The c-Jun N-terminal kinase (JNK) signaling pathway plays a crucial role in cellular responses stimulated by stress-inducing agents and proinflammatory cytokines. The group I germinal center kinase family members selectively activate the JNK pathway. In this study, we have isolated a mouse cDNA encoding a protein kinase homologous to Nck-interacting kinase (NIK), a member of the group I germinal center kinase family. This protein kinase is expressed during the late stages of embryogenesis, but not in adult tissues, and thus named NESK (NIK-like embryo-specific kinase). NESK selectively activated the JNK pathway when overexpressed in HEK 293 cells but did not stimulate the p38 kinase or extracellular signal-regulated kinase (ERK) pathways. NESK-induced JNK activation was inhibited by the dominant negative mutants of MEKK1 and MKK4. Tumor necrosis factor (TNF)-α or TNF receptor-associated factor 2 (TRAF2) stimulated the NESK activity. Furthermore, this dominant negative NESK mutant inhibited the JNK activation induced by TNF-α or TRAF2. These results suggest that NESK, a novel activator of the JNK pathway, functions in coupling TRAF2 to the MEKK1 → MKK4 → JNK kinase cascade during the late stages of mammalian embryogenesis.

The germinal center kinase (GCK) family is a subfamily of the Ste20 family of protein kinases (1). GCK family members have an N-terminal kinase domain and a C-terminal regulatory domain. The GCK family is divided into two structurally and functionally distinct groups (1). Group I GCKs include GCK itself, hematopoietic progenitor kinase-1, GCK-like kinase, GCK-related kinase, Nck-interacting kinase (NIK), kinase homologous to SPS1/STE20, hematopoietic progenitor kinase/GCK-like kinase, and TNF receptor-associated factor 2 (TRAF2)- and Nck-interacting kinase. These kinases selectively activate the c-Jun N-terminal kinase (JNK) signaling pathway when overexpressed in cultured cells but do not stimulate the p38 kinase or extracellular signal-regulated kinase (ERK) signaling pathways (2–13). Group II GCKs share catalytic domain homology with group I GCKs, but their C-terminal regulatory domains differ significantly from those of group I GCKs (14–17). Group II GCKs do not activate any of the known mitogen-activated protein kinase pathways.

The JNK pathway plays a crucial role in cellular responses stimulated by a variety of stress-inducing agents, including osmotic and heat shock, UV irradiation, and proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin 1 (18). JNKs are activated through threonine and tyrosine phosphorylations by mitogen-activated protein kinase kinases such as MKK4 and MKK7, which are in turn phosphorylated and activated by mitogen-activated protein kinase kinase kinase, including MEKK1, mixed lineage kinase 2, and mixed lineage kinase 3 (18). Many members of the group I GCK family, including GCK, GCK-related kinase, GCK-like kinase, hematopoietic progenitor kinase/GCK-like kinase, and TRAF2- and Nck-interacting kinase, have been shown to mediate the TNF-α-induced JNK activation (5, 7, 10, 11, 13). Among them, GCK, GCK-related kinase, and TRAF2- and Nck-interacting kinase have been implicated in mediating the TNF-α-induced JNK activation through TRAF2 (7, 10, 13). The JNK activation by group I GCKs such as GCK and GCK-like kinase is mediated through the MEKK1 and MKK4 kinase cascade (5, 10), whereas TAK1, but not MEKK1, functions as a mitogen-activated protein kinase kinase kinase in the hematopoietic progenitor kinase/GCK-like kinase-induced JNK activation (11). NIK and TRAF2- and Nck-interacting kinase interact with the Src homology 2-Src homology 3 domain-containing adapter protein Nck and have been proposed to link protein tyrosine kinase signals to the JNK activation (8, 13).

