Molecular cloning of MINK, a novel member of mammalian GCK family kinases, which is up-regulated during postnatal mouse cerebral development

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Abstract **A new germinal center kinase (GCK) family kinase, Misshapen/NIKs-related kinase (MINK), has been cloned and its expression has been characterized in several tissues and various developmental stages of the mouse brain. MINK encodes a 1300 amino acid polypeptide, consisting of an N-terminal kinase domain, a proline-rich intermediate region, and a C-terminal GCK homology region. The expression of MINK is up-regulated during the postnatal development of the mouse brain. MINK activates the cJun N-terminal kinase and the p38 pathways.**
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Signal transduction; Germinal center kinase family kinase;
Mitogen-activated protein kinase; Stress-activated protein kinase

1. Introduction

Mitogen-activated protein kinase (MAPK) pathways regulate a wide spectrum of cellular processes, including cell growth, stress responses, differentiation, and gene transcription. MAPK pathways have been widely conserved in eukaryote evolution. Downstream of MAPK pathways are core signaling modules consisting of MAPKs, which are activated by dual phosphorylation on threonine and tyrosine residues catalyzed by MAPK kinases (MAPKKs). These kinases are, in turn, activated by serine/threonine phosphorylation catalyzed by MAPKK kinases (MAPKKKs) [1–4]. In mammalian cells, there are at least six distinct MAPK pathways, three of which have been characterized in detail: extracellular signal-regulated kinases (ERK) [2–4], p38-MAPK [5,6], and the cJun N-terminus kinases (JNKs) [7,8].

Upstream of these pathways, there exists the Ste20 family of kinases, which can be divided into two subfamilies. The first subfamily includes p21-activated kinase 1 (PAK1), a mammalian homologue of the yeast serine/threonine kinase, Ste20. The second subfamily is represented by the germinal center kinase (GCK), and therefore it is often referred to as the GCK family of protein kinases. The first subfamily kinases contain a C-terminal catalytic domain and an N-terminal binding site for the small G proteins Rac1 and Cdc42, while the GCK family kinases have an N-terminal kinase domain and a C-terminal regulatory domain. The Nck-interacting kinase (NIK) belongs to the GCK family, and has been proposed to link the protein tyrosine kinase signals to the JNK activation via the SH2–SH3 domain of Nck [1].

In this paper, we report a novel kinase of the GCK family, Misshapen/NIKs-related kinase (MINK), which has been identified and cloned in our systematic screening of genes that are involved in postnatal mouse cerebral development. MINK was found to be involved in both the JNK and the p38 pathways. MINK is structurally similar to the kinases that are related to NIK. A phylogenetic analysis showed that these kinases are likely to form a new subfamily of GCK family kinases. The expression of MINK was found to be up-regulated during the postnatal development of the mouse brain.

2. Materials and methods

2.1. Screening and cloning of MINK
From a λ ZAP phage cDNA library constructed from P28 male ICR mouse brains, clones containing inserts larger than 4.5 kb were randomly selected and subjected to a developmental Northern blot analysis in the postnatal mouse cerebrum. Sequencing of the full length B55 clone was performed from both orientations and at least twice for each orientation.

2.2. Northern blot analysis of MINK
Total RNA samples were prepared from various tissues and developmental stages by the single step method [9]. Total RNA (20 µg) was transferred onto nylon membranes. A 1.6 kb *Sac*I fragment (3086–4696) was used as a probe. The probe DNA was randomly labeled using [α-^{32}P]dCTP (Amersham Pharmacia) and the BcaBEST Labeling Kit (Takara, Otsu, Japan). Hybridization was performed in hybridization buffer (4×SSC, 1 mM EDTA, 0.1% Ficoll type 400, 0.1% polyvinylpyrrolidone, 1% SDS, and 200 µg/ml denatured salmon sperm DNA) at 65°C. Two high stringency washes were performed for 30 min at 65°C with 0.1×SSC and 0.5% SDS. The membranes were visualized by a phosphorimager, model BAS2000 (Fujifilm, Tokyo). The membranes were subsequently re-hybridized with a glyceraldehyde-3-phosphate dehydrogenase probe.

