Rap2 to Regulate Actin Cytoskeleton

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**Rap2 belongs to the Ras family of small GTP-binding proteins, but its specific roles in cell signaling remain unknown. In the present study, we have affinity-purified from rat brain a Rap2-interacting protein of ≈155 kDa, p155. By liquid chromatography tandem mass spectrometry, we have identified p155 as Traf2- and Nck-interacting kinase (TNIK). TNIK possesses an N-terminal kinase domain homologous to STE20, the Saccharomyces cerevisiae mitogen-activated protein kinase kinase kinase kinase, and a C-terminal regulatory domain termed the citron homology (CNH) domain. TNIK induces disruption of F-actin structure, thereby inhibiting cell spreading. In addition, TNIK specifically activates the c-Jun N-terminal kinase (JNK) pathway. Among our observations, TNIK interacted with Rap2 through its CNH domain but did not interact with Rap1 or Ras. TNIK interaction with Rap2 was dependent on the intact effector region and GTP-bound configuration of Rap2. When co-expressed in cultured cells, TNIK colocalized with Rap2, while a mutant TNIK lacking the CNH domain did not. Rap2 potently enhanced the inhibitory function of TNIK against cell spreading, but this was not observed for the mutant TNIK lacking the CNH domain. Rap2 did not significantly enhance TNIK-induced JNK activation, but promoted autophosphorylation and translocation of TNIK to the detergent-insoluble cytoskeletal fraction. These results suggest that TNIK is a specific effector of Rap2 to regulate actin cytoskeleton.**

Rap2 is a member of the Ras family of small GTP-binding proteins, which regulate a range of cellular processes including cell proliferation, differentiation, and cytoskeletal rearrangement (for a review, see Ref. 1). To regulate these processes, Ras family proteins cycle between GTP-bound active and GDP-bound inactive forms. In the GTP-bound active form, Ras family proteins physically interact with downstream effectors and thereby regulate their subcellular localization and activity (1). For instance, GTP-bound Ras interacts with effectors including Raf-1, B-raf, Ras guanine nucleotide dissociation stimulator (RalGDS), and phosphoinositide 3-kinase (PI3K) (for a review, see Ref. 2). In addition, GTP-bound Ras interacts with phospholipase C (PLC) (Ref. 3 and 4, reviewed in Ref. 5). These effectors possess structurally similar regions of about 100 amino acids that mediate interaction with Ras, termed the Ras-binding domain (RBD) or Ras-associating domain (RAD) (1, 2).

During interaction with effectors, the effector regions of Ras family proteins (amino acids 32–40 in the case of Ras) serve as binding interfaces; thus, mutations within their effector regions impair interaction with effectors (Refs. 1–4, reviewed in Ref. 6). The effector regions are also critical for the differential recognition of effectors. For instance, the effector region of Rap1, a close relative of Rap2, is identical to that of Ras. Rap1 interacts with effectors of Ras, and sometimes counteracts Ras-mediated signaling (1, 2). For example, Rap1 regulates the extracellular signal-regulated kinase (ERK) pathway, the “classical” mitogen-activated protein kinase (MAPK) pathway, through Raf-1 and B-raf. Although Rap1 interacts with Raf-1 and B-raf, it only activates B-raf. In fibroblasts, Rap1 inhibits Ras-induced cellular transformation. Rap1 exerts this action presumably by trapping Raf-1 in an inactive complex, thereby inhibiting ERK activation (1, 2). In PC12 cells, Rap1 mediates the sustained activation of ERK induced by nerve growth factor through the activation of B-raf (8). However, Rap1 does not have a specific effector that does not interact with Ras. On the other hand, the effector region of Rap1, another Ras family protein, differs from that of Rap by three amino acids. Unlike Rap1, Rap has a specific effector, Rap-binding protein 1, which does not possess RBD or RAD and does not interact with Ras (1, 2).

Rap2 has been thought to be functionally analogous to Rap1. In fact, Rap2 interacts with effectors of Ras and sometimes counteracts Ras-mediated signaling, as does Rap1. Rap2 interacts with Raf-1 in HEK293T cells and inhibits the Ras-dependent activation of the transcription factor Elk1, a direct down-

