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NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain

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Nck, an adaptor protein composed of one SH2 and three SH3 domains, is a common target for a variety of cell surface receptors. We have identified a novel mammalian serine/threonine kinase that interacts with the SH3 domains of Nck, termed Nck Interacting Kinase (NIK). This kinase is most homologous to the Sterile 20 (Ste20) family of protein kinases. Of the members of this family, GCK and MSST1 are most similar to NIK in that they bind neither Cdc42 nor Rac and contain an N-terminal kinase domain with a putative C-terminal regulatory domain. Transient overexpression of NIK specifically activates the stress-activated protein kinase (SAPK) pathway. Both the kinase domain and C-terminal regulatory region of NIK are required for full activation of SAPK. NIK likely functions upstream of MEKK1 to activate this pathway; a dominant-negative MEK kinase 1 (MEKK1) blocks activation of SAPK by NIK. MEKK1 and NIK also associate in cells and this interaction is mediated by regulatory domains on both proteins. Two other members of this kinase family, GCK and HPK1, contain C-terminal regulatory domains with homology to that of NIK. These findings indicate that the C-terminal domain of these proteins encodes a new protein domain family and suggests that this domain couples these kinases to the SAPK pathway, possibly by interacting with MEKK1 or related kinases.

Keywords: MEKK1/Nck interacting kinase/Ste20-related kinase/stress-activated protein kinase

Introduction

Over the past several years it has become apparent that while different signaling molecules may contain distinct catalytic activities, most share several conserved protein domains (Schlessinger, 1994; Cohen *et al.*, 1995; Pawson, 1995). These domains are critical for regulating signal transduction pathways, and function by mediating protein–protein or protein–lipid interactions. For example, two domains frequently found on signaling molecules are SH2 and SH3 domains (Schlessinger, 1994; Cohen *et al.*, 1995; Pawson, 1995). SH2 domains bind to a phosphotyrosine moiety in the context of short amino acid sequences and also to phosphatidylinositol (3,4,5)trisphosphate (PtdIns[3,4,5]P_3) (Rameh *et al.*, 1995), while SH3 domains bind to proline-rich sequences on effector molecules.

One class of signaling molecules with SH2 and SH3 domains lack sequence homology with other proteins containing known catalytic domains (Schlessinger, 1994; Pawson, 1995). Studies over the past several years have elucidated the mechanism whereby SH2 domain-containing proteins without catalytic activity, such as Grb2- and p85-associated PI3 kinase, regulate signal transduction pathways. SH2 domain-containing proteins in this group are tightly associated with catalytic molecules, and function as adaptors linking these molecules to phosphotyrosine-containing proteins. Grb2 provides one of the best examples of how molecules in this group function to regulate signal transduction pathways (Schlessinger, 1994; Pawson, 1995). Grb2 is tightly associated via its SH3 domains to the Ras guanine nucleotide exchange factor, Son of sevenless (Sos). Following growth factor stimulation, binding of SH2 domains of Grb2 to the autophosphorylated receptor localizes Sos adjacent to Ras in the plasma membrane, thereby enabling Sos to activate Ras.

Nck is a ubiquitously expressed protein which is composed of one SH2 and three SH3 domains and, like Grb2, does not contain homology to any known catalytic domains and thus fits into the adaptor class of signaling molecules (Lehman *et al.*, 1990). Several findings have indicated that Nck is likely to be an important signaling molecule. First, Nck is an oncoprotein; cellular overexpression of Nck results in transformation of NIH 3T3 cells and 3Y1 rat fibroblasts (Chou *et al.*, 1992; Li *et al.*, 1992). Secondly, Nck is a common target for a variety of growth factor receptors and becomes phosphorylated on serine, threonine and tyrosine residues after growth factor stimulation (Chou *et al.*, 1992; Li *et al.*, 1992; Park and Goo Rhee, 1992). More recently, genetic screens in *Drosophila* have indicated that the *Drosophila* protein DOCK is related to Nck and is important in mediating one of the signals for axonal guidance (Garrity *et al.*, 1996). Nck may regulate these processes by functioning as an adaptor molecule to couple a catalytic subunit, bound to its SH3 domains, to tyrosine-phosphorylated proteins.

Over the past couple of years, several effector molecules that interact with the SH3 domains of Nck have been identified. These domains have been shown to bind Sos, Cbl, the Wiskott–Aldrich syndrome protein (WASP) and a 65 kDa serine/threonine kinase which is likely to be a member of the PAK 65 family (Rivero-Lezcano *et al.*, 1994, 1995; Bagrodia *et al.*, 1995; Chou and Hanafusa, 1995; Hu *et al.*, 1995; Galisteo *et al.*, 1996). However, for the most part, the biological significance of these interactions is still not known. For example, while overexpression of Nck under some circumstances leads to Ras activation and thus may be accounted for by the interaction

of Nck with Sos, dominant inhibitory Nck molecules do not block activation of Ras by Abl or the epidermal growth factor receptor (EGFR) (Tanaka *et al.*, 1995). In addition, the biological significance of Nck binding Cbl or WASP is still not known.