2.3. Plasmid construction
Full length mouse MINK was cloned into a pCMV expression vector, under the control of the cytomegalovirus promoter, with a

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Flag epitope tag (MDYKDDDDKR) inserted on the N-terminus by a standard PCR-mediated method. The resultant expression plasmid contains the sequence MDYKDDDDKRIPRGSSR added at the N-terminal side of the initial methionine residue of MINK. The sequence of the fusion protein was confirmed after construction. The mammalian expression vectors for Flag–TAK1 were described previously [10,11]. HA–JNK1, HA–p38, HA–ERK2, GST–cJun, and GST–ATF2 were kind gifts from Dr. E. Nishida at Kyoto University.

2.4. Phylogenetic tree creation

All sequences used were obtained through DDBJ/EMBL/GenBank. The kinase domains, the intermediate regions or the C-terminal regions, and the GCKH regions (if present) were aligned separately using the Clustal W program in the DDBJ [12]. The aligned sequences were combined together and the phylogenetic tree was created using the neighbor joining method with 10 000 bootstrap trials [13].

2.5. Transient cell expression

Transfection of 293 cells was performed as previously described [10]. For single transfection assays, 1×10^6 cells per 10 cm dish were transfected with 5 μg cDNA. For co-transfection assays, 5 μg of each cDNA was used. In the case of the Flag–TAK1 and TAB1 co-transfection, 2.5 μg of each cDNA was used. Cell lysates were prepared as previously described [10].

2.6. Antibodies

The antibodies used in this study are the following. Anti-Flag M2 monoclonal antibody (mAb) from Sigma (St. Louis, MO, USA); anti-HA.11 mAb, BAbCO (Berkeley, CA, USA); rabbit polyclonal IgG against full length JNK1, Santa Cruz Biotechnology (Santa Cruz, CA, USA); heavy and light chain (goat) peroxidase conjugated anti-mouse IgG and heavy and light chain (goat) peroxidase conjugated anti-rabbit IgG, Calbiochem (La Jolla, CA, USA).

2.7. Immunoprecipitation

For immunoprecipitation studies, 300 μl cell lysates were rotated with the desired antibodies, at the concentration recommended by the manufacturer, and 15 μl of a 50% slurry of protein G-Sepharose (Amersham Pharmacia) for 2 h. The mixtures were centrifuged at 15 000 rpm for 2 min. The pellets were washed three times with PBS and were suspended in 30 μl of PBS.

2.8. Immunoblotting

An aliquot of the cell lysate (3 μl) or the immunoprecipitate (6 μl) was subjected to SDS–PAGE and immunoblotting. For detection of the Flag-tagged and HA-tagged samples, the anti-Flag mAb and the anti-HA mAb were used as primary antibodies, respectively. Since HA–JNK1 has the same mobility as mouse IgG on the SDS–PAGE gel, for detection of the HA–JNK1 the rabbit anti-JNK1 polyclonal antibody was used instead.

2.9. Kinase assays

Kinase activities in the immune complexes were examined using myelin basic protein (MBP, from Sigma) as a kinase substrate for Flag–MINK and HA–ERK, GST–cJun for HA–JNK1, and GST–ATF2 for HA–p38. A solution (10 μl) containing 6.5 μl of the immune complex, 1 μg of a kinase substrate, 2 μl of 5×kinase buffer (50 mM HEPES pH 7.4, 5 mM DTT, and 25 mM MgCl$_2$), and 5 μCi of [γ-^{32}P]ATP was incubated for 2 min at 25°C. The reaction products were subjected to SDS–PAGE and autoradiography.

2.10. Accession number

The mouse MINK and the hypothetical human MINK sequences have been submitted to DDBJ/EMBL/GenBank; the accession numbers are AB035697 and AB035698, respectively.