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1 The abbreviations used are: RalGDS, Ral guanine nucleotide dissociation stimulator; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; RAD, Ras-binding domain; RBD, Ras-associating domain; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; GEF, guanine nucleotide exchange factor; LC-MS/MS, liquid chromatography tandem mass spectrometry; TNF, tumor necrosis factor; Traf2, TNF receptor-associated factor 2; TNIK, Traf2- and Nck-interacting kinase; JNK, c-Jun N-terminal kinase; SH, Src homology; CNH, citron homology; MAPK4, MAPK kinase kinase kinase; PKA, p21-activated kinase; GCK, germininal center kinase; GST, glutathione S-transferase; GTP-S, guanosine 5’-O-(3-thiotriphosphate); NP-40, Nonidet P-40; RIPA, radioimmune precipitation assay buffer; CIAP, calf intestine alkaline phosphatase; EGFP, enhanced green fluorescent protein; NIK, Nck-interacting kinase; MAP3K, MAPK kinase kinase; MINK, Misshapen/NIKs-related kinase; NRK, NIK-related kinase; NIKs, NIK-like embryo-specific kinase; HA, hemagglutinin.
stream target of ERK (9). Rap2 also interacts with RalGDS in COS7 cells, but this does not lead to the activation of Raf, the downstream target of RalGDS (10). Further, Rap2 interacts with PI3K in the B cell lymphoma cell line A20, and inhibits the activation of Akt, the downstream target of PI3K (11). However, contrary to these observations, Rap2 fails to suppress Ras-induced cellular transformation (12), while it suppresses v-Src-induced transformation (9). Furthermore, Rap2 is activated by GDP/GTP exchange factors (GEFs) differently from Rap1 (9). For instance, RA-GEF-1 (13), also termed nRapGEP, PDZ-GEF, or CNrasGEF (14–16), is the strongest activator of Rap2, whereas GFR/MR-GEF (17, 18) is the strongest activator of Rap1. RA-GEF-2 (19) is more effective in activating Rap2 than Rap1, whereas C3G (20) and CalDAG-GEFI/RasGRF2 (21, 22) are more effective in activating Rap1 than Rap2 (9). Furthermore, the GEF domain of PLCε acts on Rap1 but not on Rap2 (23). These observations suggest that Rap2 is not functionally analogous to Rap1, and that Rap2 and Rap1 perform overlapping but distinct signaling functions.

We hypothesized that Rap2 performs its specific signaling functions by regulating specific effectors. In support of this, the effector region of Rap2 differs from those of Ras or Rap1 by a single amino acid. Amino acid 39 in Rap2 is Phe, while in Ras and Rap1 it is Ser. This may confer on Rap2 the ability to interact with specific effectors and play signaling roles distinct from those of Ras and Rap1. In fact, Rap2 interacts with a specific effector candidate, Rap2-interacting protein 8, which does not interact with Ras or Rap1 (24). However, no signaling function for this protein is known. In the present study, we attempted to affinity-purify a specific effector(s) of Rap2 from rat brain. We searched for proteins that interact with a Rap2 affinity column but not with a Ras affinity column. We found one such protein of ~155 kDa. By liquid chromatography tandem mass spectrometry (LC-MS/MS), we identified this protein, designated p155, as tumor necrosis factor (TNF) receptor-associated factor 2 (Traf2)- and Nck-interacting kinase (TNIK) (25).

TNIK was isolated by yeast two-hybrid screening for proteins that interact with Traf2 and Nck (25). Traf2 belongs to a family of adaptor proteins that shares a common structural domain, the Traf domain. Traf2 is implicated in the regulation of c-Jun N-terminal kinase (JNK), a “stress-activated” MAPK, and the transcription factor NF-κB by TNF receptor or related receptors (for a review, see Ref. 26). Nck belongs to a family of adaptor proteins containing the Src homology (SH)2/SH3 domains. Nck is implicated in the regulation of the actin cytoskeleton by receptor or non-receptor tyrosine kinases (for a review, see Ref. 27).

TNIK does not possess RBD or RAD. TNIK consists of an N-terminal kinase domain, a C-terminal regulatory domain termed the citron homology (CNH) domain, and an intervening region between these domains. The CNH domain was named after citron kinase (28), an effector of the Rho family small GTPases. Nck is implicated in the regulation of the actin cytoskeleton (30). TNIK belongs to the GCK family, which possesses a C-terminal regulatory domain (for a review, see Ref. 29). The germinal center kinase (GCK) family, which possesses an N-terminal kinase domain (for a review, see Ref. 31). Several PAK family members serve as effectors of the Rho family small GTP-binding proteins Cdc42 and Rac and regulate JNK and the actin cytoskeleton (30). TNIK belongs to the GCK family (29), and exhibits two mutually independent functions (25). Like several other GCK family members, TNIK regulates JNK. For this function, its CNH domain is necessary and sufficient. TNIK does not activate other MAPKs such as p38, or ERK. TNIK does not activate NF-κB, either. Unlike other GCK family members, TNIK also regulates the actin cytoskeleton. TNIK induces actin fiber disassembly and consequently reverses pre-established cell spreading: with the expression of TNIK, well adherent and spread cells round up and finally lose attachment to culture dishes. However, these cells are viable and are not undergoing apoptosis (25). For this function, its kinase domain is necessary and sufficient. TNIK phosphorylates gelsolin, an F-actin fragmenting and capping enzyme, in vitro, although it is unknown whether phosphorylation takes place and influences gelsolin functions within cells (25).