In the hope of further elucidating the role of Nck in growth factor signaling, we undertook studies to identify proteins that bind the SH3 domains on Nck. Here, we report the identification of a novel serine/threonine kinase, NIK (Nck Interacting Kinase), which binds the SH3 domains of Nck *in vitro* and *in vivo*. Transient overexpression of NIK activates the MEKK1/MKK4/SAPK pathway, and full activation of this pathway requires both a functional kinase and a conserved C-terminal regulatory domain. Interestingly, we found that the C-terminal regulatory domain of NIK interacts with MEKK1. In addition, this domain is conserved in two other members of this kinase family, thereby identifying a previously undescribed binding domain. Upstream regulators of MEKK1 are still largely unknown. These findings suggest that NIK directly modulates MEKK1 activity *in vivo*. NIK and related kinases may provide a link by which adaptor proteins such as Nck couple receptors to the JNK/SAPK pathway.

Results

Identification of NIK using the yeast two-hybrid system and subsequent cDNA cloning

We screened for proteins that interact with the SH3 domains of Nck using the yeast two-hybrid system. We identified several partial cDNA clones that interacted specifically with Nck. The proteins encoded by the clones interacted with the Nck–LexA fusion, but not with LexA coupled to a variety of other cDNAs (data not shown). All clones contained at least one proline motif that matched consensus motifs previously shown to be critical for interaction of SH3 domains with target proteins (Yu *et al.*, 1994). One clone was found to encode a partial sequence of a serine/threonine protein kinase, termed NIK, which is the focus of this report.

The clone identified in the yeast two-hybrid system contained 528 nucleotides (corresponding to amino acids 443–619 of full-length NIK). To identify the full-length NIK cDNA, an adult mouse brain cDNA library (Stratagene) was screened using the DNA isolated from the two-hybrid screen. Several overlapping clones were identified, the largest of which (cl 16) was ~3 kb. Cl 16 contained a single large open reading frame followed by a polyadenylation signal and a long 3′ untranslated region of ~1000 nucleotides. Since the insert from cl 16 did not contain the 5′ end of the gene, an embryonic mouse brain cDNA library (Stratagene) was screened using the 5′ 250 nucleotides of cl 16 as a probe. This approach enabled us to identify three additional overlapping clones that extended 5′ from cl 16. One of these clones, cl 11, contained the 5′ end of the gene; this clone contained an AUG codon that met Kozak translation initiation criteria as well as a stop codon that was 5′ and in-frame to this predicted translation start site (Kozak, 1989).

Analysis of the full-length gene demonstrated three regions of homology to other genes in the database. The N-terminus of NIK contains 11 subdomains that are characteristic of serine/threonine protein kinases (Figure 1A) (Hanks and Hunter, 1995). The middle portion of NIK contains two proline-rich sequences that match consensus sequences important in mediating SH3-domain binding to protein targets and are responsible for the interaction of NIK with Nck in the yeast two-hybrid system (see Figure 4B) (Yu *et al.*, 1994). The C-terminal portion of NIK lacks any obvious sequence motifs. However, data presented below strongly suggest that this regions plays an important regulatory role in NIK function.

The NIK kinase domain is most similar to the p21 activated kinase (PAK) Ste20 family of protein kinases (Figure 1B) (Ramer and Davis, 1993; Katz *et al.*, 1994; Manser *et al.*, 1994; Creasy and Chernoff, 1995; Martin *et al.*, 1995). Of the members in this group identified thus far, NIK is structurally most similar to human GCK and MST1 and yeast Sps1 in that NIK does not bind the GTP-bound form of either Cdc42Hs or Rac1 (data not shown) and contains an N-terminal kinase domain with a putative C-terminal regulatory domain (Friesen *et al.*, 1994; Creasy and Chernoff, 1995; Pombo *et al.*, 1995).

Interestingly, NIK is most homologous to a gene identified in the *Caenorhabditis elegans* genome sequencing project and is likely the mammalian homologue of this gene (Figure 1C). No function has yet been described to the *C.elegans* gene, nor has it been characterized at the mRNA or protein level. NIK is 52% identical and 68% similar overall to the *C.elegans* gene (data not shown). Moreover, the kinase and C-terminal domains of the two proteins are even more conserved; NIK and the *C.elegans* gene are 86% similar and 72% identical between these two domains (Figure 1C and data not shown). The C-terminal domain of NIK is also similar to the regulatory domains of two other kinases in this group, GCK and HPK1 (Figure 1C) (Katz *et al.*, 1994; Pombo *et al.*, 1995; Hu *et al.*, 1996). The strong conservation of the NIK C-terminus with the *C.elegans* gene and with GCK and HPK1 suggested to us that this domain plays an important regulatory role in NIK function.

Northern hybridization analysis was performed on various human tissues using cl 16 as a probe (Figure 2A). This analysis demonstrated the presence of two major bands of 4.5 and 6.5 kb in most tissues, with highest