The Drosophila Misshapen and C. elegans Mig-15 proteins are highly homologous to mammalian NIK, both within and outside of the kinase domain (19). The Misshapen protein functions upstream of basket, the Drosophila homologue of JNK, and hemipterous, a homologue of MKK7, to stimulate dorsal closure in the Drosophila embryo (19). Dorsal closure occurs during the later stages in Drosophila embryogenesis and involves cell migrations and shape changes that position and fuse the lateral epidermal primordia over the aminoserosa. The Drosophila TRAF binds Misshapen in vitro, and coexpression of Misshapen and Drosophila TRAF leads to the synergistic activation of JNK (20). The C. elegans Mig-15 is necessary for several developmental processes in C. elegans, mig-15 mutants.
have a variety of developmental defects including defects in Q-neuroblast migration and muscle arm targeting (19). Thus, GCKs may be responsible for some of the developmental processes in mammals.

We report here a novel mammalian group I GCK family kinase isolated from mouse embryo by a PCR-based screen for cDNA clones of protein kinases. It is expressed during the late stages of mouse embryogenesis, but not in various adult tissues, and has the highest homology to NIK. Thus, this newly identified kinase was designated Nesk (NIK-like embryo-specific kinase). We demonstrate in this report that Nesk, like other group I GCK family members, can selectively activate the JNK pathway when overexpressed in HEK 293 cells. Furthermore, dominant negative forms of Mekk1 and Mkk4 inhibited the Nesk-induced JNK activation, and a dominant negative Nesk mutant inhibited Traf2-induced JNK activation, suggesting that Nesk functions downstream of Traf2 and upstream of Mekk1 and Mkk4 in the JNK signaling pathway.

**EXPERIMENTAL PROCEDURES**

**PCR Cloning of Partial cDNAs for Protein Kinases**—Degenerate oligonucleotide primers (cDNA library corresponding to subdomains Vb and VIa of GCK acid sequences VHRDL and D/V/MW(S/A)(F/Y), respectively) of the catalytic domain of protein kinases were chemically synthesized. The sequences of the primers were as follows: forward primer, 5′-G(T/G/T/C/A/G/C/A/G/C/T/C/T/C-T′; reverse primer, 5′-G/A/T/A/G(T/G/C)/A/T/C/C/A/G/A/T/C/T/C-G′-Tc). Double-stranded cDNA from mouse 11-day embryo (QuickClone; Clontech) was amplified by PCR with the primers, using a GeneAmp® DNA amplification reagent kit (Takara Shuzo Co.). Thirty cycles were performed with a step cycle profile of 30 s at 94 °C, 30 s at 35 °C, and 2 min 30 s at 72 °C. The PCR products were purified by agarose gel electrophoresis and subcloned into pT7Blue vector (Novagen) for DNA sequencing.

**Library Screening and Sequence Analysis**—The cloned PCR product was used as a probe to screen a Agt11 mouse whole embryo (15 days) cDNA library (Clontech) to obtain a full-length cDNA clone. Hybridization to nylon replica membranes (Hybond-N®; Amersham Pharmacia Biotech) was performed at 42 °C for 16 h with 32P-labeled probe in a solution containing 50% formamide, 5× Denhardt’s solution (0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, and 0.1% Ficoll), 5× SSPE (0.75 M NaCl, 50 mM sodium phosphate, pH 7.4, 6 mM EDTA), 0.5% SDS, and 0.1 mg/ml salmon sperm DNA. The probe was labeled using the Rediprime DNA labeling system (Amersham Pharmacia Biotech). The membranes were washed twice with 1× SSPE containing 0.1% SDS at 65 °C for 15 min. Hybridization-positive phage clones were isolated by repeated plaque purification. The DNA sequences were determined from both strands by the chain termination method.

**Northern Blot Analysis**—Mouse adult and embryo multiple-tissue Northern blots (Clontech) were hybridized in same hybridization solution used for library screening at 42 °C for 16 h with the 32P-labeled probe. The membranes were washed twice with 1× SSPE containing 0.1% SDS at 65 °C for 15 min. The hybridization probe was the PCR clone of Nesk.

