that the intermediate domain of TNIK is sufficient for TNIK to interact with Traf2 (Fig. 3C, top panel, lanes 1, 3, 4, and 6). However, TNIK(C2) consistently showed weak interaction with Traf2 (lane 5), suggesting that the GCKH domain was also involved in the interaction with Traf2. TNIK(N1), the TNIK mutant with only the kinase domain, failed to interact with Traf2 (lane 2). Expression levels of the transfected proteins were controlled by immunoblotting cell lysates with anti-HA and anti-FLAG antibodies (Fig. 3C, middle and bottom panels). In addition, TNIKs, the shortest form of TNIK, was still able to interact with Traf2 (data not shown), suggesting that the three alternatively spliced exons were not required for TNIK to interact with Traf2.

We then mapped the domains on Traf2 that mediated the interaction with TNIK. FLAG-tagged Traf2 mutants (Fig. 3D) were co-expressed with HA-TNIK, and the lysates were subjected to anti-HA immunoprecipitation. The immune complexes were then blotted with an anti-FLAG antibody. Traf2(WT), Traf2(87–501), and Traf2(272–501) were all able to co-immunoprecipitate with HA-TNIK, whereas Traf2(272–501) failed to interact with HA-TNIK (Fig. 3E, top panel). Immunoblotting cell lysates with anti-HA and anti-FLAG antibodies showed comparable expression levels of the transfected proteins (Fig. 3E, middle and bottom panels). This result suggested that the Traf domain is required for Traf2 to interact with TNIK. However, because the interaction of full-length Traf2 with TNIK is stronger than that of either Traf2(87–501) or Traf2(272–501), the N-terminal ring finger may directly contribute to the interaction or may stabilize the configuration of the Traf2 molecule to facilitate this interaction.

**Interaction of TNIK with NCK**—The interaction of TNIK with NCK was investigated in a similar fashion. Following transient expression of HA-TNIK in Phoenix-A cells, the cell lysates were immunoprecipitated with an anti-HA antibody and blotted with an anti-NCK antibody. Endogenous NCK specifically co-immunoprecipitated with HA-TNIK (Fig. 4A, top panel). To map the domains on TNIK required for this interaction, HA-tagged TNIK mutants were co-expressed with FLAG-tagged NCK, and the HA-TNIK mutants were immunoprecipitated with an anti-HA antibody. The immune complexes were then blotted with an anti-FLAG antibody. TNIK(WT), TNIK(N2), TNIK(C1), and TNIK(KM) were all able to associate with NCK, suggesting that the GCKH domain was also sufficient for TNIK to bind NCK (Fig. 4B, top panel, lanes 1, 3, 4, and 6). Neither the GCKH domain nor the kinase domain showed any detectable binding to NCK (lanes 2 and 5). Immunoblotting cell lysates with anti-HA and anti-FLAG antibodies showed equivalent levels of expression of the transfected proteins (Fig. 4B, middle and bottom panels).

**Activation of JNK2 by TNIK**—We next examined whether TNIK was able to activate the JNK pathway. 1, 2, or 3 μg of TNIK expression plasmid was co-transfected into Phoenix-A cells with Myc-JNK2. 24 h after transfection, Myc-JNK2 was
FLAG-Traf2 mutants, and the cell lysates were analyzed as in with TNIK. HA-TNIK was co-transfected into Phoenix-A cells with E, mutants.

Middle precipitated with an anti-HA pAb and blotted with an anti-FLAG mAb.

cells with HA-TNIK mutants.

interaction with Traf2. FLAG-Traf2 was co-transfected into Phoenix-A

immunoprecipitated from cell lysates and its kinase activity measured using GST-c-Jun-(1–79) as a substrate. Co-transfection of TNIK enhanced JNK2 kinase activity in a dose-dependent fashion (Fig. 5A, top panel, lanes 1 and 3–5). When 3 μg of

TNIK was transfected, JNK2 activity was enhanced 3–4-fold. A similar magnitude of JNK2 activation was observed when cells were treated for 15 min with 100 ng/ml of TNF (Fig. 5A, top panel, lanes 1, 2, and 5). Also consistent with published result (16), Traf2 potently activated JNK2 activity (lane 6). The expression levels of Myc-JNK2 were controlled by immunoblotting cell lysates with an anti-Myc antibody (Fig. 5A, bottom panel).