In the present study, we show that TNIK interacts with Rap2 but not with Rap1 or Ras. The interaction requires GTP-bound configuration of Rap2 and the intact effector region of Rap2. TNIK co-localizes with Rap2 when co-expressed in cells. Rap2 potently enhances TNIK-induced loss of cell spreading. Furthermore, Rap2 promotes the autophosphorylation and translocation of TNIK to the detergent-insoluble cytoskeletal fraction. These observations suggest that TNIK serves as a specific effector of Rap2 to regulate actin cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Affinity Purification and Mass Spectrometry**—To generate affinity columns, glutathione S-transferase (GST)-fusion proteins of Rap2A and -B and Ras were expressed in SF9 insect cells using a baculovirus expression system, extracted, and immobilized on glutathione-Sepharose resin (Amersham Biosciences) as previously described (7, 32). Rat brain (10 g) was homogenized in buffer A (20 mM Tris/HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 5 mM MgCl2) containing 0.1 mM phenylmethylsulfonyl fluoride and 10% sucrose. This and all subsequent steps were performed at 4 °C. The homogenate was centrifuged at 100,000 × g for 1 h, and the resultant supernatant was dialyzed against buffer A three times and applied to a 2.5-ml glutathione-Sepharose column. The flow-through fraction was applied to a 0.1-ml affinity column containing GST-Rap2A or GST-Ha-Ras fusion proteins preloaded with GTP-S or GDP as previously described (7, 32). After washing the columns with 2 ml of buffer A, bound proteins were eluted with buffer A containing 10 mM glutathione. The eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized with Coomassie Brilliant Blue staining. A gel piece containing Rap2A was excised, and the proteins were in-gel digested with trypsin. The resultant peptides were analyzed using an LC-MS/MS system (LCQ DecaXP, ThermosQuest Inc., San Jose, CA), and the data were used to search against the NCBI data base with MASCOT software (Matrix Science Ltd., London, UK) for protein identification.

**In Vitro Binding Assay with Recombinant TNIK**—The cDNA clone KIAA0551 (GenBank™ accession number AB011123) containing the full-length coding sequence for the largest isoform of human TNIK (1360 amino acids) was kindly provided by Dr. Takahiro Nagase (Kazusa DNA Research Institute, Chiba, Japan). TNIK and its C-terminal deletion mutant TNIKΔC NH (amino acids 1–1041) lacking the CNH domain were expressed in HEK293T cells with the HA epitope tag. To this end, the HA coding sequence was inserted into the mammalian expression vector pCIneo (Promega) to yield pCIneo-HA. From the clone KIAA0551, the TNIK and TNIKΔC NH coding sequences were amplified by PCR and inserted into pCIneo-HA to yield pCIneo-HA-TNIK-and TNIKΔC NH, respectively. 293T cells were then transfected with pCIneo-HA-TNIK or pCIneo-HA-TNIKΔC NH using Polyfect reagent (Qiagen) and harvested at 24 h post-transfection. The cells were homogenized in buffer A containing 1% Nonidet P-40 (Nonidet P-40) and protease inhibitors (Roche Applied Science), incubated for 30 min at 4 °C, and then centrifuged at 10,000 × g for 4 h. The supernatant (Nonidet P-40-soluble fraction) was next incubated with glutathione-Sepharose resin carrying immobilized GST-Rap2A, GST-Rap2A and Rap2A preloaded with GTP-S or GDP as previously described (7, 32). After incubation at 4 °C for 1 h, the resin was washed four times with the same buffer, and bound proteins were eluted with 10 mM glutathione and subjected to SDS-PAGE followed by Western immunoblot detection with monoclonal anti-HA antibody (12CA5;
EDTA was applied to a glutathione-Sepharose column carrying nucleotide-free resin carrying GTP of GST-Rap2A. In a parallel experiment, the extract was applied to the resin carrying GTP.

**Fig. 1.** Purification and identification of p155/TNIK. A, affinity purification of the Rap2-interacting protein p155. Rat brain extract was subjected to Western immunoblotting with polyclonal anti-Myc antibody (9E10; Cell Signaling Technology, Beverly, MA) and polyclonal anti-HA antibody (sc-805; Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (4, 23, 32, 35).

**JNK Activation Assay—**pFLAG-CMV2-JNK2 used for the expression of JNK2 tagged with the FLAG epitope was kindly provided by Dr. Katsuji Yoshioka (Kanazawa University, Ishikawa, Japan) (36). 293T cells were transfected with pFLAG-CMV2-JNK2 alone or together with pCIneo-Myc-Rap2A, pCIneo-HA-TNIK or both plasmids. At 18-h post-transfection, the cells were harvested and homogenized as previously described (32). The cells were harvested at 18-h post-transfection because all cells were well adherent at this time point, allowing easy collection of cytoskeleton proteins. The Nonidet P-40-insoluble fraction was prepared by centrifugation as mentioned above. The Nonidet P-40-soluble fraction was then solubilized in RIPA buffer (buffer A containing 1% Nonidet P-40 and protease inhibitors). Total cell homogenates were subjected to Western immunoblotting with polyclonal anti-phospho-JNK (Thr-183/Tyr-185) antibody (no. 9251; Cell Signaling Technology), monoclonal anti-HA antibody, and monoclonal anti-Myc antibody as previously described (32). The cells were harvested at 18-h post-transfection because all cells were well adherent at this time point.