3. Results and discussion

3.1. Molecular cloning of MINK

The MINK gene was cloned in our effort to find genes involved in postnatal mouse cerebral development. We obtained a clone, B55, which exhibited an up-regulated expression pattern in postnatal cerebral development (Fig. 1A). The
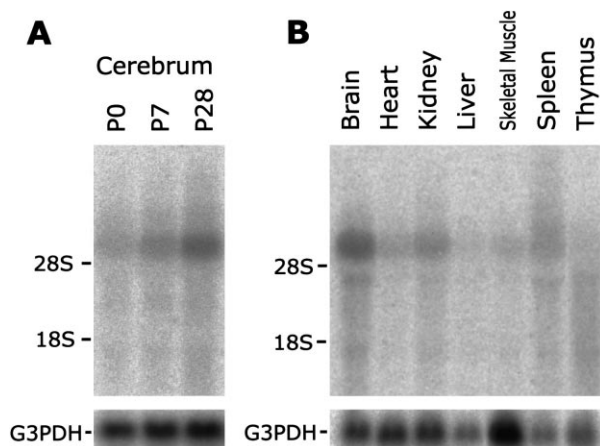

Fig. 1. Expression patterns of MINK. A: Northern blot analysis of MINK in the developing postnatal mouse cerebrum. B: Multi-tissue Northern blot analysis of MINK. Total RNAs were hybridized with a MINK cDNA probe labeled by [α-^{32}P]dCTP. The same blot was later re-hybridized with a glyceraldehyde-3-phosphate dehydrogenase probe as a control.

expression of B55 started at birth and increased as the brain developed. The clone harbored a 4848 bp transcript with a Kozak consensus initiation sequence followed by an open reading frame encoding a polypeptide of 1300 amino acids with a predicted molecular mass of 146 kDa (Fig. 2) [14]. B55 is highly homologous to several members of the group I GCK family kinases, namely, NIK, TRAF2/Nck-interacting kinase (TNIK), NIK-related kinase (NRK), as well as their *Caenorhabditis elegans* orthologue, MIG-15, and the *Drosophila* homologue, Misshapen [15–18]. Therefore, we designated this clone <u>Mi</u>sshapen/<u>NIK</u>s-related <u>k</u>inase (MINK).

3.2. Sequence analysis of MINK

MINK consists of an N-terminal kinase domain comprising 11 kinase subdomains, an intermediate region, and a C-terminal GCK homology (GCKH) region [1,19,20]. Its kinase domain and GCKH region share up to 90% amino acid identity with those of the NIK-related kinases (Fig. 3) [15–18]. These regions are also distantly related to those of GCK. In the intermediate region, however, the sequence homology was less striking, and MINK displayed less than 45% amino acid identity with other NIK-related kinases. Like the intermediate regions of other group I GCK family kinases, MINK also contains eight proline-rich motifs (PXXP): the putative Src homology 3 (SH3) domain binding regions [15,16]. Especially, PKVPQR (amino acids 669–674), which is also conserved in NIK and TNIK, satisfied the PXXPXR criterion, and RSRPGRP (amino acids 761–767) satisfied the RXXPXXP criterion [21,22]. MINK has two Q-rich motifs that contain nine and 16 glutamine residues. The Q-rich motifs are also found in Misshapen, but their functions remain unknown [18].

In the case of NIK, the PXXPXR motif has been demonstrated to interact with the SH3 domain of Nck [15]. Also, TNIK has been shown to bind to Nck via its intermediate region [16]. Considering these observations, it is likely that MINK may also interact with Nck. However, the sequence deviation of MINK in its intermediate region suggests that there may be unknown upstream binding partners.