To determine whether TNIK can also activate the ERK and p38 pathways, Myc-ERK1 and FLAG-p38 were co-transfected into Phoenix-A cells with different doses of TNIK. The transfected kinases were then immunoprecipitated from cell lysates and the kinase activities measured using myelin basic protein and GST-ATF2 as exogenous substrates. In contrast to JNK2, neither ERK1 nor p38 was activated by TNIK overexpression, whereas co-transfection of MEKK1 potently activated both kinases (Fig. 5, B and C). In addition, TNIK did not activate NF-κB (data not shown).

To further investigate the mechanism of this activation, the cohort of TNIK mutants were co-transfected into Phoenix-A cells with Myc-JNK2, and the ability of these mutants to up-regulate JNK2 kinase activity was examined by the in vitro kinase assay. TNIK(WT), TNIK(KM), TNIK(C1), and TNIK(C2) were all able to activate Myc-JNK2, whereas TNIK(N1) and TNIK(N2) were not (Fig. 5D). This result suggested that the C-terminal GCKH region is both necessary and sufficient for activation of the JNK pathway, whereas the kinase domain is dispensable.

Regulation of the Cytoskeleton by TNIK—When TNIK was overexpressed in Phoenix-A cells, the cells showed a striking morphological change. In control GFP-transfected cells, more than 80% of GFP-positive cells were adherent and well spread (Fig. 6A, top row, left panel). In contrast, in TNIK and GFP co-transfected cells, more than 80% of GFP-positive cells showed inhibited cell spreading. These cells rounded up and lost attachment to the plate (Fig. 6A, top row, right panel). Similar morphologic change was also observed in Hela and NIH-3T3 cells transfected with TNIK (data not shown). We then transfected the cohort of TNIK mutants into Phoenix-A cells to determine which domain of TNIK was involved in inducing the morphologic change. TNIK(KM), TNIK(C1), and TNIK(C2), which lacked the kinase activity, failed to induce the morphologic change (Fig. 6A, left column, middle and bottom panels and data not shown), whereas TNIK(N1) and TNIK(N2) were both competent in inducing the inhibition of cell spreading (Fig. 6A, right column, middle panel, and data not shown). Therefore, the kinase domain, rather than the GCKH domain required for JNK activation, was both necessary and sufficient for TNIK to regulate cell spreading. This result suggested that the JNK pathway was not involved in this regulation. Consistent with this hypothesis, overexpression of Myc-JNK failed to inhibit cell spreading (Fig. 6A, right column, bottom panel). Because JNK has been implicated in inducing apoptosis in some cells (17), we examined whether cells transfected with TNIK were undergoing apoptosis. Nuclei of phenix-A cells transfected with control vector, TNIK(WT), TNIK(KM), or RIP were stained with Hoechst 33258 (Fig. 6B). No apoptotic body was observed in vector, TNIK(WT)- or TNIK(KM)-transfected cells, whereas apoptotic bodies were readily detected in greater than 60% of cells transfected with the control RIP cDNA (Fig. 6B). In addition, no activation of caspases was observed in TNIK-transfected cells (data not shown). Taken together, these results suggested that TNIK induced cell morphology change but not apoptosis in transfected Phoenix-A cells.

These observations raised the possibility that overexpression of TNIK might have disrupted intracellular F-actin structure. We therefore examined actin distribution in the Triton X-100-
soluble (G-actin) and -insoluble (F-actin) fractions in control vector, TNIK- and TNIK(KM)-transfected Phoenix-A cells. Overexpression of wild type TNIK, but not empty vector or TNIK(KM), resulted in the enhanced distribution of actin in Triton X-100-soluble fraction, consistent with the reduced spreading observed in these cells (Fig. 6C). We hypothesized that overexpression of TNIK may lead to phosphorylation of cytoskeletal components. Recently, a GCK family protein kinase that could phosphorylate the actin-fragmenting protein Severin was purified and cloned from Dictyostelium (14). We therefore decided to test whether TNIK was able to phosphorylate the mammalian Severin homologue, Gelsolin (18). TNIK and TNIK(KM) were expressed in Phoenix-A cells, immunoprecipitated and incubated in an in vitro kinase assay. Wild type TNIK, but not the kinase mutant form of TNIK, phosphorylated Gelsolin in vitro (Fig. 6D).
DISCUSSION