**Analyses of Autophosphorylation and Cytoskeletal Translocation—**pCIneo-HA-TNIK(K54R) used for the expression of a kinase-deficient mutant of TNIK (25) was constructed using an oligonucleotide-directed mutagenesis technique. 293T cells were transfected with pCIneo-HA-TNIK or pCIneo-HA-TNIK(K54R) alone or in combination with pFLAG-CMV2-JNK2. At 18-h post-transfection, the cells were harvested and a Nonidet P-40-insoluble fraction was prepared by centrifugation as mentioned above. The Nonidet P-40-insoluble pellet was then solubilized in radioimmunoprecipitation assay (RIPA) buffer (buffer A containing 1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate, and protease inhibitors) for 30 min at 4 °C. The resultant supernatant was used as the Nonidet P-40-insoluble/RIPA buffer-soluble fraction (cytoskeletal fraction). From these fractions, HA-TNIK or HA-TNIK(K54R) was immunoprecipitated as previously described (35). For alkaline phosphatase treatment, the beads carrying HA-TNIK or HA-TNIK(K54R) were washed three times with RIPA buffer and then incubated with 1 unit of alkaline phosphatase (Roche Applied Science) or Coomassie Brilliant Blue staining as previously described (32).

**Yeast Two-hybrid Assay—**a cDNA fragment encoding amino acids 995–1360 of TNIK was amplified by PCR and inserted into plasmid pACT2 (Clontech, Palo Alto, CA). The resultant plasmid pACT2-CN was used to express the N-terminal portion of Raf1 containing RBD as a fusion protein with the GAL4 activation domain. pGAD-Raf1 was used to express the C-terminal portion of TNIK containing the CNH domain as a fusion protein with the GAL4 activation domain. pGAD-Raf1 was used to express the N-terminal portion of Raf1 containing RBD as a fusion protein with the GAL4 activation domain as previously described (33). pBTM116-Rap2A, -Rap2A-G12V, -Rap2A-S17N, and Rap2A-F395S, -Rap1A, and -Ha-Ras expressed the LexA DNA binding domain fusion proteins as previously described (32). Various combinations of pACT2, pGAD, and pBTM116 constructs were co-transformed into the S. cerevisiae L40 reporter strain, and transformants were examined for HIS3 and β-galactosidase expression as previously described (3, 4, 32, 34).

**Immunofluorescence Microscopy—**pCIneo-Myc-Rap2A and -Ha-Ras expressed Rap2A and Ha-Ras, respectively, with the Myc epitope tag as previously described (32). These plasmids were transfected alone or in combination with pCIneo-HA-TNIK or pCIneo-HA-TNIK/K54R into NIH3T3 cells cultured on glass coverslips. At 24-h post-transfection, the cells were fixed with formaldehyde and processed for immunofluorescence confocal microscopy (LSM 5 PASCAL, Carl Zeiss) with monoclonal anti-Myc antibody (9E10; Cell Signaling Technology, Beverly, MA) and polyclonal anti-HA antibody (sc-805; Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (4, 23, 32, 35).

**GST-Ha-Ras.** Bound proteins were co-eluted with the respective GST fusion proteins by the addition of glutathione. Aliquots (30 μl each) from the eluates were resolved by SDS-PAGE (7% gel) followed by Coomassie Brilliant Blue staining. The molecular mass (kDa) is indicated above the sequence.

**TNIK as an Effector of Rap2**

**Fig. 1.** Purification and identification of p155/TNIK. A, affinity purification of the Rap2-interacting protein p155. Rat brain extract was applied to a glutathione-Sepharose column carrying nucleotide-free (EDTA, lane 1), GTP-γ-S-bound (lane 2), and GDP-bound (lane 3) forms of GST-Rap2A. In a parallel experiment, the extract was applied to the resin carrying GTP-γ-S-bound (lane 4), and GDP-bound (lane 5) forms of GST-Ha-Ras.
washed twice with buffer B (50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, and 1 mM dithiothreitol) and then incubated with 20 units of calf intestine alkaline phosphatase (CIAP) in the same buffer for 50 min at 37 °C. The beads were washed twice with buffer B, and proteins were eluted from the beads by boiling in SDS sample buffer and subjected to Western immunoblot analysis with monoclonal anti-HA antibody.

**Cell Rounding Assay—**EGFP-C1 (Clontech) expressing the enhanced green fluorescent protein (EGFP) was transfected with various pCIneo constructs into 293T cells cultured in 35-mm dishes. At 18-h post-transfection, live cells were observed using an inverted fluorescent microscope (Axiovert 135, Zeiss) with >10 objective under a GFP filter, and images were collected using a CCD camera (DP70, Olympus). EGFP-positive cells were examined for their round or spread morphology, and the percentage of cells that maintained a spread morphology was determined. The cells were then harvested to assess the expression of various HA- or Myc-tagged proteins by Western immunoblot analyses.

**RESULTS**

**p155/TNIK Is a Novel Rap2-interacting Protein—**To search for proteins that specifically interact with Rap2, rat brain extract was applied to GST-Rap2A and -Ha-Ras affinity columns. After extensive washing, proteins bound to the affinity columns were eluted with GST-Rap2A by glutathione and subjected to SDS-PAGE. As shown in Fig. 1A, a protein with a molecular mass of ~155 kDa was detected in the eluate from the affinity column carrying GTPγS-bound GST-Rap2A and was thus designated p155 (Fig. 1A, lane 2). A smaller amount of p155 was also eluted from the column carrying GDP-bound GST-Rap2A (lane 3). However, it was not eluted from the column carrying nucleotide-free GST-Rap2A (lane 1). Further, p155 was not eluted from columns carrying GTPγS- or GDP-bound GST-Ha-Ras, either (lanes 4 and 5).