**Expression Plasmids**—Full-length Nesk and the kinase domain of Nesk (ΔNesk) were cloned into mammalian FLAG tag expression vectors (pME18S-FLAG and pCMV-FLAG) by PCR using two oligonucleotide primers. A catalytically inactive Nesk mutant (pME18S-FLAG-NESSK(K54E)) and pCMV-FLAG-NESSK(K54E)) was created with the QuickChange™ mutagenesis kit (Stratagene). Complementary DNAs for human TRAF2 and a region of human MEKK1 corresponding with other known protein kinases. We previously reported one of them (24). We designated the other clone NESK.

**Cell Culture and Transfection**—COS7 and HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2. Plasmid DNAs were transfected into COS7 or HEK 293 cells by FuGene™ transfection Reagent (Roche Molecular Biochemicals). The final amount of transfected DNA was adjusted with empty vector, pME18S or pCMV.

**RESULTS**

**Isolation of a cDNA Clone Encoding a Novel Protein Kinase, Nesk**—To isolate cDNA clones encoding novel protein kinases, we used the strategy of PCR amplification with degenerative oligonucleotide primers corresponding to conserved amino acid sequences in the catalytic domain of protein kinases. The amplified PCR products of approximately 200 base pairs from mouse 11-day embryo cDNA were cloned in the plasmid vector and identified by DNA sequencing. Sequences of 20 different mouse 11-day embryo cDNA were cloned in the plasmid vector.

**In Vitro Kinase Assays**—At 48 h after transfection, the cells were lysed with 400 μl of lysis buffer A (20 mM HEPES-NaOH, pH 7.5, 3 mM MgCl2, 100 mM NaCl, 1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM EDTA, 1 mM Na3VO4, 10 mM NaF, 20 mM β-mercaptoethanol, and 0.5% Triton X-100). The lysates were centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was incubated with 0.2 μg of an anti-FLAG antibody (M2 monoclonal antibody; Eastman kodak Co.) and 20 μl of a 50% slurry of protein A-Sepharose (Amersham Pharmacia Biotech) for 1.5 h at 4 °C. The immune complexes were precipitated and washed twice with lysis buffer A and twice with reaction buffer A (20 mM HEPES-NaOH, pH 7.5, 1 mM diithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml leupeptin, and 0.1 mM EDTA). The precipitates were incubated in 30 μl of reaction buffer A containing 5 μg of myelin basic protein (MBP) (Sigma), 20 μM ATP, and 5 μCi of [γ-32P]ATP (NEN Life Science Products) at 30 °C for 20 min. The reaction was stopped by adding 10 μl of 4× Laemmli sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 30% diithiothreitol, and 10% glycerol). The mixture was heated at 95 °C for 5 min. The proteins were separated by SDS-polyacrylamide gel electrophoresis. The radioactivity incorporated into MBP was detected by autoradiography. Assays were performed at least three times, and representative results are shown in the figures.

**Immunoblotting**—Aliquots of cell lysates were boiled in Laemmli sample buffer. The boiled samples were electrophoresed on polyacrylamide gels, and the proteins were electrophoretically transferred to nitrocellulose membranes. After the membranes were blocked, the separated proteins were visualized by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech), using anti-rabbit or anti-mouse Ig antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) as a secondary antibody.

**Northern Blot Analysis**—Mouse adult and embryo multiple-tissue Northern blots were hybridized in same hybridization solution used for library screening at 42 °C for 16 h with the 32P-labeled probe. The membranes were washed twice with 1× SSPE containing 0.1% SDS at 65 °C for 15 min. Hybridization-positive phage clones were isolated by repeated plaque purification. The DNA sequences were determined from both strands by the chain termination method.

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**RESULTS**

**Isolation of a cDNA Clone Encoding a Novel Protein Kinase, Nesk**—To isolate cDNA clones encoding novel protein kinases, we used the strategy of PCR amplification with degenerative oligonucleotide primers corresponding to conserved amino acid sequences in the catalytic domain of protein kinases. The amplified PCR products of approximately 200 base pairs from mouse 11-day embryo cDNA were cloned in the plasmid vector and identified by DNA sequencing. Sequences of 20 different protein kinases were cloned. Two of them were distinctly related with other known protein kinases. We previously reported one of them (24). We designated the other clone Nesk and characterized it further.