We describe in this report the cloning of a novel member of the GCK family, TNIK. TNIK shares strong homology (90%) with NIK in both the kinase domain and the C-terminal GCKH domain. However, it deviates significantly from NIK in the intermediate region, where only 53% of amino acids are conserved. In addition, three regions in the intermediate domain can be alternatively spliced, resulting in a total of eight spliced variants.

FIG. 6. Regulation of the cytoskeleton by TNIK. A, inhibition of cell spreading by TNIK. 0.4 μg of GFP was co-transfected into Phoenix-A cells with 3 μg of Vector, TNIK(WT), TNIK(KM), TNIK(N1), TNIK(C1), or JNK2. 24 h after transfection, cells were examined under fluorescent microscope. B, TNIK overexpression did not induce apoptosis. 3 μg of Vector, TNIK(WT), TNIK(KM), or RIP was transfected into Phoenix-A cells for 24 h. Transfected cells were stained with Hoechst 33258 and examined under fluorescent microscope as described under “Experimental Procedures.” C, TNIK overexpression induced redistribution of actin. Phoenix-A cells were transfected with 3 μg of vector, HA-TNIK(WT), or HA-TNIK(KM) and lysed with 1% Triton X-100 as described under “Experimental Procedures.” Top panel, cell lysates (4 × 10⁵ cells) from the Triton X-100-soluble (lanes 1–3) or -insoluble (lanes 4–6) fractions were resolved on SDS-PAGE and immunoblotted with an anti-β-actin mAb. Bottom panel, total cell lysates were blotted with an anti-HA mAb to control for expression levels of TNIK(WT) and TNIK(KM). D, phosphorylation of Gelsolin by TNIK in vitro. Phoenix-A cells were transiently transfected with 3 μg of HA-TNIK(WT) (lane 1) or HA-TNIK(KM) (lane 2). Cell lysates were subjected to anti-HA immunoprecipitation and an in vitro kinase assay using Gelsolin (Sigma) as an exogenous substrate.
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isoforms. The functional differences among the eight isoforms are currently unknown.

Like many other GCK family kinases, overexpression of TNIK specifically activated the JNK pathway (Fig. 5A). It had no effect on either the ERK pathway or the p38 pathway (Fig. 5, B and C). However, unlike any other GCK family members, both the kinase mutant form of TNIK and the GCKH domain of TNIK were as effective as the wild type protein in JNK2 activation, and the kinase domain alone of TNIK was virtually ineffective (Fig. 5D). This result suggested that the C-terminal GCKH domain was solely responsible for the activation. This is in contrast to other GCK family kinases, which activate the JNK pathway either using the kinase domain alone, as is seen with GCKR, HPK/GCK-like kinase, and hematopoietic protein kinase 1, or using the kinase domain plus the GCKH region, as is seen with GCK, GCK-like kinase, and NIK (7–12). The GCKH domain of NIK interacted with MEKK1, and the dominant negative mutant of MEKK1 inhibited NIK-induced JNK activation (12). Given the high level of sequence identity between the GCKH of NIK and the GCKH of TNIK, TNIK likely activated the JNK pathway through MEKK1.

NIK was cloned by its ability to interact with the adapter protein NCK. It associated with NCK SH3 domains via two PXXPXR sequences in the intermediate domain, PCPPPSR (amino acids 574–579) and PRVPR (amino acids 611–616). Both sequences were required for efficient interaction (12). Similar to NIK, TNIK also interacted with NCK via the intermediate domain. However, PCPPPSR is not conserved in TNIK. Instead, TNIK contained two other PXXPXR sequences, PN-LPXR (amino acids 562–567) and PPLPXR (amino acids 647–652), in addition to the conserved PKVPQR (amino acids 670–675). TNIK likely interacted with NCK through the cooperative interaction with these three PxxPxr sequences. NCK is an adapter protein involved in many growth factor receptor-mediated signal transduction pathways (19). It has been proposed that the NIK-NCK interaction may recruit NIK to receptor or nonreceptor tyrosine kinases to regulate MEKK1 (12). TNIK may be recruited in a similar fashion.