A

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   1  MANDSPAKSL VDIDLSSLRD PAGIFELVEV VGNGTYGQVY KGRHVKTVTA
  51  AIKVMDVTED EEEEITLEIN MLKKYSHHRN IATYYGAFIK KSPPGHDDQL
 101  WLVMEFCGAG SITDLVKNTK GNTLKEDWIA YISREILRGL AHLHIHHVIH
 151  RDIKGQNVLL TENAEVKLVD FGVSAQLDRT VGRRNTFIGT PYWMAPEVIA
 201  CDENPDATYD YRSDLWSCGI TAIEMAEGGP PLCDMHPMRA LFLIPRNPPP
 251  RLKSKKWSKK FFSFIEGCLV KNYMQRPSTE QLLKHPFIRD QPNERQVRIQ
 301  LKDHIDRTRK KRGEKDETEY EYSGSEEEEE EVPEQEGEPS SIVNVPGEST
 351  LRRDFLRLQQ ENKERSEALR RQQLLQEQQL REQEEYKRQL LAERQKRIEQ
 401  QKEQRRRLEE QQRREREARR QQEREQRRRE QEEKRRLEEL ERRRKEEEER
 451  RRAEEEKRRV EREQEYIRRQ LEEEQRHLEI LQQQLLQEQA MLLHDHRRPH
 501  AQQQPPPPQQ QDRSKPSFHA PEPKPHYDPA DRAREVQWSH LASLKNNVSP
 551  VSRSHSFSDP SPKFAHHHLR SQDPCPPSRS EGLSQSSDSK SEVPEPTQKA
 601  WSRSDSDEVP PRVPVRTTSR SPVLSRRDSP LQGGGQQNSQ AGQRNSTSSI
 651  EPRLLWERVE KLVPRPGSGS SSGSSNSGSQ PGSHPGSQSG SGERFRVRSS
 701  SKSEGSPSPR QESAAKKPDD KKEVFRSLKP AGEVDLTALA KELRAVEDVR
 751  PPHKVTDYSS SSEESGTTDE EEEDVEQEGA DDSTSGPEDT RAASSPNLSN
 801  GETESVKTMI VHDDVESEPA MTPSKEGTLI VRQTQSASST LQKHKSSSSF
 851  TPFIDPRLLQ ISPSSGTTVT SVVGFSCDGL RPEAIRQDPT RKGSVVNVNP
 901  TNTRPQSDTP EIRKYKKRFN SEILCAALWG VNLLVGTESG LMLLDRSGQG
 951  KVYPLISRRR FQQMDVLEGL NVLVTISGKK DKLRVYYLSW LRNKILHNDP
1001  EVEKKQGWTT VGDLEGCVHY KVVKYERIKF LVIALKSSVE VYAWAPKPYH
1051  KFMAFKSFGE LLHKPLLVDL TVEEGQRLKV IYGSCAGFHA VDVDSGSVYD
1101  IYLPTHIQCS IKPHAIIILP NTDGMELLVC YEDEGVYVNT YGRITKDVVL
1151  QWGEMPTSVA YIRSNQTMGW GEKAIEIRSV ETGHLDGVFM HKRAQRLKFL
1201  CGRNDKVFFS SVRSGGSSQV YFMTLGRTSL LSW*
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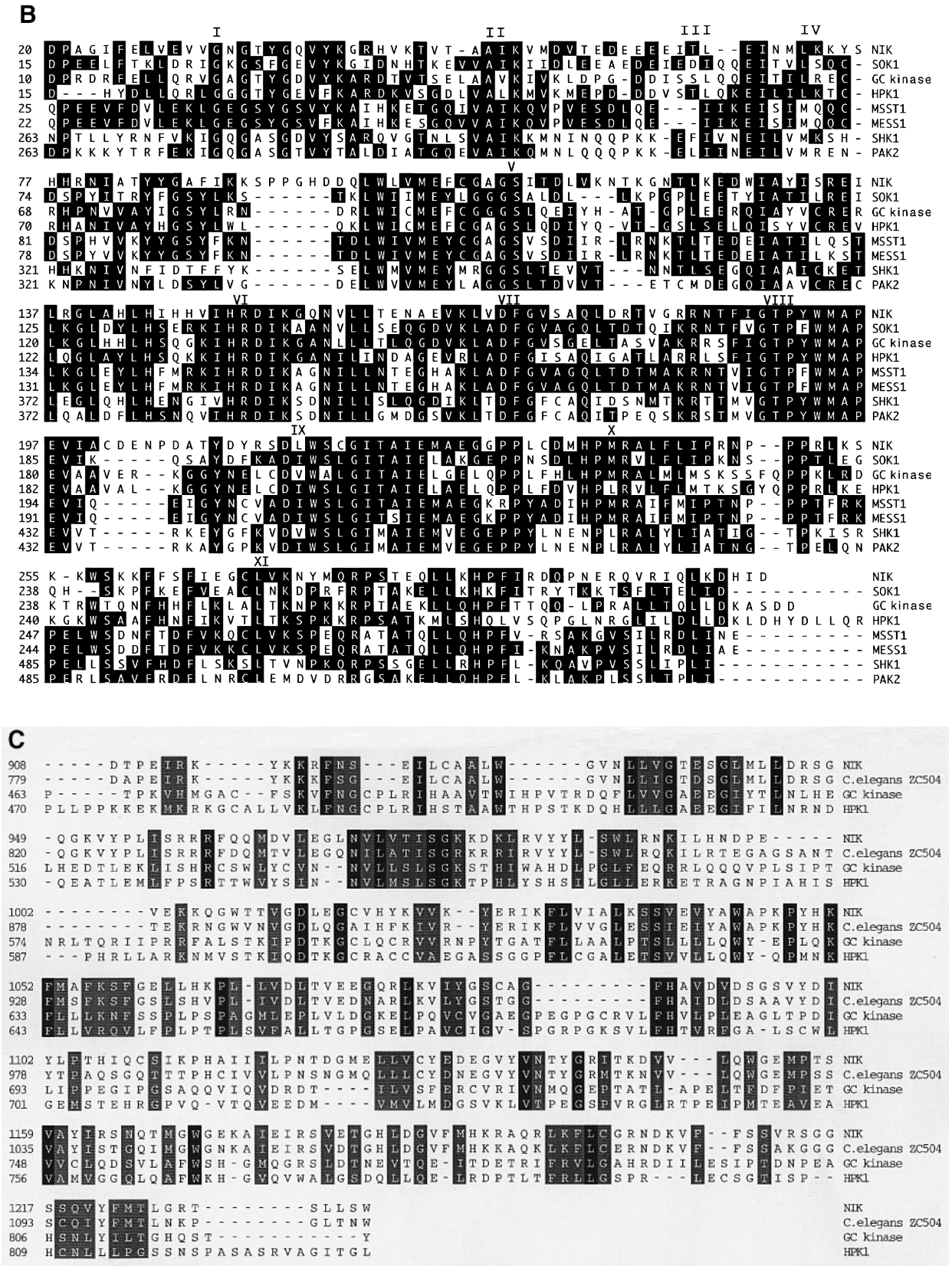

