Identification and Functional Characterization of a Novel Human Misshapen/Nck Interacting Kinase-related Kinase, hMINKβ*

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Misshapen/NIKs-related kinase (MINK) is a member of the germinal center family of kinases that are homologous to the yeast sterile 20 (Ste20) kinases and regulate a wide variety of cellular processes, including cell morphology, cytoskeletal rearrangement, and survival. Here, we present the cloning and functional characterization of a novel human Misshapen/NIKs-related kinase β (hMINKβ) that encodes a polypeptide of 1312 amino acids. hMINKβ is ubiquitously expressed in most tissues with at least five alternatively spliced isoforms. Similar to Nck interacting kinase (NIK) and Traf2 and Nck-interacting kinase (TNIK), hMINKβ moderately activates c-Jun N-terminal kinase (JNK) and associates with Nck via the intermediate domain in the yeast two-hybrid system and in a glutathione S-transferase (GST) pull-down assay. Interestingly, overexpression of the kinase domain deleted and kinase-inactive mutants of hMINKβ in human fibrosarcoma HT1080 cells enhanced cell spreading, actin stress fiber formation, and adhesion to extracellular matrix, as well as decreased cell motility and cell invasion. Furthermore, these mutants also promoted cell-cell adhesion in human breast carcinoma MCF7 cells, evidenced with cell growth in clusters and increased membrane localization of β-catenin, a multifunctional protein involved in E-cadherin-mediated cell adhesion. Finally, hMINKβ protein was found to colocalize with the Golgi apparatus, implicating that hMINKβ might exert its functions, at least in part, through the modulation of intracellular protein transport. Taken together, these results suggest that hMINKβ plays an important role in cytoskeleton reorganization, cell adhesion, and cell motility.

The Ste20 kinase family consists of more than 30 serine/threonine kinases whose catalytic domains are homologous to yeast Ste20 kinase and can be divided into two distinct classes: p21-activated protein kinases and germinal center kinases (GCKs)1 (1). The first class of kinases contains a C-terminal catalytic domain and an N-terminal binding site for Rac1 and Cdc42. The second class of kinases has an N-terminal kinase domain and a C-terminal regulatory domain. Like their yeast counterparts, most of mammalian Ste20 kinases activate mitogen-activated protein kinase pathways that control diverse cellular processes including gene transcription, cytoskeletal reorganization, cell growth, and apoptosis (2).

The GCK family kinases can be further subdivided into eight subfamilies using a phylogenetic-based classification scheme (2). The GCK-IV subfamily (also named the MSN subfamily) includes NIK, TNIK, MINK, HPK/GCK-like kinase (HGK), and Nck-interacting kinase (NIK)-related kinase/NIK-like embryospecific kinase as well as Drosophila melanogaster Misshapen (Ms) and Caenorhabditis elegans ortholog Mig-15 (2). These kinases share high sequence similarity in their N-terminal kinase domains and C-terminal citron homology (CNH) domains, whereas the intermediate domains are less conserved. The CNH domain, originally described in citron Rho-interacting kinase (3), was also found in GCK-I kinases (2), vacuolar protein sorting factors including human Vam6 and yeast Vam6p/Vps39, as well as yeast Rho GDP-exchange factor Rop1p (4). This domain has been shown to play a role in protein–protein interactions and activation of the JNK pathway. For example, the CNH domain of NIK interacted with the cytoplasmic domain of β1-integrin receptor and MEKK1 (5, 6). In addition, the association with MEKK1 is required to activate the JNK pathway. MAP4K4, a member of GCK-IV subfamily kinases, interacted with small GTP-binding protein Rap2 through its CNH domain (7).

The intermediate domains of GCK-IV kinases contain multiple proline-rich motifs (PXXP), which are putative SH3 domain binding sites. These sequences in NIK and TNIK kinases are required for efficient interaction with Nck, an adaptor protein consisting of one SH2 and three SH3 domains (6, 8). Nck is known to interact with a large number of proteins including phosphorylated receptor-tyrosine kinases and proteins involved in actin cytoskeleton organization, DNA synthesis, and gene expression (9). The SH3 domains of Nck mediate interactions with a variety of binding partners, whereas the SH2 domain binds to phosphotyrosine-containing proteins such as activated receptor-tyrosine kinases (9, 10). Nck has been proposed to link GCKs to the upstream tyrosine-receptor kinases (11).