A DNA database search revealed that human MINK is

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              I                                II                III                      IV
  1 MGDPAPARSL DDIDLSALRD PAGIFELVEV VGNGTYGOVY KGRHVKTGOL AAIKVMDVTE DEEEEIKOEI NMLKKYSHHR NIATYYGAFI KKSPPGNDDO 100
              V                                VI                        VII                         VIII
101 LWLVMEFCGA GSVTDLVKNT KGNALKEDCI AYICREILRG LAHLHAHKVI HRDIKGONVL LTENAEVKLV DFGVSAOLDR TVGRRNTFIG TPYWMAPEVI 200
              IX                               X                         XI
201 ACDENPDATY DYRSDIWSLG ITAIEMAEGA PPLCDMHPMR ALFLIPRNPP PRLKSKKWSK KFTDFIDTCL IKTYLSRPPT EOLLKFPFIR DOPTEROVRI 300
301 OLKDHIDRSR KKRGEKEETE YEYSGSEEED DSHGEEGEPS SIMNVPGEST LRREFLRLQQ ENKSNSEALK OOOOLOOOOO RDPEAHIKHL LHQRQRRIEE 400
401 QKEERRRVEE QQRREREQRK LQEKEQQRRL EDMQALRREE ERRQAEREQE YKRKQLEEQR QSERLQRQLQ QEHAYLKSLO OOOOOOOLOK OOOOOOOOILP 500
501 GDRKPLYHYG RGINPADKPA WAREVEERAR MNKQQNSPLA KRSQAGAGPE PPISQASPSP PGPLSQTPPM QRPVEPOEGP HKSLVAHRVP LKPYAAPVPR 600
601 SQSLQDQPTR NLAAFPASHD PDPAAVPTPT ATPSARGAVI RQNSDPTSEG PGPSPNPPSW VRPDNEAPPK VPQRTSSIAT ALNTSGAGGS RPAQAVRASN 700
701 PDLRRSDPGW ERSDSVLPAS HGHLPQAGSL ERNRNRVGAS TKLDSSPVLS PGNKAKPEDH RSRPGRPADF VLLKERTLDE APKPPKKAMD YSSSSEEVES 800
801 SEEEEEEGDG EPSEGSRDTP GGRSDGDTDS VTTMVVHDVE EISGTQPSYG GGTMVVQRTP EERSLLLAD SNGYTNLPDV VQPSHSPTEN SKGQSPPTKD 900
901 GGSDYQSRGL VKAPGKSSFT MFVDLGIYQP GGSGDTIPIT ALVGGEGGRL DQLQFDVRKG SVVNVNPTNT RAHSETPEIR KYKKRFNSEI LCAALWGVNL 1000
1001 LVGTENGLML LDRSGOGKVY GLIGRRRFQQ MDVLEGLNLL ITISGKRNKL RVYYLSWLRN KILHNDPEVE KKQGWTTVGD MEGCGHYRVV KYERIKFLVI 1100
1101 ALKNSVEVYA WAPKPYHKFM AFKFSFADLPH RPLLVDLTVE EGQRLKVIYG SSAGFHAVDV DSGNSYDIYI PVHIQSQITP HAIIFLPNTD GMEMLLCYED 1200
1201 EGVYVNTYGR IIKDVVLQWG EMPTSVAYIC SNQIMGWGEK AIEIRSVETG HLDGVFMHKR AQRLKFLCER NDKVFFASVR SGGSSQVYFM TLNRNCIMNW 1300
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Fig. 2. Deduced amino acid sequence of MINK. The kinase domain is underlined. The conserved 11 kinase subdomains are indicated by Roman numerals and they are alternatingly shadowed. The eight proline-rich motifs are in bold type. Glutamine-rich motifs are in black boxes. The GCKH region is shadowed in a box.

encoded by the clone hRPK177_H_5 from chromosome 17. By sequence comparison, we predicted a hypothetical human MINK protein. The human MINK gene covers over 63 kb of genomic DNA. The coding region of the MINK transcript consists of 31 exons. All exon/intron boundaries matched the consensus sequences for splicing [23].