Fig. 1. Deduced amino acid sequence of NIK and alignment with other proteins containing similar sequence motifs. (**A**) NIK contains an open reading frame of 1234 amino acids. The kinase domain is marked by a broken underline (.....). Two proline-rich motifs that match consensus SH3 binding motifs and mediate the association of NIK with Nck are shown in bold. The C-terminal domain of NIK that contains homology to GCK and the *C.elegans* gene ZC504 is marked by a double underline (===). The initial clone identified in the yeast two-hybrid screen that interacted with Nck is denoted by single underline (__). (**B**) Amino acid alignment of NIK kinase domain with other Ste20/Pak family members. Identical amino acids are shown in black and conserved amino acids substitution are shown in gray. (**C**) Alignment of the C-terminal domain of NIK with GCK and the *C.elegans* gene ZC504. Identical amino acids are shown in black and conserved amino acids substitution are shown in gray.

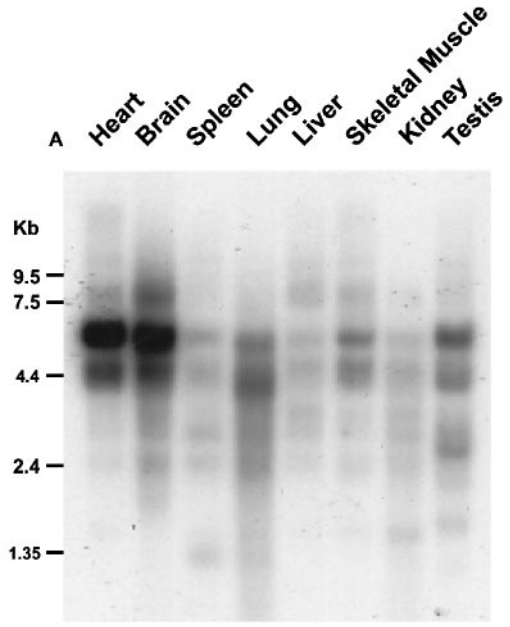

Fig. 2. Northern analysis of NIK. (**A**) An adult tissue mouse Northern blot (purchased from Clontech) was hybridized with the [α-^{32}P]dCTP-labeled clone identified in the yeast two-hybrid system.

expression in the brain and heart. The 4.5 kb message corresponds to the expected size which codes for the clone that we isolated.

NIK encodes a protein kinase of 140 kDa

The predicted molecular mass of full-length NIK was ~140 kDa. To confirm that the NIK cDNA encoded a protein of this molecular mass, the full-length NIK cDNA was transfected into 293 cells and transfected cell lysates were immunoblotted with antibodies to NIK. Antibodies to NIK specifically recognized a protein of 140 kDa in cells transfected with NIK, but not in cells transfected with a vector control (Figure 3A), thereby confirming that the NIK cDNA encodes a protein of the predicted molecular mass.

To determine whether NIK has kinase activity, 293 cells were transfected with NIK and an immune complex kinase assay was performed on NIK immunoprecipitates using myelin basic protein (MBP) as a substrate. While kinase activity was detected in NIK immunoprecipitates from 293 cells transfected with vector alone, kinase activity was markedly increased in cells transfected with NIK (Figure 3B). The kinase activity immunoprecipitated by the anti-NIK antibody was specific; preimmune serum did not immunoprecipitate kinase activity (Figure 3B). In addition to MBP, two other proteins of 140 and 35 kDa were also phosphorylated in the immune complex kinase assay. The band at 140 kDa is autophosphorylated NIK, while the band at 35 kDa has not been identified. To rule out the possibility that a kinase co-precipitating with NIK accounted for the kinase activity, a Myc epitope-tagged kinase-defective NIK mutant in which the conserved aspartic acid at position 152 was mutated to asparagine [NIK(D152N)] was also transiently expressed in 293 cells. Kinase activity was detected only in anti-Myc immunoprecipitates from cells transfected with wild-type NIK (Figure 3C). A truncated NIK protein containing only the kinase domain also had full kinase activity (Figure 3C). These findings confirm that NIK is a protein kinase.