Activation of the JNK pathway, one of well defined mitogen-activated protein kinase modules, is a common feature in many of GCKs, such as GCK (12), hematopoietic progenitor kinase 1 (HPK1) (13, 14), NIK (6), GCK-like kinase (15), TNIK (8), HGK (16), and mouse MINK (17). Some of these kinases directly phosphorylate MAP3Ks and are classified as MAP4Ks (6, 13, reverse transcriptase; GST, glutathione S-transferase; GFP, green fluorescent protein; TRITC, tetramethylrhodamine isothiocyanate.

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‡ The abbreviations used are: GCK, germinal center kinase; MINK, Misshapen/Nck interacting kinase-related kinase; NIK, Nck interacting kinase; TNIK, Traf2 and Nck-interacting kinase; HPK1, hematopoietic progenitor kinase 1; HGK, HPK/GCK-like kinase; Ste20, sterile 20 kinase; CNH, C-terminal citron homology; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein; Ms, Misshapen; SH3, Src homology domain 3; RT, reverse transcriptase; GST, glutathione S-transferase; GFP, green fluorescent protein; TRITC, tetramethylrhodamine isothiocyanate.

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Mutations and Methods

**Materials and Methods**

*Cell Lines and Reagents—Phoenix A cells (a derivative of 293T cells with integrated viral packaging genes) (20) were grown in Dulbecco’s modified Eagle’s medium. Human fibrosarcoma HT1080 and human breast carcinoma cells MCCP were maintained in minimal essential medium and RPMI 1640, respectively. All media were supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin. A rabbit polyclonal antibody was raised against a recombinant hMINKβ fragment (from amino acid 581 to 780) (Zymed Laboratories, South San Francisco, CA). Anti-Myc monoclonal antibody was purchased from Santa Cruz Biotechnology.*

**Cloning of Full-length hMINK**—Phoenix A cells were co-transfected with vector expressing hMINKβ and Nck and/or hMINK with the Golgi apparatus. Thus, hMINKβ appears to function in cellular and developmental processes through modulation of cytoskeleton reorganization, cell adhesion, and cell migration, possibly in part via the control of intracellular protein transport.

**In Vitro Kinase Assays—** For cell adhesion assays, HT1080 cells expressing GFP and various hMINKβ proteins were transfected with plasmids expressing the full-length or truncated hMINK proteins by the calcium phosphate precipitation method. 40 h post-transfection, cells were lysed in lysin buffer as described above. Equal amounts of the cell lysates were incubated with 2 μg of purified GST-Nck or GST-Nck-(1-270) plus 40 μl of glutathione-Sepharose beads (Sigma) at 4 °C for 2 h. The pellets were washed three times with lysin buffer. hMINKβ proteins bound to GST-Nck fusions were detected by Western blot analysis using anti-FLAG antibody.

**Immunofluorescence Staining—** Cells were seeded onto sterile glass coverslips placed in 6-well plates 18 h before the staining. Cells were fixed with 4% paraformaldehyde for 10 min and processed for indirect immunofluorescence using anti-hMINKβ or anti-tubulin antibody (Sigma). Cells were stained with TRITC-phalloidin (Sigma) at a final concentration of 1 μg/ml and washed with phosphate-buffered saline. The remaining adhesive cells were quantified with a CyQuant cell proliferation assay kit (Molecular Probes).

**Cell Adhesion Assays—** For cell adhesion assays, HT1080 cells were transfected with plasmids expressing the full-length or truncated hMINKβ proteins. For the cell adhesion assay, HT1080 cells expressing GFP and various hMINKβ proteins were immunoprecipitated with anti-FLAG beads (Sigma) and subjected to an in vitro kinase reaction in the presence of [γ-32P]ATP and 5 μg of myelin basic protein (MBP, Upstate Biotechnology).

**Immunofluorescence Staining—** Cells were seeded onto sterile glass coverslips placed in 6-well plates 18 h before the staining. Cells were fixed with 4% paraformaldehyde for 10 min and processed for indirect immunofluorescence using anti-hMINKβ or anti-tubulin antibody (Sigma). Cells were stained with TRITC-phalloidin (Sigma) at a final concentration of 1 μg/ml and washed with phosphate-buffered saline. The remaining adhesive cells were quantified with a CyQuant cell proliferation assay kit (Molecular Probes).