**Northern Blot Analysis**—Using the PCR clone as a probe revealed that the mouse embryo produced significant amounts of Nesk mRNA. Thus, to obtain a full-length cDNA clone for mouse Nesk, a cDNA library from the mouse embryo was screened using the PCR clone as a probe. Eight hybridization-positive clones were obtained from about 8×105 plaques. The clone that contained the largest cDNA was sequenced to determine the primary structure of mouse Nesk.

**Amino Acid Sequence of Nesk**—The nucleotide sequence of the cDNA predicted an open reading frame of 1455 amino acids with a calculated molecular mass of 167,325 (Fig. 1A). The deduced amino acid sequence contained a kinase catalytic do
main in the N terminus that included 12 kinase subdomains (Fig. 1B). Comparison of the amino acid sequence of the kinase domain of NESK with other sequences showed it to be most similar to that of NIK (8), a member of the group I GCK family, with 57% identity (Fig. 1B). In addition, NESK had a C-terminal germinal center kinase homology region. It shared 38% amino acid identity with NIK (Fig. 1C). However, NESK was only 20% identical to NIK in the intermediate region. While NIK has two potential Src homology 3 domain binding sites (8), NESK contained three possible Src homology 3 binding sites in the intermediate region. NESK shared 46 and 56% amino acid identity in the kinase domain and 33 and 32% identity in the germinal center kinase homology region with C. elegans Mig-15 and Drosophila Misshapen proteins, respectively (Fig. 1, B and C).

Expression Pattern of NESK mRNA—The expression of NESK mRNA was examined in a variety of mouse adult tissues and at various stages of mouse embryogenesis by Northern blot analysis using the first PCR product of NESK as a probe. The probe hybridized to a transcript of approximately 9.0 kilobases in the late stages of embryogenesis. However, no hybridized transcript was detected in any adult tissues examined (Fig. 2). These results suggest that NESK functions in the late stages of embryogenesis.

Kinase Activity of NESK—To determine whether NESK has kinase activity, COS7 cells were transfected with the FLAG-tagged full-length NESK or the FLAG-tagged truncated NESK containing only the kinase domain, and an immune complex kinase assay was performed on NESK immunoprecipitates using MBP as a substrate. Although the immunoprecipitates from cells transfected with vector alone phosphorylated MBP, the kinase activity was markedly increased in immunoprecipi-
tates from cells transfected with the full-length NESK (Fig. 3, lanes 1 and 2). The kinase activity was much higher in immunoprecipitates from cells transfected with the kinase domain of NESK (Fig. 3, lane 4). To rule out the possibility that an associated kinase coprecipitating with NESK may account for the kinase activity, a FLAG-tagged kinase-defective NESK mutant in which Lys54 in the ATP binding domain was replaced with a glutamic acid (K54E) was expressed in COS7 cells. The level of phosphorylation of MBP by the K54E mutant was much lower than that of the kinase-active NESK (Fig. 3, lane 3). These results indicate that NESK is a functional protein kinase.

NESK Activates JNK, but Not p38 Kinase or ERK, in Transfected HEK 293 Cells—To examine whether NESK can activate the mammalian JNK cascade, we co-transfected HEK 293 cells with mammalian expression vectors encoding the full-length NESK, the kinase-inactive NESK (K54E), or the kinase domain of NESK (∆NESK) and an HA epitope-tagged JNK1. Recombinant JNK was then immunoprecipitated from cell lysates and used in a protein kinase assay with GST-c-Jun as a substrate. Transfection of cells with the full-length NESK resulted in JNK1 activation (Fig. 4A, lane 2), while cells transfected with vector alone showed little activation (Fig. 4A, lane 1). Transfection of cells with the kinase-inactive form of NESK (K54E) did not result in JNK1 activation (Fig. 4A, lane 3). Transfection of cells with the kinase domain of NESK (∆NESK) resulted in strong JNK1 activation (Fig. 4A, lane 4). Thus, the kinase activity of NESK is required for JNK activation.