NIK binds Nck in cells and interacts specifically with the SH3 domains of Nck via two proline-rich motifs

To confirm that NIK interacts with the SH3 domains of Nck, as well as to assess whether NIK binds SH3 domains present in other proteins, we determined whether NIK bound GST fusions containing the SH3 domains of a variety of proteins. Myc-epitope-tagged NIK was transfected into 293 cells and binding of NIK in the transfected cell lysates to several GST fusions was assessed. NIK bound a GST fusion protein containing either full-length or the three SH3 domains of Nck (Figure 4A). In contrast to the association of NIK with Nck, NIK interacted only weakly with GRB2 and we were unable to detect association of NIK with SH3 domains from a variety of other proteins (Figure 4A). While several of these SH3-domain fusion proteins did not bind NIK, they were able to bind proline motifs present in a related kinase, human PAK1 (data not shown), indicating that these fusion proteins were functional.

We also identified the proline motifs that mediated the association of NIK with the SH3 domains of Nck. NIK contains two proline motifs that match consensus SH3 binding sites (Figure 1A, shown in bold) (Yu *et al.*, 1994; Mayer and Eck, 1995). Both of these motifs were also contained in the partial cDNA isolated using the yeast two-hybrid screen. To determine which of these proline-rich sequences mediates the association of NIK with Nck, the two proline residues in each motif were mutated to alanine and binding to Nck was determined. Mutations in either proline motif markedly decreased binding of NIK to an Nck–GST fusion protein *in vitro* (Figure 4B), although the second motif was more critical for binding Nck. These findings are consistent with the idea that high-affinity binding of Nck to NIK requires the simultaneous engagement of two of Nck's SH3 domains with the two proline-rich sequences in NIK.

In addition to binding Nck *in vitro*, NIK also bound Nck in cells. 293 cells were transiently transfected with Myc-epitope-tagged NIK and the ability of NIK to associate with endogenous Nck in cells was assessed by determining whether the two proteins co-immunoprecipitate. Transfection of 293 cells with NIK led to the association of the two proteins as demonstrated by the co-immunoprecipitation of Nck with NIK using either anti-Myc or anti-Nck antibodies (Figure 4C). In summary, these findings indicate that NIK and Nck interact in cells and that the interaction is mediated via the SH3 domains of Nck. The inability of a variety of other SH3 domains to bind NIK suggests that NIK may be a specific downstream target for Nck.

NIK activates the MKK4–JNK/SAPK MAP kinase pathway

Several distinct MAP kinase modules have been identified in yeast and mammalian cells (Minden *et al.*, 1994; Sanchez *et al.*, 1994; Yan *et al.*, 1994; Derijard *et al.*, 1995; Herskowitz, 1995). Previous studies have indicated that kinases related to NIK are involved in activation of the JNK/SAPK family of MAP kinases (Bagrodia *et al.*,

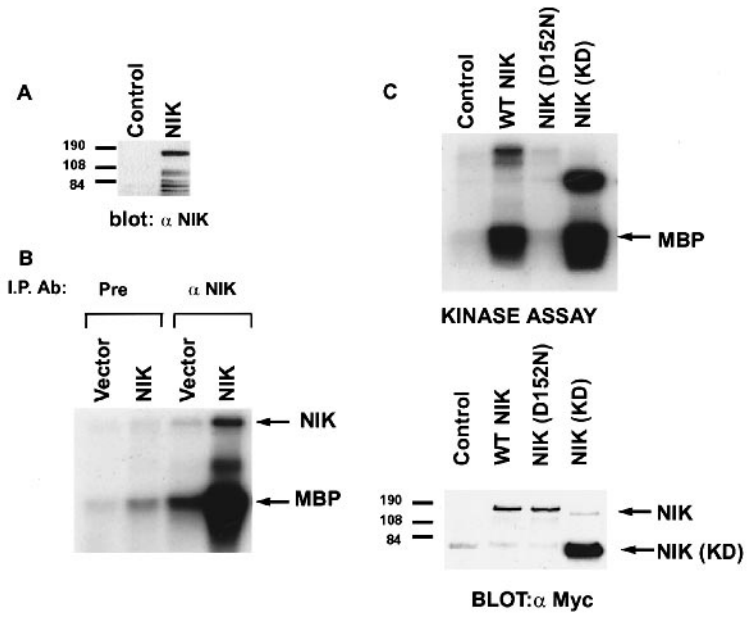

Fig. 3. Immunoblot and immune complex kinase assays of NIK in 293 cells transfected with NIK cDNA. (**A**) Lysates from 293 cells transfected with 2 μg NIK or vector control were separated by SDS–PAGE (8%) and, after transfer to nitrocellulose filters, immunoblotted with antibodies to NIK. (**B**) Lysates from 293 cells transfected as in (A) were immunoprecipitated with anti-NIK or preimmune antibodies and subjected to an *in vitro* kinase reaction using [γ-^{32}P]ATP and MBP as substrate. Reactions products were separated by SDS–PAGE (12%) and subjected to autoradiography. (**C**) Lysates from 293 cells transfected with Myc-epitope-tagged wild-type NIK, NIK(D152N) or NIK(KD) were immunoprecipitated with the anti-Myc antibody 9E10. The immunoprecipitates were subjected to an *in vitro* kinase assay as described in (A). To ensure that equal amounts of protein were immunoprecipitated between samples, one-half of the immunoprecipitate was immunoblotted with the anti-Myc antibody 9E10. NIK(KD) encodes only the kinase domain of NIK (amino acids 1–310).