**RESULTS**

**Identification and Cloning of hMINKβ—** In an effort to discover novel human germinal center kinases, we identified a cDNA clone TR5 with about 89% sequence homology to mouse MINK1 cDNA. The TR5 clone was isolated from a Jurkat cDNA library by a functional screen designed to isolate genes that confer resistance to Taxol-induced cell death. The yeast cells were grown in nutrient-depleted medium (Leu-, His-, Trp-) plus 3-aminotriazole and tested for β-galactosidase activity 10 days post-transformation (22).
clone carries an antisense fragment of the human MINK homolog corresponding to nucleotides 2804 to 3187 of mouse MINK1 (17). Using TR5 as a probe, we cloned a 3951-bp cDNA, encoding a polypeptide of 1312 amino acids. This human MINK cDNA represents a novel spliced isoform of the human MINK gene and displays more than 98% sequence identity in the N-terminal kinase domain and the C-terminal CNH domain with its mouse homolog. The intermediate domain shares at least 80% sequence similarity with mouse MINK1 with short deletions and/or insertions from alternative splicing. Northern blot analysis with a probe covering the least homologous region among all GCK family members revealed that this cDNA is ubiquitously expressed in most tissues tested with relative high levels in brain, skeletal muscle, pancreas, and testis (Fig. 1A). This cDNA clone was named human MINKβ (hMINKβ, see full description below).

In the process of cloning the full-length human MINK, we found that like other GCKs, hMINK also exists as multiple spliced versions. Because the probe used for Northern blots cannot distinguish the expression pattern of these variations, we conducted RT-PCR experiments using primers covering the alternative spliced junctions. The amplified PCR products were separated on agarose gel and individual bands were isolated and sequenced (Fig. 1B). Five alternative spliced isoforms of hMINK were obtained (Fig. 1C). Comparing with human genomic sequences, all exon/intron boundaries matched the consensus splicing sequences. The longest alternative spliced form designated as hMINKα encodes a polypeptide of 1332 amino acids. hMINKβ corresponding to the cDNA that we isolated encodes a protein of 1312 amino acids missing a 20-amino acid region between amino acids 581 and 600 compared with hMINKα. The third and fourth isoforms equivalent to human MINK2 (1303 amino acids) and MINK1 (1295 amino acids) were named hMINKγ and hMINKδ, respectively. hMINKγ has a 37-amino acid deletion from amino acid 696 to 732 plus an 8-amino acid insertion between amino acids 800 and 801. hMINKδ only contains the 37-amino acid deletion described in hMINKγ. The fifth spliced form (hMINKε) lacks both regions described above and encodes a polypeptide of 1275 amino acids. It is interesting to note that the tissue distribution patterns of each spliced isoform are very different. hMINKα is ubiquitously expressed in most tissues tested at relative higher levels. hMINKγ is predominantly expressed in brain, whereas hMINKδ is most abundant in skeletal muscle. hMINKβ and hMINKε are expressed as minor forms in almost every tissue.

Kinase Activity and JNK Activation—To evaluate the kinase activity of hMINKβ, FLAG-tagged hMINKβ and hMINKβ-KD with a point mutation in the catalytic domain (K548R) were expressed in Phoenix-A cells, and the proteins were then immunoprecipitated and subjected to in vitro kinase assays using MBP as a substrate. As shown in Fig. 2A, hMINKβ strongly phosphorylated MBP and hMINKβ autophosphorylation was also shown. In contrast, hMINKβ-KD failed to phosphorylate MBP and itself (Fig. 2A), indicating that this mutant is devoid of catalytic activity. Equal expression of hMINKβ proteins were shown in the lower panel of Fig. 2A. To determine whether hMINKβ activates the JNK pathway, Myc-tagged JNK2 along with various constructs of hMINKβ were cotransfected into Phoenix-A cells, and JNK2 proteins were immunoprecipitated and subjected to in vitro kinase assays using GST-c-Jun as a substrate. MEKK1, a well established MAP3K for JNK activation (6), was used as a positive control. Surprisingly, both wild-type and the kinase-inactive mutant of hMINKβ enhanced GST-c-Jun phosphorylation by JNK2 as compared with the control, whereas the kinase domain (amino acids 1–547) alone failed to do so (Fig. 2B). Comparable expressing levels of Myc-JNK2 in each transfection were confirmed by immunoblotting the cell lysates with anti-Myc antibody (Fig. 2B). These results suggest that hMINKβ can activate the JNK pathway by a mechanism independent of its kinase activity. hMINKβ was unable to activate the extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways in similar cotransfection assays (data not shown), suggesting that hMINKβ specifically activates the JNK pathway.