To examine whether NESK can also activate p38 kinase or ERK, HEK 293 cells were transiently co-transfected with the full-length NESK, NESK(K54E), or ∆NESK along with HA epitope-tagged p38 kinase or ERK2. p38 kinase or ERK2 was then immunoprecipitated, and its activity was assayed by phosphorylation of either GST-ATF2 or MBP. Treatment of cells with anisomycin strongly activated p38 kinase (Fig. 4B, lane 5). However, no increase in p38 kinase activity was observed when NESK and its mutants were overexpressed in HEK 293 cells (Fig. 4B, lanes 1–4). Similarly, although expression of Ras(G12V) in HEK 293 cells strongly activated ERK2 (Fig. 4C, lane 5), ERK2 activity was not increased when NESK and its mutants were overexpressed in HEK 293 cells (Fig. 4C, lanes 1–4). These results suggest that NESK does not play a role in the p38 kinase and ERK pathways and that the activation of the JNK pathway by NESK is specific.

Inhibition of NESK-induced JNK Activation by the Dominant Negative Mutants of MKK4 and MEKK1—M KK4 is an
The GCK Family Kinase NESK

Inhibition of TNF-α or TRAF2-induced JNK Activation by the Dominant Negative Mutant of NESK—To determine whether TNF-α can regulate NESK kinase activity, HEK 293 cells were transfected with an expression vector encoding NESK and exposed to TNF-α for various periods of time. Then an immune complex kinase assay was performed using MBP as a substrate. NESK immune complexes from TNF-α-treated cells exhibited an elevation in in vitro protein kinase activity (Fig. 6A). Next, to determine whether NESK is involved in the TNF-α-induced JNK activation, HEK 293 cells were transfected with an expression vector encoding a dominant negative mutant of NESK. Reverse transcriptase-PCR analysis showed that the NESK mRNA was expressed in HEK 293 cells (data not shown), suggesting that the endogenous NESK protein is present in HEK 293 cells. We expected that the dominant negative mutant of NESK inhibited the activity of the endogenous NESK. The expression of the dominant negative mutant of NESK inhibited the JNK activity induced by TNF-α (Fig. 6B). upstream activator of JNK, which phosphorylates and activates JNK. To determine whether NESK activates JNK through MKK4, HEK 293 cells were co-transfected with expression vectors encoding NESK and a dominant negative mutant of MKK4 to determine whether the dominant negative mutant of MKK4 could inhibit the NESK-induced JNK activation. The expression of the dominant negative form of MKK4 inhibited the JNK activity induced by NESK (Fig. 5, lanes 2 and 4), suggesting that NESK functions upstream of MKK4. MKK1 is a physiological activator of MKK4, which phosphorylates and activates MKK4. To determine whether NESK activates JNK through MEKK1, HEK 293 cells were co-transfected with expression vectors encoding NESK and a dominant negative mutant of MEKK1. The expression of the dominant negative form of MEKK1 inhibited the JNK activity induced by NESK (Fig. 5, lane 3), suggesting that MEKK1 is a downstream target for the NESK activity in the JNK signaling cascade.

FIG. 4. Activation of JNK, but not p38 or ERK, by NESK in transfected HEK 293 cells. A, HEK 293 cells (5 × 10⁶ cells/60-mm dish) were transfected with 1 μg of SRα-HA-JNK1 (lanes 1–5) plus 2 μg of the empty vector pCMV alone (lane 1), 2 μg of pCMV-FLAG-NESK (lane 2), 2 μg of pCMV-FLAG-ΔNESK (lane 3), 2 μg of pCMV-FLAG-MEKK1 (lane 4), and 2 μg of pCMV-FLAG-MEKK1Δ (lane 5). The cells were collected 48 h after transfection, and immune complex kinase assays were performed with an anti-HA antibody using GST-c-Jun–(1–223) as a substrate (upper panel). B, HEK 293 cells were transfected with 1 μg of pCMV-HA-p38 (lanes 1–5), plus 2 μg of the same expression vectors (lanes 1–4) as described in A. Otherwise, the cells were treated with anisomycin (20 μg/ml) for 20 min (lane 5). The cells were collected 48 h after transfection or after anisomycin treatment, and immune complex kinase assays were performed with an anti-HA antibody using GST-ATF2 as a substrate (upper panel). C, HEK 293 cells were transfected with 1 μg of SRα-HA-ERK (lanes 1–5), plus 2 μg of the same expression vectors (lanes 1–4) as described in A and 2 μg of pCMV-Ras(G12V) (lane 5). The cells were collected 48 h after transfection, and immune complex kinase assays were performed with an anti-HA antibody using MBP as a substrate (upper panel). Expression levels of proteins were verified equivalent by immunoblotting using an anti-HA antibody (middle panel) and an anti-FLAG antibody (bottom panel).