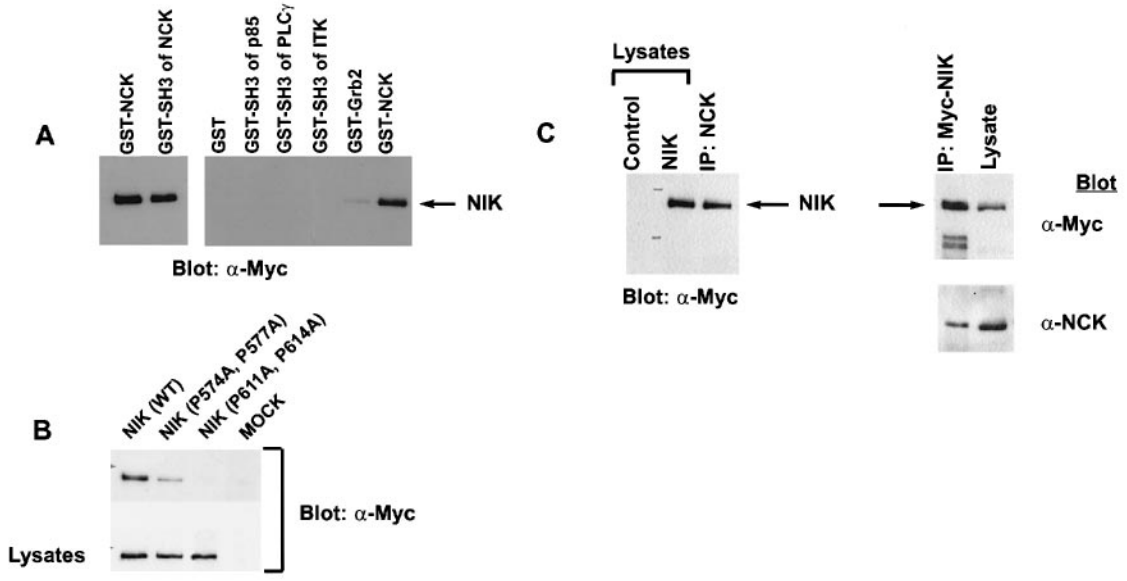

Fig. 4. Association of NIK with Nck *in vitro* and *in vivo*. (**A**) Various GST fusion proteins (as indicated) were incubated with lysates from 293 cells transfected with Myc-epitope-tagged NIK. After washing, proteins were separated by SDS–PAGE (8%), and bound NIK was visualized by immunoblotting with the anti-Myc antibody 9E10. (**B**) 293 cells were transfected with Myc-epitope-tagged wild-type NIK or NIK containing point mutations in the two sequences that match consensus SH3 binding sites in which the two conserved prolines were mutated to alanine. Binding of each construct to GST–Nck was assessed as in (A). (**C**) NIK or Nck were immunoprecipitated from 293 cells transfected with 2 μg of Myc-epitope-tagged NIK. The immunoprecipitated proteins were then separated by SDS–PAGE (8%) and immunoblotted as indicated. As a control, 25 μg of lysates from 293 cells transfected with NIK or vector control were run alongside the immunoprecipitates.

1995; Pombo *et al.*, 1995). To determine whether NIK activates this pathway, we assessed whether overexpression of NIK activates MKK4 and JNK. MKK4 is one of the kinases responsible for directly phosphorylating and activating JNK/SAPK (Sanchez *et al.*, 1994; Derijard *et al.*, 1995). 293 cells were transfected with NIK, together with an epitope-tagged SAPK or MKK4, and kinase activity was performed on SAPK and MKK4 precipitates. Overexpression of NIK led to a 3- to 4-fold activation of both MKK4 and SAPK compared with cells transfected with vector control (Figure 5A and B). This magnitude of SAPK activation by NIK was similar to the activation

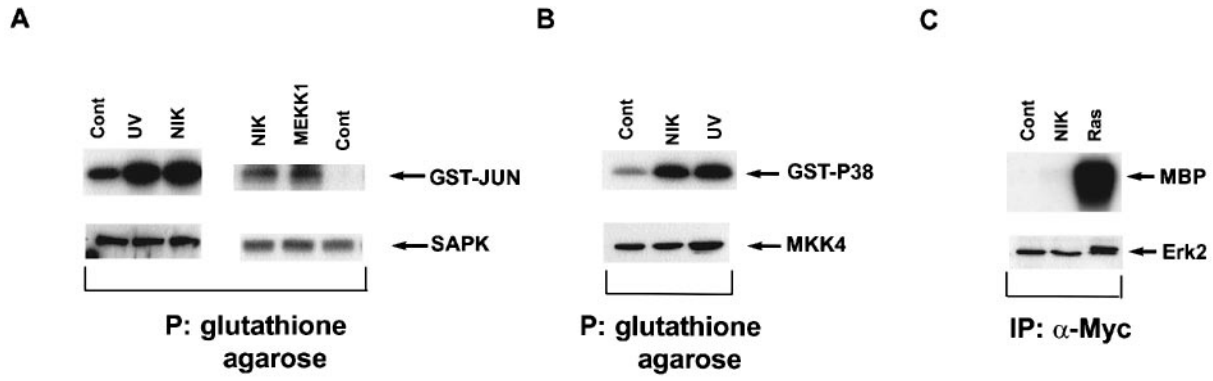

Fig. 5. Activation of JNK/SAPK and MKK4 by NIK. 1 μg of NIK was transfected into 293 cells together with: (**A**) 1 μg GST-tagged JNK/SAPK, (**B**) 1 μg of FLAG-tagged MKK4 or (**C**) 1 μg of HA-tagged ERK2. To assess activation, the various kinase were precipitated from 500 μg of cell lysates and subjected to an *in vitro* kinase reaction using GST–Jun (A), GST–p38 (B) or MBP (C) as substrates (upper panel). Reaction products were separated by SDS–PAGE and visualized by autoradiography. To ensure that equal amounts of kinases were immunoprecipitated between samples, one-half of the immunoprecipitate was immunoblotted with αGST (A and B) or αMyc (C) antibodies (lower panel).