Interaction with Nck—Because hMINKβ, like NIK and TNIK, also contains multiple proline-rich motifs (PXXP) in its kinase domain, we asked whether hMINKβ interacts with the Nck adaptor protein. To address this question, we first performed a yeast two-hybrid assay. As shown in Fig. 3A, hMINKβ interacts with Nck in the yeast two-hybrid system, manifesting with both cell growth in selective media and β-galactosidase expression. Control cells expressing either Nck or hMINKβ failed to grow (Fig. 3A).

To confirm the interaction and further delineate the domains of hMINKβ required for this interaction, we transfected Phoenix-A cells with plasmids expressing FLAG-tagged wild-type or deletion mutants of hMINKβ as shown in Fig. 3B, and conducted GST pull-down assays. The full-length hMINKβ and a truncated mutant (296–959) containing the intermediate domain specifically bound to GST-Nck and GST-Nck-(1–270) with three SH3 domains (Fig. 3C), but not to the GST control (data not shown); whereas the mutant encoding the kinase domain (1–547), failed to interact with both GST-Nck and GST-Nck-(1–270) (Fig. 3C). Whether the CNH domain (960–1312) is involved in the association with Nck currently cannot be concluded because of its low levels of expression. The levels of hMINKβ proteins in cell lysates are shown in Fig. 3C. Together, these data indicate that hMINKβ associates with the SH3 domains of Nck mainly through its intermediate domain. This interaction was also confirmed with endogenous proteins (data not shown).

Role of hMINKβ in Cytoskeleton Organization and Cell-Extracellular Matrix Adhesion—We have observed that overexpression of hMINKβ in Phoenix-A cells induced cell rounding and detachment, suggesting that hMINKβ might affect cytoskeleton organization (data not shown). To delineate the functional domains of hMINKβ that might be involved in the regulation of cell morphology, we generated a number of retroviral constructs expressing wild-type and mutant hMINKβ proteins as indicated in Fig. 4A. The viruses were introduced into human fibrosarcoma HT1080 cells and the high expressing cells were enriched via fluorescence-activated cell sorting based on GFP signals. The expression levels of hMINKβ proteins in those cells were revealed by Western blot analysis with an antibody specifically raised against the intermediate domain of hMINKβ or anti-FLAG antibody (Fig. 4B). The endogenous hMINKβ protein was shown as two bands in GFP-infected cells. Both wild-type and the kinase-deficient mutant of hMINKβ were expressed at higher levels relative to the endogenous protein, whereas the truncated mutants tended to be less stable and expressed at lower levels than the full-length protein.

Actin filament rearrangement plays a major role in cellular morphological changes. The actin cytoskeleton in those infected cells was examined by TRITC-phalloidin staining. As reported earlier (26), HT1080 cells expressing GFP were devoid of noticeable stress fibers and exhibited marked actin staining in the cell margins with membrane ruffles (Fig. 4C). Strikingly, the cells expressing the kinase-inactive and kinase domain deleted (296–1312) mutants of hMINKβ displayed a more flat and spreading morphology with profound actin stress fiber formation (Fig. 4C). The wild-type protein also induced cell
spreading, but with little actin stress fiber formation (Fig. 4C). Of interest, more spreading cells tended to have fewer markedly stained membrane ruffles and more well organized actin stress fibers. The fact that hMINKβ-H9252 and hMINKβ-KD had similar effects on cell morphology suggests that the phenotypical changes of HT1080 cells are mediated by overexpression of the C-terminal regulatory domain of hMINKβ. Other truncated mutants had little effect on either cellular morphology or actin stress fiber formation (Fig. 4C). Some of these proteins with low levels of expression might limit their abilities to cause the phenotypical changes.