FIG. 5. Inhibition of NESK-induced JNK activation by dominant negative kinase mutants of MEKK1 and MKK4 in HEK 293 cells. HEK 293 cells (5 × 10⁶ cells/60-mm dish) were transfected with 1 μg of SRα-HA-JNK1 (lanes 1–4) plus 2 μg of pCMV-FLAG-NESK (lanes 2–4), 2 μg of pCMV-FLAG-MEKK1(KR) (lane 3), and 2 μg of pCMV-FLAG-MKK4(KR) (lane 4). The final amount of DNA was adjusted to 5 μg with empty vector. The cells were collected after 48 h, and immune complex kinase assays were performed with an anti-HA antibody using GST-c-Jun–(1–223) as a substrate (top panel). Expression levels of proteins were verified equivalent by immunoblotting using an anti-HA antibody (middle panel) and an anti-FLAG antibody (bottom panel).

Inhibition of TNF-α or TRAF2-induced JNK Activation by the Dominant Negative Mutant of NESK—To determine whether TNF-α can regulate NESK kinase activity, HEK 293 cells were transfected with an expression vector encoding NESK and exposed to TNF-α for various periods of time. Then an immune complex kinase assay was performed using MBP as a substrate. NESK immune complexes from TNF-α-treated cells exhibited an elevation in in vitro protein kinase activity (Fig. 6A). Next, to determine whether NESK is involved in the TNF-α-induced JNK activation, HEK 293 cells were transfected with an expression vector encoding a dominant negative mutant of NESK. Reverse transcriptase-PCR analysis showed that the NESK mRNA was expressed in HEK 293 cells (data not shown), suggesting that the endogenous NESK protein is present in HEK 293 cells. We expected that the dominant negative mutant of NESK inhibited the activity of the endogenous NESK. The expression of the dominant negative mutant of NESK inhibited the JNK activity induced by TNF-α (Fig. 6B).
The GCK Family Kinase NESK

FIG. 6. Inhibition of TNF-α-induced JNK activation by dominant negative mutants of NESK in HEK 293 cells. A, HEK 293 cells (5 × 10⁵ cells/60-mm dish) were transfected with 2 μg of pCMV-FLAG-NESK. The cells were treated with TNF-α (50 ng/ml) for 0, 5, 15, 30, and 60 min (lanes 1–5) at 48 h after transfection, and immune complex kinase assays were performed with an anti-FLAG antibody using MBP as a substrate (upper panel). Expression levels of NESK were verified equivalent by immunoblotting using an anti-FLAG antibody (bottom panel). B, HEK 293 cells (5 × 10⁵ cells/60-mm dish) were transfected with 1 μg of SRA-HA-JNK1 (lanes 1–3), plus 2 μg of pCMV-FLAG-NESK(K54E) (lane 3). The final amount of DNA was adjusted to 3 μg with empty vector. The cells were treated with (lanes 2 and 3) and without (lane 1) TNF-α (50 ng/ml) for 10 min at 48 h after transfection, and immune complex kinase assays were performed with an anti-HA antibody using GST-c-Jun-(1–223) as a substrate (upper panel). Expression levels of proteins were verified equivalent by immunoblotting using an anti-HA antibody (middle panel) and an anti-FLAG antibody (bottom panel).