To clarify the molecular identity of p155, peptides resulting from in-gel tryptic digestion of the 155 kDa gel slice were analyzed by LC-MS/MS. As shown in Fig. 1B, twelve peptides matched human TNIK, mouse TNIK or both (human and mouse TNIK share 99.9% amino acid identity). TNIK possesses an N-terminal kinase domain, C-terminal CNH domain, and an intervening region where Tra2- and Nck-binding sites are located. TNIK consists of multiple isoforms resulting from alternative splicing within the intervening region. Human TNIK consists of 8 isoforms (TNIKα to TNIKβ) with different combinations of 3 alternatively spliced modules of 29, 55, and 8 amino acids (25), for which one peptide matched a part of the 55 amino acid module. TNIK consists of 8 isoforms (TNIKα to TNIKβ) with different combinations of 3 alternatively spliced modules of 29, 55, and 8 amino acids (25), for which one peptide matched a part of the 55 amino acid module. TNIK is the largest isoform containing all of these modules with a predicted molecular mass of 155,361 Da, which was close to the apparent molecular mass of p155 estimated by SDS-PAGE. Therefore, we concluded that p155 corresponds to rat TNIK, most likely the counterpart of TNIK. Accordingly, TNIK was used in the following experiments.

The CNH Domain of TNIK Mediates Specific Interaction with Rap2—We next confirmed that recombinant TNIK interacts with Rap2 in a manner similar to that of p155. For this purpose, HA-tagged TNIK (HA-TNIK) was expressed in 293T cells and was examined for interaction with immobilized GST-Rap2A (Fig. 2A). Similar to p155, HA-TNIK preferentially interacted with the GTPγS-bound form of GST-Rap2A (lane 2) compared with the GDP-bound form of GST-Rap2A (lane 3), but did not interact with the nucleotide-free form of GST-Rap2A (lane 4). In another experiment (Fig. 2B), HA-TNIK did not interact with GTPγS-bound GST-Rap1A or GST-Ha-Ras (lanes 5 and 6), providing further support for the interaction specificity between TNIK and Rap2. Unlike HA-TNIK, HA-TNIKΔCNH, a C-terminal deletion mutant that lacks the CNH domain, failed to interact with GST-Rap2A (lane 4), suggesting that the CNH domain is required for this interaction.

To test whether the CNH domain mediates the specific interaction of TNIK with Rap2, we examined the interaction of the CNH domain with Rap2, its mutants, Rap1, and Ras using a yeast two-hybrid assay (Table I). The CNH domain strongly interacted with Rap2 and its activated mutant Rap2(G12V), while it weakly interacted with the dominant negative mutant Rap2(S17N), and did not interact with Rap2(F39S), in which the effector region of Rap2 was mutated to resemble those of Ras and Rap1. As expected, the CNH domain did not interact with Rap1 or Ras, either. On the other hand, Raf-1, which possesses an RBD, failed to interact only with the dominant negative mutant, Rap2(S17N).

To determine whether the CNH domain mediates interaction with Rap2 in cells, the subcellular localizations of Rap2 and TNIK expressed in NIH3T3 cells were examined by immunofluorescence microscopy (Fig. 3). When expressed alone, Myc-
Strikingly, when co-expressed with Myc-Rap2A (panel b), HA-TNIK was evenly distributed throughout the cytoplasm when expressed alone (panel b). Unlike Myc-Rap2A, HA-TNIK was almost completely co-localized with Myc-Rap2A and HA-TNIK lost their flat, spread out morphology, and rounded up (panels f–h). Myc-Rap2A and HA-TNIK were co-localized in these rounded cells as well. In contrast to full-length HA-TNIK, HA-TNIKΔCNH failed to co-localize with Myc-Rap2A (panels i–k). Myc-Rap2A again showed a perinuclear staining pattern (panel i), while HA-TNIKΔCNH showed a cytoplasmic staining pattern (panel g). This staining pattern for HA-TNIKΔCNH in cells co-expressing Myc-Rap2A was the same as that in cells expressing HA-TNIKΔCNH alone (data not shown). The absence of co-localization was also observed when HA-TNIK was co-expressed with Myc-Ha-Ras (panels l–n). Again, HA-TNIK showed a cytoplasmic staining pattern (panel m), while Myc-Ha-Ras exhibited a perinuclear and plasma membrane-associated staining pattern (panel l). This staining pattern for Myc-Ha-Ras in cells co-expressing HA-TNIK was the same as that in cells expressing Myc-Ha-Ras alone (data not shown).