of this pathway by two known activators of JNK, MEKK1 and UV light (Figure 5A). Although NIK activates MKK4 and SAPK, it does not activate the Ras–p42 MAP kinase pathway; while overexpression of an activated Ras led to a marked increase in p42 MAP kinase activation, overexpression of NIK did not (Figure 5C). We did not detect activation of either p38, MKK3 or MKK6 by NIK overexpression as assessed by co-transfection of epitope-tagged versions of these proteins with NIK (Han *et al.*, 1996; data not shown).

NIK activation of JNK requires the activation of MEKK1 and MKK4

To confirm that NIK signals through MKK4 to activate JNK, we determined whether overexpression of a kinase-defective MKK4(K129R) blocks NIK activation of JNK (Sanchez *et al.*, 1994; Han *et al.*, 1996). MKK4(K129R) has previously been shown to function as a dominant-negative molecule to inhibit JNK activation (Sanchez *et al.*, 1994). In support of the idea that NIK signals through MKK4 to activate JNK, co-expression of MKK4(K129R) together with NIK and epitope-tagged JNK inhibited JNK activation by NIK (Figure 6A).

MEKK1 is a MAP kinase kinase kinase that has been shown to activate MKK4. ΔMEKK1 containing an arginine for lysine substitution in the ATP binding domain [ΔMEKK1(K-R)] is kinase-defective and functions as a dominant-negative molecule to inhibit endogenous MEKK1 (Yan *et al.*, 1994). Co-expression of a ΔMEKK1(K-R) together with NIK inhibited the activation of JNK by NIK (Figure 6B). ΔMEKK1(K-R) also inhibited the activation of JNK by UV light (data not shown). These findings raise the possibility that NIK functions upstream of MEKK1 to activate JNK. However, we cannot rule out the possibility that ΔMEKK1(K-R) is functioning as a dominant-negative by binding and inactivating MKK4; under this scenario, inhibition by ΔMEKK1(K-R) would only confirm that NIK signals through MKK4 to activate JNK.

MEKK1 and NIK associate in vivo

To begin to address whether NIK may directly regulate MEKK1 we determined whether MEKK1 and NIK co-immunoprecipitate. 293 cells were transiently transfected

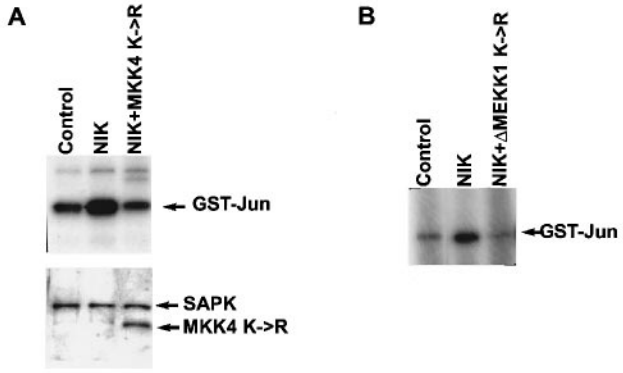

Fig. 6. Dominant-negative MKK4 (**A**) and ΔMEKK1 (**B**) block activation of JNK/SAPK by NIK. 293 cells were transfected as in Figure 5A, with the exception that either kinase dead MKK4(K→R) or ΔMEKK1(K→R) were included in the transfections as indicated. Activation of GST-tagged JNK/SAPK and immunoblotting were assessed as described in Figure 5.

with HA-tagged full-length MEKK1 (Xu *et al.*, 1996) together with Myc-epitope-tagged NIK and the ability of NIK and MEKK1 to associate was determined by assessing whether the two proteins co-immunoprecipitate. We found that MEKK1 co-immunoprecipitated with NIK as demonstrated by the co-immunoprecipitation of HA-epitope-tagged MEKK1 with anti-myc antibodies (Figure 7A). Moreover, the association of MEKK1 with NIK was mediated through the interaction of the N-terminus of MEKK1 with the C-terminus of NIK. The N-terminal 719 amino acids of MEKK1 and the C-terminal regulatory domain (Figure 1C) of NIK were sufficient to mediate the association of the two proteins (Figure 7A). These interactions were specific; we did not detect association of NIK with the C-terminal 320 amino acids of MEKK1 (ΔMEKK1) (Figure 7B; Yan *et al.*, 1994).