To examine the evolutionary relationship of MINK among the GCK family of kinases, we created a phylogenetic tree. Conventionally, the GCK family kinases have been structurally classified into two distinct groups: group I contains an N-terminal catalytic domain and a C-terminal regulatory domain, and group II contains a catalytic domain but lacks the other characteristic domains [1]. The dendrogram in Fig. 4 shows that MINK, TNIK, NIK, MIG-15, Misshapen, and NRK form a distinct branch, which is separated from a branch comprising the other group I GCK family kinases and that of the group II kinases. Interestingly, each branch contains a particular putative kinase in *C. elegans*, MIG-15, zc404.9, and T19A5.2. This characteristic grouping of the NIK-related kinases has its structural basis within their highly conserved GCKH regions and moderately conserved, long intermediate regions (Fig. 3). This result suggests that the NIK-related kinases, the other group I GCK family kinases, and the group II GCK family kinases have distinct evolutionary roots. Taken together, we propose that MINK, NIK, TNIK, and NRK form a NIK subfamily that is distinct from other group I GCK family kinases.

3.3. Tissue distribution of MINK mRNA

We examined the expression pattern of the MINK mRNA by mouse multi-tissue Northern blotting (Fig. 1B). A major band of 5.0 kb was detected. This band corresponds to the size of the cDNA clone we obtained. The MINK transcript was most abundant in the brain. It was also detected in kidney, spleen, and heart. A longer exposure revealed that it was ubiquitously expressed in all tissues examined. Its expression pattern was different from those of NIK, TNIK, and NRK [15–17]. While NIK and TNIK are most abundant in the heart, MINK is less abundant. In adult skeletal muscle, NIK and TNIK are abundant, while MINK is not. NRK's expression is limited to embryonic muscle. In contrast, MINK

is moderately expressed in kidney and spleen, where NIK and TNIK are expressed at low levels. These different tissue expression patterns among the NIK subfamily kinases imply functional distinctions among them.

Among the NIK subfamily kinases, MINK is the most abundant species in the brain and its expression is up-regulated in postnatal cerebral development. This is reminiscent of the expression pattern of the *C. elegans* orthologue, MIG-15, which is required for migration of central nerve cells (Q cells).
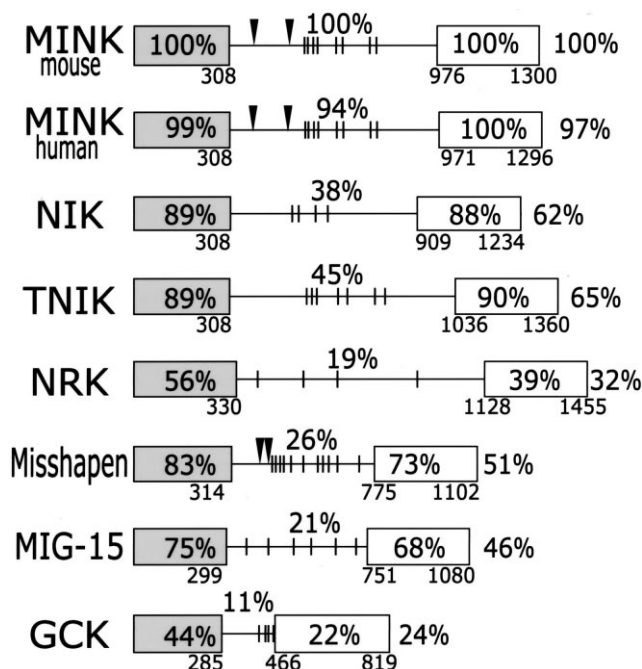