Rap2 Does Not Enhance TNK-induced JNK Activation—Next, the effect of interaction with Rap2 on TNIK function was tested. TNIK activates JNK when expressed in Phoenix-A cells, derivatives of 293 cells (25). We therefore tested whether Rap2 enhances TNK-induced JNK activation in 293T cells (Fig. 4A). FLAG-tagged JNK (FLAG-JNK2) was expressed alone, with HA-TNIK, Myc-Rap2A, or with both. The activation of JNK was examined by assessing the extent of JNK phosphorylation within whole cell homogenates by Western immunoblotting with anti-phospho-JNK antibody that detects activated JNK (top panel). The expression of FLAG-JNK2 alone resulted in no detectable JNK phosphorylation (lane 1), whereas co-expression of HA-TNIK with FLAG-JNK2 resulted in substantial phosphorylation (lane 2). This phosphorylation most likely reflected that of FLAG-JNK2, since the expression of HA-TNIK alone did not result in phosphorylation (data not shown). The co-expression of Myc-Rap2A with FLAG-JNK2 did not result in appreciable phosphorylation (lane 3), whereas co-expression of HA-TNIK and Myc-Rap2A with FLAG-JNK2 again resulted in substantial phosphorylation (lane 4). However, the extent of phosphorylation did not greatly exceed that attained with the co-expression of HA-TNIK and FLAG-JNK2 (compare with lane 2).

Rap2 Promotes Autophosphorylation and Translocation of TNIK to the Cytoskeletal Fraction—In the above experiment where the quantities of HA-TNIK were assessed with anti-HA antibody (Fig. 4A, third panel), we noted that Rap2 affected electrophoretic mobility of HA-TNIK. HA-TNIK in cells that co-expressed HA-TNIK with FLAG-JNK2 (lane 2) migrated as a broader band than that in cells which co-expressed HA-TNIK plus Myc-Rap2A with FLAG-JNK2 (lane 4). HA-TNIK in the former cells (lane 2) consisted of two forms with higher and lower mobility, while HA-TNIK in cells co-expressing Myc-Rap2A and FLAG-JNK2 (lane 3) migrated as a single band. Moreover, the band corresponding to lower mobility HA-TNIK was more prominent in lane 3 than in lane 2. These results suggested that Rap2 may enhance autophosphorylation of HA-TNIK, possibly at tyrosine residues.

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lower mobility, while that in the latter cells (lane 4) consisted of a single lower mobility form. We speculated that in the latter cells, the higher mobility form was converted to the lower mobility form by Myc-Rap2A.

To test whether this Rap2-induced conversion occurred in the absence of co-expressed FLAG-JNK2, cells that expressed HA-TNIK alone were compared with cells that co-expressed HA-TNIK and Myc-Rap2A (Fig. 4B). Again, HA-TNIK in the former cells (lane 1) migrated as a broad band consisting of lower and higher mobility forms, while HA-TNIK in the latter cells (lane 2) migrated as a narrow band consisting of the lower mobility form. To determine whether this Rap2-induced conversion was related to the kinase activity of TNIK itself, we expressed the kinase-deficient mutant HA-TNIK(K54R), which fails to undergo autophosphorylation in vitro (25) (lanes 3 and 4). Notably, HA-TNIK(K54R) migrated as a narrow band (lane 3) with a similar mobility to that of the higher mobility form of wild-type HA-TNIK. Co-expressed Myc-Rap2A reduced the mobility of HA-TNIK(K54R) only slightly (lane 4), suggesting that the kinase activity of TNIK is important for the Rap2-induced conversion. In addition, HA-TNIKΔCNH migrated as a narrow band, and Myc-Rap2A did not affect the mobility of HA-TNIKΔCNH detectably, thus suggesting that effect of Rap2 on the mobility of TNIK requires the CNH domain (lanes 5 and 6).

Contrary to the above observations, wild-type HA-TNIK migrated as a narrow band in our in vitro binding assay (Fig. 2A). In the in vitro binding assay, we used the Nonidet P-40-soluble fraction, while in the above experiment (Fig. 4, A and B) we used total cell extract. This difference led us to examine the Nonidet P-40-solubility of the lower and higher mobility forms of HA-TNIK (Fig. 4C). HA-TNIK was immunoprecipitated with anti-HA antibody from the Nonidet P-40-soluble fraction (Nonidet P-40 soluble) or the Nonidet P-40-insoluble/RIPA buffer-soluble fraction (Nonidet P-40 insoluble), incubated with or without CIAP (phosphatase), and subjected to Western immunoblotting with anti-HA antibody.

Collectively, these data suggest that the lower mobility form of HA-TNIK represents an autophosphorylated form of HA-TNIK, which is mainly associated with the cytoskeletal fraction, and that interaction of HA-TNIK with Myc-Rap2 promotes the autophosphorylation and translocation of HA-TNIK to the cytoskeletal fraction.