Cell spreading requires both actin reorganization and integrin receptor engagement with ECM ligands. We next tested the ability of the cells to adhere to various extracellular matrix

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**Fig. 1. Expression of hMINKβ in human tissues.** A, a random-primed hMINKβ probe of the intermediate domain was hybridized to human poly(A⁺) RNA blots (top panels). Equal loading of RNAs was verified by hybridizing with an actin probe (low panels). B, one-step RT-PCR was performed using total RNA from the indicated tissues. The PCR products were separated on agarose gel. A 100-bp DNA molecular weight ladder was included at the right. Four distinct DNA bands could be identified in the gel. Bands 1, 2, and 4 represent hMINKα, hMINKβ, and hMINKε isoforms, respectively (see below). Sequencing analysis of these bands indicates that band 3 from brain represents isoform γ, whereas band 3 from lung represents isoform δ. C, a schematic representation of hMINK isoforms. hMINKβ has 20 amino acids missing from 581 to 600 compared with hMINKα. A 37-amino acid region from 696 to 732 is missing from hMINKδ. hMINKε lacks both regions. hMINKγ, the major form expressed in brain, has an additional 8-amino acid insertion between amino acid 800 and 801 in addition to the 37-amino acid deletion (696–732). GenBank accessions numbers of hMINK isoforms are: hMINKα, AAH34673; hMINKβ, NP-733763; hMINKδ, NP-056531.
proteins. Consistent with the cell spreading results, hMINKβ, hMINKβ-KD, and hMINKβ-(296–1312) enhanced the speed of HT1080 cell attachment to fibronectin with about 60% cells attached after a 15-min incubation; whereas in the GFP expressing cells, only 58% became attached after 30 min incubation (Fig. 4D). Furthermore hMINKβ-KD and hMINKβ-(296–1312) also promoted HT1080 cell adhesion to laminin, whereas the wild-type protein did not show any effect (Fig. 4D). hMINKβ-(1–547) encoding the kinase domain alone showed reduced adhesion to both fibroenectin and laminin, suggesting that the kinase activity of hMINKβ has an opposite effect from the C-terminal regulatory domain, and hMINKβ-KD and hMINKβ-(296–1312) may act in a dominant-negative manner. Neither hMINKβ nor its mutants affected the adhesion of HT1080 cells to collagen I (Fig. 4D). These results suggest that hMINKβ is involved in modulating cell morphology, presumably through affecting actin organization and cell adhesion to extracellular matrix.

**Effect on Cell-Cell Adhesion—**To extend the observations, we examined the effect of hMINKβ on cell-cell adhesion in epithelial cells. hMINKβ and various mutants were introduced into human breast carcinoma MCF7 cells by retroviral infection. The expression levels of various hMINKβ proteins in MCF7 cells were similar as in HT1080 cells except for hMINKβ-(1–959), which was only detectable when overexpressing the film (Fig. 5A). Because of the extremely low expression, we omitted hMINKβ-(1–959) infected cells in our following experiments. As shown in Fig. 5B, overexpression of wild-type hMINKβ in MCF7 cells decreased cell-cell adhesion, and the cells tended to scatter throughout the tissue culture plate, whereas the cells expressing GFP grew in clusters (Fig. 5B). In sharp contrast, hMINKβ-KD and hMINKβ-(296–1312) markedly increased cell-cell adhesion and promoted the cells to grow in more compact clusters (Fig. 5B). hMINKβ-(1–547) expressing cells grew in a similar pattern as the control cells except with more floating cells in the culture medium (Fig. 5B).

To study the underlying molecular mechanism whereby hMINKβ regulates cell-cell adhesion, we sought to examine the intracellular distribution of β-catenin in confluent MCF7 cells that express various hMINKβ proteins. β-Catenin is an essential intermediary factor of adhesion junctions by linking the plasma membrane-bound E-cadherin proteins to the α-catenin-actin cytoskeleton complex. The linkage between E-cadherin and the actin cytoskeleton determines the strength and rigidity of cell-cell adhesion (27). Correlated with the observations in cell-cell adhesion, overexpression of hMINKβ greatly decreased the membrane localization of β-catenin compared with the GFP control, with significant β-catenin staining in the cytoplasm and nuclei (Fig. 5C). Conversely, hMINKβ-KD and hMINKβ-(296–1312) expressing cells displayed marked membrane staining of β-catenin with weak cytoplasmic and no nuclear staining (Fig. 5C). As expected, hMINKβ-(1–547) had no noticeable effect on β-catenin staining (Fig. 5C). These results support the idea that hMINKβ regulates cell-cell adhesion by affecting the subcellular localization of β-catenin. The different localizations of β-catenin are not because of the alterations of the protein expression. β-Catenin in all cells tested were expressed at comparable levels (Fig. 5D), which suggests that hMINKβ regulating β-catenin localization is through post-translational modification.