Fig. 3. Homologies of MINK to other NIK subfamily kinases and GCK. The kinase domain is represented by gray boxes, the intermediate region by horizontal lines, and the GCKH region by white boxes. Locations of proline-rich motifs (PXXP) are indicated by vertical bars and glutamine-rich motifs are denoted by vertical arrowheads. Amino acid identities relative to mouse MINK within each domain (indicated in the corresponding domain in the figure) and in the whole molecule (on the right) are shown. The human MINK sequence was predicted from a genomic sequence.
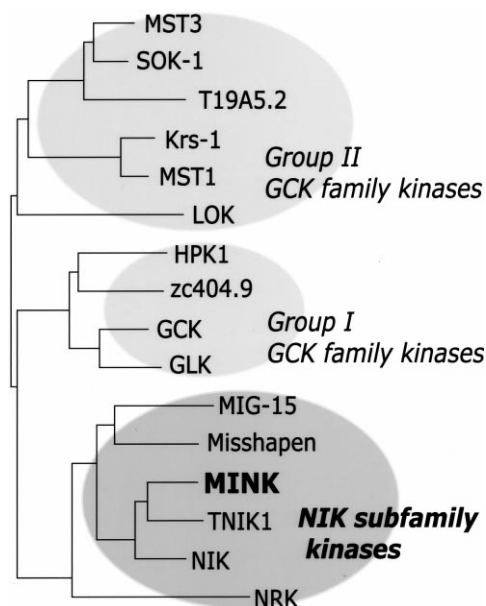
Fig. 4. A phylogenetic tree of the GCK family of protein kinases. Each family of kinases is symbolically represented with an ellipse. *C. elegans* zc404.9 and T19A5.2 are hypothetical proteins predicted from their genomic sequences. Abbreviations not explained in the text are: GLK, GCK-like kinase; HPK1, hematopoietic progenitor kinase-1; LOK, lymphocyte-oriented kinase; MST1 or 3, mammalian sterile twenty-like 1 or 3; Krs-1, kinase responsive to stress-1; SOK-1, Ste20-like oxidant stress-activated kinase-1.

It is expressed in migrating Q cells and their descendants, and is likely to play an important role in the maturation of central nerve cells [18]. We are currently examining the expression of the MINK transcript in various parts and stages of the developing mouse brain.