FIG. 7. Inhibition of TRAF2-induced JNK activation by dominant negative mutants of NESK. A, HEK 293 cells (5 × 10⁵ cells/60-mm dish) were transfected with 2 μg of the empty vector pCMV alone (lane 1), pCMV-FLAG-NESK (lanes 2 and 3), and pCMV-VSVG-TRAF2 (lane 3). The final amount of DNA was adjusted to 4 μg with empty vector. After 48 h, the cells were collected, and immune complex kinase assays were performed with an anti-FLAG antibody using MBP as a substrate (upper panel). Expression levels of proteins were verified equivalent by immunoblotting using an anti-FLAG antibody (middle panel) and an anti-TRAF2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (bottom panel). B, HEK 293 cells (5 × 10⁵ cells/60-mm dish) were transfected with 1 μg of SRA-HA-JNK1 (lanes 1–3), 2 μg of pCMV-VSVG-TRAF2 (lanes 2 and 3), 2 μg of pCMV-FLAG-NESK(K54E) (lane 3). The final amount of DNA was adjusted to 5 μg with empty vector. The cells were collected after 48 h, and immune complex kinase assays were performed with an anti-HA antibody using GST-c-Jun-(1–223) as a substrate (upper panel). Expression levels of proteins were verified equivalent by immunoblotting using an anti-HA antibody (middle panels) and an anti-FLAG antibody (bottom panel).

6B), suggesting that NESK is involved in the TNF-α-induced signaling pathway.

Because TNF-α-induced JNK activation requires TRAF2 (25), the effect of TRAF2 on NESK activity was examined by co-transfecting expression vectors encoding NESK and TRAF2. The presence of TRAF2 markedly increased NESK kinase activity (Fig. 7A). To determine whether NESK is involved in the TRAF2-induced JNK activation, HEK 293 cells were co-transfected with expression vectors encoding TRAF2 and a dominant negative mutant of NESK. The expression of the dominant negative NESK inhibited the JNK activity induced by TRAF2 (Fig. 7B). These results suggest that NESK is a downstream target of TRAF2 in TNF-α signaling.

DISCUSSION

In this study, we identified a novel protein kinase, NESK, from mouse embryo. NESK shares high sequence homology with members of the group I GCK family in both the N-terminal kinase domain and the C-terminal putative regulatory domain. All members of the group I GCK family selectively activate the JNK pathway when overexpressed in cultured cells but do not stimulate the p38 kinase or ERK signaling pathways (1). NESK also activates the JNK pathway when overexpressed in HEK 293 cells but does not activate the p38 kinase or ERK pathways. Based on these structural and functional properties, it can be concluded that NESK is a novel member of the group I GCK family.

NESK mRNA is expressed exclusively during the late stages of mouse embryogenesis. Further, NESK is highly homologous to the Drosophila Misshapen and C. elegans Mig-15 proteins, both in the kinase domain and in the C-terminal regulatory domain. The Misshapen protein functions upstream of the JNK pathway to stimulate dorsal closure in the Drosophila embryo (19). Dorsal closure occurs during the late stages of Drosophila embryogenesis. The Mig-15 protein is necessary for several developmental processes in C. elegans (19). Thus, NESK is suggested to function as an intracellular signaling molecule in a developmental process during the late stages of mammalian embryogenesis. More recently, a novel member of the group I GCK family, named NRK, was cloned from mouse embryo (26). NESK is 99% identical to NRK. Moreover, the expression pat-
The GCK Family Kinase NESK

The present study, we have demonstrated that TNF-α and TRAF2 stimulated the NESK activity, and the dominant negative mutant of NESK blocked TNF-α or TRAF2-induced JNK activation. Thus, NESK may interact with TRAF2 and play a role in coupling TRAF2, as well as TNF-α, to JNK activation.

In this study, we have found candidates of components functioning upstream and downstream of NESK in mammalian cells. However, NESK seems to be expressed in specific types of cells at the late stages of mouse embryogenesis. Thus, the identification of upstream and downstream signaling molecules of NESK in these cells will provide valuable insights into the signaling pathway regulated by NESK.

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