**Involvement in Cell Migration and Invasion—**Alteration of cell adhesion is a hallmark of tumor progression, which leads to a more invasive and metastatic phenotype in cancer cells. To address the role of hMINKβ in cell migration, we conducted wound-healing experiments. HT1080 cells expressing GFP and hMINKβ exhibited a similar migration pattern, with many cells moving toward the gap and filling the gap by 24 h (Fig. 6A). However, the cells expressing hMINKβ-KD migrated noticeably slower with fewer migrating cells seen in the gap by 24 h (Fig. 6A). Interestingly, hMINKβ-KD expressing cells tended to migrate together with very few detached and scattered cells, which may reflect the stronger adhesion feature of those cells. Next, we investigated whether hMINKβ also affects the invasion potential of the highly invasive HT1080 cells. As shown in Fig. 6B, overexpression of hMINKβ influenced cell invasion, and the cells migrating through the Matrigel decreased by 49% compared with the GFP control. Remarkably, the kinase-inactive mutant dramatically reduced cell invasion by 92%. Thus, our findings in both wound healing and cell invasion assays established a key role of hMINKβ in HT1080 cell migration and invasion.

**Localization of Endogenous hMINKβ—**To gain a better understanding of the physiological roles of hMINKβ in cells, we examined the subcellular localization of endogenous hMINKβ using anti-hMINKβ antibody. Data shown in Fig. 4B and Fig. 5A indicated that this antibody could specifically detect endogenous and overexpressed hMINKβ proteins with little cross-reactivity to other cellular proteins. Immunofluorescence staining revealed that hMINKβ is located in a perinuclear region in HeLa cells with a bright localized staining near the nuclei. To precisely determine the subcellular localization of hMINKβ, a double staining of HeLa cells was performed using antibodies specifically against hMINKβ (Fig. 7A) and golgin-97 (Fig. 7B), a Golgi apparatus marker protein (28). hMINKβ staining overlaps with that of golgin-97 (Fig. 7C), suggesting that a major portion of the hMINKβ protein is associated with the Golgi apparatus. Consistent with this result, hMINKβ also
showed perinuclear localization in transiently transfected HeLa cells (data not shown).

**DISCUSSION**

In the present study we describe the identification and functional characterization of hMINK\(^\beta\), the human ortholog of mouse MINK. Northern blot and RT-PCR analysis indicated that hMINK\(^\beta\) is ubiquitously expressed, and that several alternatively spliced forms are expressed in a tissue-specific manner. Among the spliced isoforms, alternative splicing junctions are all located in the intermediate domain, which shares the least homology among GCKs (2). The biological significance of these isoforms is currently unknown. One explanation is that these different isoforms could interact with distinct sets of intracellular proteins, therefore carrying out different functions and eliciting distinct outcomes in different tissues. Our yeast two-hybrid screen using the intermediate domain as bait showed that this domain interacts with a variety of proteins implicated in cytoskeleton organization and regulation (29).

Like most of GCKs, overexpression of hMINK\(^\beta\) in mammalian cells activates the JNK pathway. Mouse MINK was previously reported to strongly activate JNK and weakly activate p38 kinase (17). Here we demonstrated that the kinase activity of hMINK\(^\beta\) is dispensable for activating JNK2 in a transient transfection assay. Similar observations have also been shown with GCK-I group kinases such as GCK and GLK, and GCK-IV group kinases: NIK and TNIK (2). The molecular mechanism by which GCKs activate the JNK pathway independent of their
FIG. 4. hMINKβ is involved in cell spreading, stress fiber formation, and cell-matrix adhesion. A, schematic diagram of wild-type and mutant hMINKβ constructs in pBabeMN-IGFP retroviral vector. hMINKβ-(1–547) and hMINKβ-(296–1312) were constructed with FLAG tag. The truncated mutants were labeled as numbers of amino acids. B, the expression of wild-type and mutant hMINKβ proteins in HT1080 cells was determined by Western blot analysis with anti-hMINKβ (top left panel) and anti-FLAG (top right panel) antibodies. The blots were re-probed for α-tubulin (low panels) as a loading control. C, HT1080 cells expressing hMINKβ wild-type or mutant proteins were stained with TRITC-phalloidin and photographed under a fluorescent microscope. Photographs are shown at ×100 magnification. D, HT1080 cells expressing wild-type or mutant proteins were seeded onto 96-well plates coated with type I collagen or fibronectin, or 24-well plates coated with laminin and allowed to adhere to the plates for the indicated times. The attached cells were then measured with a CyQuant cell proliferation assay kit and calculated as the percentage of total input cells. The numbers represent mean ± S.D. for three experiments. The results shown here are representative of several independent experiments.
Kinase activities is largely unknown. The CNH domain of NIK was shown to interact with MEKK1, which is required for the full activation of JNK (5). In the case of GCK and TNIK, the CNH domains are sufficient to activate the JNK pathway (8, 12). Recent studies further shed light on this signal pathway (30–32). GCK was shown to interact with and activate MEKK1 by promoting oligomerization and autophosphorylation of MEKK1, which is independent of the kinase activity of GCK.
Considering that the CNH domains are highly conserved among GCK-IV kinases, it is reasonable to speculate that hMINKβ/H9252 might activate JNK2 through binding to MEKK1 or MEKK1-like kinases via its CNH domain.