Rap2 Enhances TNIK-induced Cell Rounding—We then
tested whether Rap2 enhanced TNIK-induced cytoskeletal re-arrangement. TNIK induces actin fiber disassembly and consequently disrupts pre-established cell spreading in Phoenix-A, HeLa and NIH3T3 cells (25). As shown in Fig. 5, using EGFP as a co-marker, we compared the effects of HA-TNIK expression and HA-TNIKΔCNH expression on cell morphology in the absence or presence of co-expressed Myc-Rap2A. We also used the kinase-deficient mutant HA-TNIK(K54R), since the F-actin disrupting function of TNIK is dependent on its kinase activity (25). We also used Myc-Ha-Ras as another negative control. 293T cells that expressed HA-TNIK, HA-TNIKΔCNH, or HA-TNIK(K54R) (Fig. 5A, panels b–d; Fig. 5B, columns 2–4) were well spread and looked similar to control cells co-transfected with empty vectors and pEGFP-C1 (a spread morphology was noted for more than 90% of the EGFP-positive cells) (panel a, column 1). Similarly, cells that expressed Myc-Rap2A alone did not look significantly different from the control cells (panel e, column 5). However, the majority of cells that expressed HA-TNIK in the presence of co-expressed Myc-Rap2A exhibited a distinctly rounded morphology (a spread morphology was observed for less than 40% of the EGFP-positive cells) (panel f, column 6). In contrast, cells that expressed HA-TNIKΔCNH, which is incapable of interacting with Rap2, maintained a spread morphology even in the presence of co-expressed Myc-Rap2A (panel g, column 7). As expected, cells that expressed HA-TNIK(K54R) did not round up in the presence of co-expressed Myc-Rap2A (panel h, column 8). In addition, cells that expressed Myc-Ha-Ras (panel I, column 9) as well as cells that expressed HA-TNIK plus Myc-Ha-Ras (panel j, column 10) looked similar to the control cells.Nearly equal amounts of HA-TNIK were present in cells that expressed HA-TNIK, HA-TNIKΔ, and Myc-Rap2A, or HA-TNIK and Myc-Ha-Ras as examined by Western immunoblotting (data not shown). Similar observations were made during the immunofluorescence microscopy analysis of NIH3T3 cells (Fig. 3A, panels f–h, and data not shown).2

**DISCUSSION**

There is limited information on the role of Rap2 in the regulation of the actin cytoskeleton. It inhibits spontaneous cell migration in mouse embryonic fibroblasts (MEFs) deficient in C3G (37), but promotes spontaneous and chemokine-induced cell migration in the B cell line 2PK3 (38). Cell migration involves complex regulation of the actin cytoskeleton (39). However, the effectors that mediate these Rap2 actions remain unidentified. We believe that TNIK serves as an effector of Rap2 to regulate actin cytoskeleton. TNIK reverses pre-established cell spreading by inducing the disassembly of F-actin through its kinase domain (25), and our data demonstrated that Rap2 potently enhances this TNIK function. How the kinase activity of TNIK is involved in actin fiber disassembly is unknown. One possibility is through autophosphorylation. TNIK undergoes autophosphorylation in vitro (25), and our results indicated that autophosphorylation also takes place in cells and is promoted by Rap2. The interaction of Rap2 with TNIK may promote autophosphorylation by recruiting and accumulating TNIK to specific membrane domain. This accumulation may in turn allow juxtaposed TNIK molecules to transphosphorylate each other. This speculation is consistent with the observation that kinase-deficient TNIK(K54R) undergoes autophosphorylation to a small extent in cells that co-express Rap2, where endogenous wild-type TNIK could phosphophorylate TNIK(K54R). Autophosphorylation might then trigger a conformational change in TNIK necessary for interaction with or phosphorylation of the downstream molecule that mediates F-actin disassembly. Autophosphorylation might also permit the translocation of TNIK to cellular compartments where the downstream molecule resides. Consistent with this, autophosphorylated TNIK was found in the cytoskeletal fraction.

TNIK is the first identified Rap2 effector that mediates regulation of the actin cytoskeleton by Rap2. It is also the first identified Rap2-interacting protein isolated by the affinity purification-mass spectrometry approach. In a previous report, we carried out yeast two-hybrid screening for Rap2-interacting proteins and isolated another GCK family kinase, the isoform 3 of human MAP4K4 (32). MAP4K4 consists of multiple isoforms resulting from alternative splicing (40), and other isoforms of MAP4K4 in humans and mice are known as hematopoietic progenitor kinase (HPK)/GCK-like kinase (HGK) (41) and Nck-interacting kinase (NIK) (42), respectively. Similar to TNIK, MAP4K4 possesses an N-terminal kinase domain, C-terminal CNH domain, and an intervening region, and interacts with Rap2 through its CNH domain. MAP4K4 shares 90% amino
acid identity with TNIK in its kinase and CNH domains. However, this homology drops to 50% in the intervening region, suggesting potentially different signaling roles for MAP4K4 and TNIK. Expression of MAP4K4 weakly activated JNK in cultured cells and co-expression of Rap2 markedly enhanced this activation (32). Thus, we proposed that MAP4K4 serves as an effector of Rap2 to regulate the JNK pathway. On the other hand, in the present study, expression of TNIK alone activated JNK substantially, and co-expression of Rap2 did not markedly enhance this activation. Thus, TNIK does not appear to serve as an effector of Rap2 to regulate the JNK pathway. Rap2 may regulate the actin cytoskeleton and JNK separately through TNIK and MAP4K4, respectively.