3.4. MINK kinase activity

To examine the kinase activity of MINK, a Flag-tagged MINK cDNA was transfected into 293 cells. The vector alone was also transfected as a negative control. An immunoblot of
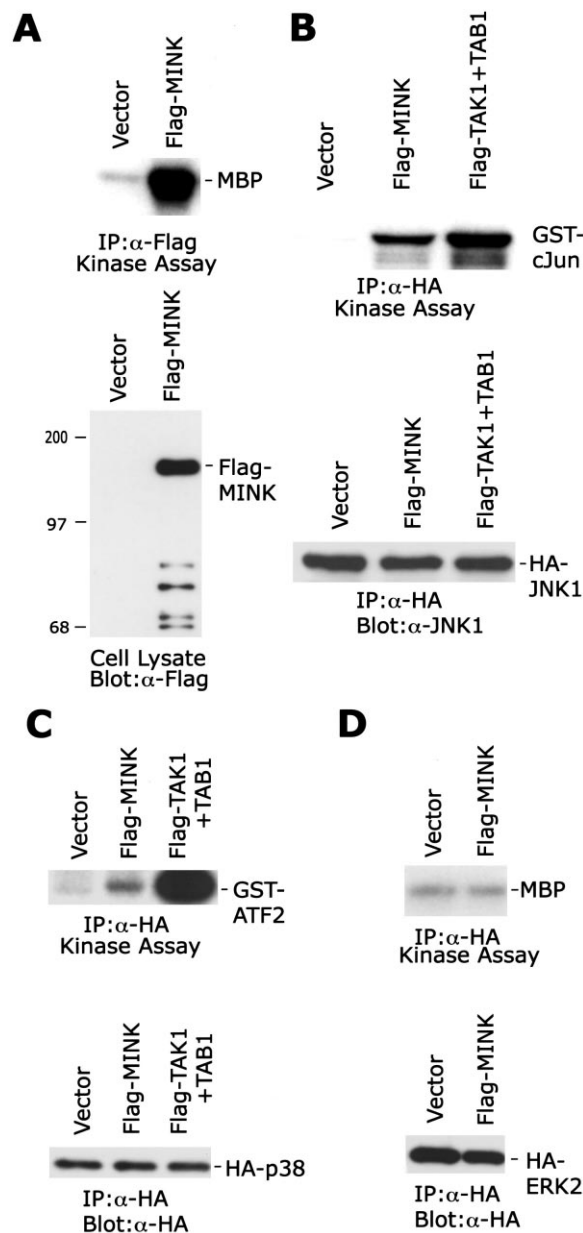
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Fig. 5. A: Kinase activity of MINK. Lysates from 293 cells transfected with Flag–MINK or a vector control were immunoprecipitated using an anti-Flag antibody, and their kinase activities were assayed using [γ-^{32}P]ATP and MBP as substrates. Reaction products were separated by SDS–PAGE (10%) and were subjected to autoradiography (upper row). Lysates from 293 cells transfected as in A were separated by SDS–PAGE (7%) and were immunoblotted with the anti-Flag antibody (lower row). B: Activation of JNK1 by MINK. Lysates from 293 cells co-transfected with HA–JNK1 and a vector control, Flag–MINK or Flag–TAK1+TAB1, were immunoprecipitated with the anti-HA antibody and were subjected to an in vitro kinase assay using [γ-^{32}P]ATP and GST–cJun as substrates. Reaction products were separated by SDS–PAGE (10%) and were subjected to autoradiography. The immune complexes were immunoblotted with the anti-JNK1 antibody to determine if the same amount of HA–JNK1 was immunoprecipitated (lower row). C: Activation of p38 MAPK by MINK. 293 cells were co-transfected with HA–p38 and a vector control, Flag–MINK or Flag–TAK1+TAB1. The immunoprecipitation and the in vitro kinase assay using GST–ATF2 as a substrate were performed as in B. The immune complexes were immunoblotted with the anti-HA antibody (lower row). D: Effect of MINK on ERK. 293 cells were co-transfected with HA–ERK2 and a vector control or Flag–MINK. The immunoprecipitation, the in vitro kinase assay using MBP as a substrate, and the immunoblot analysis (lower row) were performed as in C.

the whole cell lysates using an anti-Flag antibody detected a 150 kDa protein that roughly corresponds to the predicted molecular mass of full length MINK only in the MINK transfected cells (Fig. 5A, lower row). The transiently expressed proteins were immunoprecipitated with the anti-Flag antibody, and an in vitro kinase assay was performed using MBP as a substrate. A strongly phosphorylated band corresponding to MBP was detected only in the MINK transfected lane (Fig. 5A, upper row). These results demonstrate that MINK exhibits kinase activities.

3.5. MINK activates the JNK and the p38 pathways

To determine which portion of the mammalian MAPK pathway may be activated by MINK, 293 cells were co-transfected with Flag–MINK and HA epitope-tagged JNK1, p38, or ERK2. The respective HA–MAPKs were immunoprecipitated and subjected to in vitro kinase assays. Transfection with Flag–MINK resulted in JNK1 activation as compared to the vector control (Fig. 5B). TAK1 MAPKKK with its

activator TAB1 is known as a strong activator of the JNK and the p38 pathways, and transfection with Flag–TAK1+-TAB1 activated JNK1, in agreement with previous reports [10,11]. This result demonstrates that MINK activates the JNK pathway. In addition, MINK activated the p38 pathway as compared to the vector control (Fig. 5C). However, the degree of activation was lower than that by Flag–TAK1+-TAB1. In contrast, the ERK activity was not increased when MINK was overexpressed in 293 cells (Fig. 5D).

In conclusion, we identified and cloned a novel GCK family kinase, MINK. Its expression is up-regulated in the course of postnatal mouse cerebral development and may be involved in brain development. A phylogenetic analysis suggests that MINK and other NIK-related kinases form a distinct subfamily within the GCK family of kinase. MINK activates the JNK and the p38 pathways.

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