Like NIK and TNIK, hMINKβ/H9252 also interacts with the SH3 domains of Nck via its intermediate domain. Previous studies suggest that Nck plays a pivotal role in coupling receptors to reorganization of the cytoskeleton (33). The SH3 domains of Nck were originally used to isolate NIK (6), and subsequently Nck was shown to link NIK to Eph receptors that are known to function in patterning of the nervous and vascular systems (11). Consistent with these findings, NIK knockout mice display a mesodermal patterning defect, suggesting a role for NIK in cell migration and morphogenesis (34). Dock, the Drosophila homolog of Nck, has been proposed to transduce signals to the actin cytoskeleton via Msn to guide axon targeting (35). hMINKβ may act in a similar fashion to regulate cytoskeleton changes and cell migration.

Cytoskeleton reorganization plays a critical role in cell morphology changes and cell motility. Overexpression of wild-type but not the kinase-inactive mutant of hMINKβ in Phoenix-A cells induced cell rounding (data not shown). Reduced cell spreading was also observed with other GCKs, such as TNIK (8) and HGK (16). These findings suggest that the regulation of cytoskeleton is a common feature among GCKs. In HT1080 fibroblast cells, kinase-inactive and kinase domain deleted mutants of hMINKβ markedly induced cell spreading, along with actin stress fiber formation. Unexpected, overexpression of wild-type hMINKβ in HT1080 cells also moderately promoted cell spreading, whereas the kinase domain alone did not show any effect. These results suggest that the C-terminal regulatory domain of hMINKβ plays a dominant role in the regulation of cell morphology in HT1080 cells. Because this portion of hMINKβ contains two domains that have been shown to be...
involved in protein-protein interactions (5–8), the observed effects may be mediated by hMINKβ interacting with and sequestering the cellular factors essential for its functions. Cell morphology changes are coincident with the alterations of cell-matrix adhesion. hMINKβ-KD and hMINKβ-(296–1312) strengthened cell adhesion to both laminin and fibronectin, whereas hMINKβ only enhanced cell adhesion to fibronectin. HT1080 cells are known to attach to collagen I, fibronectin, and laminin via α5β1, α4β1, and α6β1 integrin receptors, respectively (36). HGK has been shown to up-regulate the cell surface expression of α2 integrin (37). Moreover, NIK was demonstrated to physically associate with the cytoplasmic domain of β1 integrin and colocalize with actin and β1 integrin (6). It is possible that hMINKβ modulates cell adhesion by affecting integrin signaling.

Alteration of cell-matrix adhesion has a direct impact on cell motility and cell invasion. HT1080 cells expressing the kinase-inactive mutant of hMINKβ exhibited marked retardation in both cell migration and invasion compared with the control cells. Wild-type hMINKβ also decreased the cell invasion, but to a lesser extent. It is notable that the increased actin stress fiber formation and cell adhesion are closely correlated to the impaired cell migration and invasion. hMINKβ-(1–547) was shown to reduce cell adhesion to both laminin and fibronectin, but it has no effect on cell migration (data not shown), which suggests that reduced cell adhesion is not necessary to promote cell migration.