The differential effect of Rap2 on TNIK- and MAP4K4-induced JNK activation could be related to different requirements for kinase activity for JNK activation between TNIK and MAP4K4. Unlike several other GCK family members, TNIK does not require its kinase activity for JNK activation. During this process, kinase-deficient TNIK(K54R) and the CNH domain of TNIK are as effective as wild-type full-length TNIK, and the kinase domain of TNIK is ineffective (25). In contrast, MAP4K4 requires its kinase activity for JNK activation. For instance, neither the kinase-deficient mutant nor the CNH domain of mouse NIK can fully activate JNK (42). Similarly, a kinase-deficient mutant of human HGK cannot activate JNK (41). The question as to why TNIK does not require kinase activity for JNK activation and whether this is related to the differential effect of Rap2 on TNIK- and MAP4K4-induced JNK activation awaits further study. The role of kinase activity in JNK activation by GCK family members is not fully understood. GCK family members are thought to activate downstream MAP kinase kinases (MAP3Ks) largely by binding through their noncatalytic regions, rather than by phosphorylating MAP3Ks through their kinase domains (29). Kinase activity might be used to induce conformational changes to make their noncatalytic regions more accessible to MAP3Ks through autophosphorylation (29). Autophosphorylation might not be important for TNIK to bind to its downstream MAP3K, while it may be important for MAP4K4. Rap2 likely promotes the autophosphorylation of MAP4K4, since we observed a lowered-mobility form of HA-MAP4K4 in cells that co-expressed Myc-Rap2 in an experiment similar to that shown in Fig. 4A (32). It could be that Rap2 promotes the autophosphorylation of TNIK and MAP4K4 but this promotion enhances JNK activation only in the case of MAP4K4.

The CNH domain is also present in GCK family kinases other than TNIK and MAP4K4. The GCK family consists of 8 subfamilies (29), and members of the GCK-I and -IV subfamilies possess CNH domains in their C termini. TNIK and MAP4K4 belong to the GCK-IV subfamily, while GCK and HPK belong to the GCK-I subfamily. The CNH domain of GCK-I subfamily members is only distantly related to those of GCK-IV subfamily members (less than 20% amino acid identity). On the other hand, CNH domains of GCK-IV subfamily kinases, except for one member, are conspicuously homologous to each other. The GCK-IV subfamily includes a single member in nematode Caenorhabditis elegans, MIG-15 (43), a single member in Drosophila melanogaster, Missaphael (43); and four members in mammals, TNIK, MAP4K4, Missaphael/NIK-related kinase (MINK) (44), and NIK-related kinase (NRK/NIK)-like embryo-specific kinase (NESK) (45, 46). The CNH domains of TNIK, MAP4K4, and MINK are highly homologous (about 90% amino acid identity). These CNH domains are also highly homologous to those of MIG-15 and Missaphael (about 70% identity). On the other hand, the CNH domain of NRK/NESK shares less homology with those of all other GCK-IV subfamily members (about 30–40% identity). NRK/NESK is also divergent from other GCK-IV subfamily members with respect to its kinase domain (29).

CNH domains of GCK-IV subfamily kinases, except that of NRK/NESK, may define an evolutionally conserved subclass of CNH domain that mediates specific interaction with Rap2. The CNH domains of TNIK and MAP4K4 exhibit the same properties during interaction with Rap2 (32). Moreover, MINK likely interacts with Rap2 in a GTP-dependent manner but not with Ras. In the affinity purification-mass spectrometry experiment, several peptides matching mouse or human MINK was contained in a minor band that co-eluted with p155. Furthermore, our two-hybrid screening for proteins that interact with C. elegans Rap2 (C25D7.7 protein) isolated a clone that contained the CNH domain of MIG-15. MIG-15 did not interact with C. elegans Rap1 (C27B7.8 protein) or Ras (LET-60). The C. elegans Rap2 possesses Phe-39, and C. elegans Rap1 and Ras instead possess Ser-39 within their effector regions. In addition, the CNH domain of MIG-15 interacted with human Rap2A but not with Rap1A or Ha-Ras in a two-hybrid assay.

The present study shows that Rap2 serves as a direct upstream regulator of TNIK. A well-accepted model for the activation of GCK-I and -IV subfamily kinases involves the recruitment of these kinases to a specific membrane region (29, 31). In this model, stimulation of receptor-tyro sine kinases (through SH2/SH3 adaptor proteins) or cytokine receptors (through Traf family of adaptor proteins) results in the recruitment of GCK-I and -IV subfamily kinases to membrane-associated receptor complexes thereby initiating the activation. We hypothesize that Rap2 also recruits members of GCK-IV subfamily kinases, except for NRK/NESK, to a specific membrane region. Rap2 can be activated by a variety of extracellular stimuli that regulate protein tyrosine phosphorylation or second messengers such as cAMP, Ca2+, and diacylglycerol (20–22, 47, 48). The potential roles of these diverse stimuli in the activation of GCK-IV subfamily kinases through Rap2 deserve further study.

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The Traf2- and Nck-interacting Kinase as a Putative Effector of Rap2 to Regulate Actin Cytoskeleton

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