TNIK also interacts via its intermediate domain with the Traf domain of Traf2. Both GCK and GCKR have been previously reported to interact with Traf2, and it has been suggested that they mediate Traf2-induced JNK activation (7, 10, 13). More recently, a Drosophila GCK family member, Misshapen (Msn), has been reported to interact with D-Traf1 and mediate D-Traf1-induced JNK activation (20). Msn has highest homology to NIK and TNIK. Similar to NIK and TNIK, Msn also interacted with Dock, the Drosophila homologue of NCK (20). In Drosophila, deficiency in Dock results in defective photoreceptor guidance (21), and in mammalian cells, NCK interacts with WASP, a CDC42 effector protein involved in the regulation of cytoskeleton (22, 23). These findings strongly suggest that the NCK pathway is closely linked to the cytoskeletal changes. Consistently, Msn deficiency leads to defective dorsal closure that requires extensive cell migration and cell shape changes in addition to the activation of the JNK pathway (24). Interaction of Man with Dock may regulate these cell shape changes. TNIK may participate in the regulation of a similar pathway in mammalian cells.

Supporting this hypothesis, overexpression of TNIK inhibited cell spreading in Phoenix-A cells, NIH-3T3 cells and Hela cells (Fig. 6A and data not shown). This effect is likely due to the disruption of filamentous actin structure. No F-actin fiber could be detected by staining with TRITC-Phalloidin of NIH-3T3 cells transfected with a GFP-TNIK fusion protein, whereas F-actin fibers were abundant in cells transfected with GFP alone (data not shown). Consistent with this notion, overex- pression of TNIK resulted in a decreased proportion of actin in the Triton X-100-insoluble fraction (Fig. 6C). The Triton X-100-insoluble fraction contains the filamentous actin pool, whereas the Triton X-100-soluble fraction contains the globular actin monomers. This is the first evidence that a mammalian GCK family member exerts an effect on cytoskeletal organization. A Dictyostelium GCK member was recently cloned that can phosphorylate the Dictyostelium actin fragmenting protein, Sevin, in vitro (14). Interestingly, TNIK can phosphorylate the mammalian Sevin homologue, Gelsolin, in vitro (Fig. 6D).

Gelsolin is also an F-actin fragmenting and capping enzyme that can reduce the content of F-actin. Although it is not known whether Gelsolin phosphorylation affects its activity, this result raises a possibility that TNIK may regulate F-actin assembly through Gelsolin or other related actin severing enzymes. This is consistent with the result that the kinase domain of TNIK is responsible for the regulation of cell spreading (Fig. 6A). The mammalian p21-activated kinase, PAK1, which is distantly related to GCK family members and an effector protein of small G proteins Rac1 and CDC42, has been demonstrated to regulate actin cytoskeleton organization. One proposed mechanism of the regulation is through phosphorylation and inhibition of the myosin light chain kinase (25). Interestingly, overexpression of a constitutively active form of PAK1 also resulted in the inhibition of cell spreading (21), an effect similar to that caused by overexpression of TNIK (Fig. 6, A and B). It is therefore of interest to test whether TNIK can also phosphorylate the myosin light chain kinase.

Evidence provided in this study suggests that GCK family kinases may participate in regulating the cytoskeleton organization, in addition to their roles in regulating the JNK pathway. It will be of interest to examine whether NIK has a similar activity. Because of the high level of homology between TNIK and NIK in the kinase domain and GCKH domain, these two kinases may serve redundant functions. Alternatively, the diverse sequence in the intermediate domain may dictate the specificity of these two kinases. We are currently using yeast two-hybrid to identify additional proteins that bind to the intermediate domain of TNIK, which may give us more information on its physiological function.

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