In addition to playing a role in cell-matrix adhesion, hMINKβ also modulates cell-cell adhesion in MCF7 epithelial cells. Wild-type hMINKβ tended to reduce cell-cell adhesion, which is accompanied by down-regulation of membrane-associated β-catenin. In contrast, hMINKβ-KD and hMINKβ-(296–1312), acting as dominant-negative mutants, induced the membrane localization of β-catenin and consequently promoted cell-cell adhesion. hMINKβ kinase domain alone had no effect on the distribution of β-catenin. Moreover this mutant also lost perinuclear localization in transiently transfected HeLa cells (data not shown). These results suggest that the kinase activity of hMINKβ is not sufficient to induce the translocation of β-catenin, and that the appropriate subcellular localization of hMINKβ is also required for its function. β-Catenin plays dual roles in cells. It becomes plasma membrane associated when bound to the transmembrane adhesive receptor E-cadherin, whereas in the presence of a Wnt signal, β-catenin can translocate to the nucleus where it acts as a transcription factor and activates a series of target genes, some of them involved in cell proliferation, such as cyclin D1 and c-Myc (38). How hMINKβ regulates β-catenin localization remains elusive. hMINKβ might directly phosphorylate proteins that are involved in assembly of the actin-catenin-cadherin complex, therefore affecting complex formation and resulting in reduced cell-cell adhesion. Another possibility is that hMINKβ acts downstream of the Wnt signal pathway like its Drosophila homolog Msn (39), to regulate nuclear localization of β-catenin.

The subcellular distribution of cellular proteins provides clues to understand how these proteins function in cells. The finding that hMINKβ is localized to the Golgi complex raises the intriguing possibility that hMINKβ may be involved in the regulation of vesicle biogenesis and transport. Intracellular protein transport is a complex process that is regulated by multiple mechanisms including phosphorylation. Protein kinases that associate with the Golgi membrane and modulate vesicle transport processes have been demonstrated with several protein kinase C family members (40). A germinal center kinase has also been implicated in vesicle transport through interaction with Rab8, a small GTP-binding protein (41). hMINKβ may associate with the Golgi membrane through the CNH domain that has been found in a number of vacuolar protein sorting factors including human Vam6 and yeast Vam6p/Vps39 (4). In human Vam6, the CNH domain is required for inducing lysosome clustering and fusion as well as its ability to localize to lysosomes (4). Thus, hMINKβ might regulate cell adhesion and cell migration by affecting the protein transport process. The fact that a majority of the newly synthesized β-catenin arrives at the plasma membrane in a complex with the E-cadherin precursor via vesicle transport (42) also supports this hypothesis.

The present study demonstrates that hMINKβ interacts...
with Nck and activates the JNK pathway, which is independent of its kinase activity. We also provide evidence indicating that hMINKβ is involved in the regulation of actin cytoskeleton, cell-matrix adhesion, and cell-cell adhesion, leading to changes in cell morphology, cell migration, and cell invasion. The observations that the kinase-inactive mutant of hMINKβ enhanced cell-cell adhesion, and blocked cell migration and invasion suggest that perturbing the hMINKβ activity may prevent tumor progression. Together, hMINKβ, like the Droso phila ortholog Msn, might participate in the regulation of its kinase activity. We also provide evidence indicating that hMINKβ, like the Dro sophila ortholog Man, might participate in the regulation of multiple signaling pathways. Further studies to identify upstream and downstream signals will be necessary for a full understanding of the biological role of hMINKβ.

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Functional Characterization of a Novel Human MINK Isoform 54397
Identification and Functional Characterization of a Novel Human Misshapen/Nck Interacting Kinase-related Kinase, hMINK β
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Identification and functional characterization of a novel human Misshapen/Nck interacting kinase-related kinase, hMINKβ.

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Page 54390, Fig. 1: There was an error in the legend to Fig. 1B. The numbers for the DNA fragments seen in the agarose gel were labeled incorrectly. The correct legend should read as follows:

B, one-step RT-PCR was performed using total RNAs from indicated tissues. The PCR products were separated on agarose gel. A 100-bp DNA molecular weight ladder was included at the right. Five distinct DNA bands could be identified in the gel. Bands 1, 2, and 5 represent hMINKα, hMINKβ, and hMINKε isoforms, respectively (see below). Sequencing analysis of these bands indicates that band 3 from the brain represents isoform γ, whereas band 4 from the lung represents isoform δ.

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