Both NIK's kinase activity and C-terminus are required for full activation of the JNK/SAPK pathway

As discussed above, we found that the C-terminal domain of NIK and GCK are 22% identical over 330. Expression of the regulatory region of GCK without the kinase domain activates SAPK (Pombo *et al.*, 1995). Therefore, we were

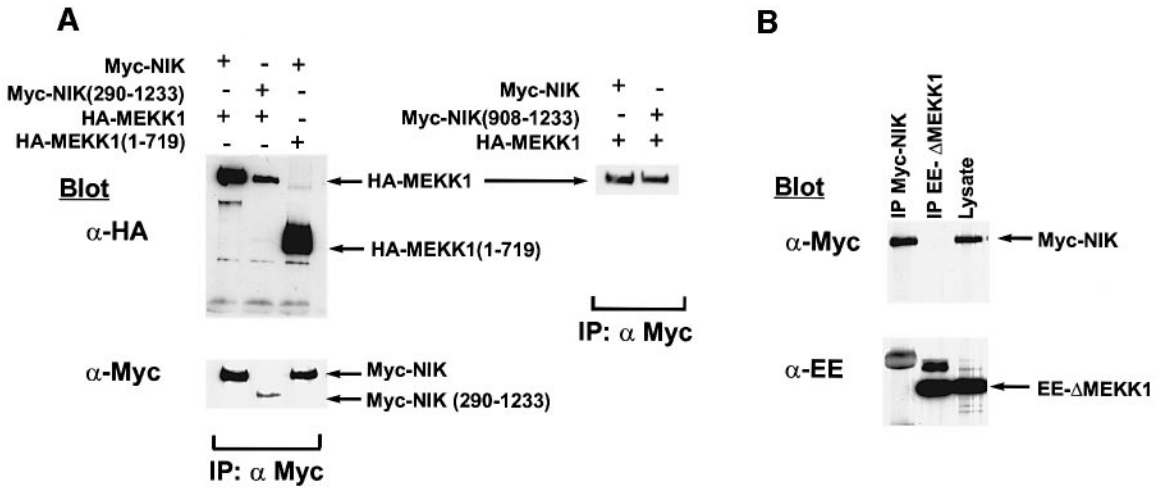

Fig. 7. Association of NIK and MEKK1 in 293 cells transfected with NIK and MEKK1. (**A**) 293 cells were transfected with Myc-epitope-tagged full-length or truncated versions of NIK together with HA-tagged full-length or truncated versions of MEKK1. To assess whether MEKK1 and NIK associate in cells, NIK was immunoprecipitated from transfected cell lysates using the anti-myc antibody 9E10. The washed immunoprecipitates were separated by SDS–PAGE and associated MEKK1 was assessed by immunoblotting with antibodies to HA. (**B**) As a control, an EE-tagged MEKK1 containing only the C-terminal domain of MEKK1 (ΔMEKK1) was co-transfected with NIK. The ability of NIK and EE-tagged ΔMEKK1 to co-immunoprecipitate was assessed as described in (A), with the exception that ΔMEKK1 was detected using a monoclonal antibody to the EE epitope tag (Babco).

interested in determining whether the C-terminus of NIK and GCK function similarly to activate the SAPK pathway. In addition, we wanted to determine whether NIK required a functional kinase and C-terminus to activate this pathway. To address this, several truncated NIK proteins as well as a kinase-defective NIK [NIK(D152N)] were expressed in 293 cells and JNK activation was determined. In agreement with the results obtained with GCK, expression of the C-terminal domain of NIK alone stimulated a small increase in SAPK activity when compared with full-length NIK (Figure 8A). However, full activation of SAPK by NIK required both NIK's kinase activity and C-terminus; while overexpression of NIK resulted in a ~5-fold increase in SAPK activity, expression of the kinase domain alone (data not shown) or NIK(D152N) consistently resulted in only a 2- to 2.5-fold increase in SAPK activity.

We were able to confirm these results by examining whether these same NIK constructs activate an ATF2-stimulated luciferase reporter gene (Figure 8C); SAPK has been shown to phosphorylate and activate several transcription factors including ATF2 (Gupta *et al.*, 1995). NIK or the various NIK constructs described above were co-transfected into 293 cells together with a plasmid expressing a fusion protein consisting of the activation domain of ATF2 and the DNA binding domain of GAL4 and a plasmid expressing a GAL4 luciferase reporter (Gupta *et al.*, 1995). Overexpression of NIK led to a ~20-fold increase in the transcriptional activity of ATF2 (Figure 8B). In agreement with the results presented above, overexpression of the NIK C-terminus, the kinase domain or NIK(D152N) also consistently stimulated ATF2 transcriptional activity, although the increase in luciferase activity was 5- to 10-fold less than that obtained with wild-type NIK. The increase in ATF2 transcriptional activity was dependent upon phosphorylation of ATF2; increased ATF2 transcriptional activity by NIK was markedly decreased when threonine at position 71 was mutated to alanine (Gupta *et al.*, 1995). These findings indicate that both NIK's kinase activity and C-terminal domain are required for NIK to fully activate the SAPK pathway in cells. The finding that the C-terminal domain of NIK by itself is capable of activating SAPK and ATF2 reporter activity, together with the finding that a truncated NIK protein lacking the C-terminus fails to fully activate this pathway, suggests that the C-terminal domain of NIK plays an important regulatory role, possibly via its interaction with MEKK1 or related molecules.

Discussion

We have identified a novel serine/threonine kinase, NIK, that interacts with the SH3 domains of Nck. Overexpression of NIK constitutively activated the JNK/SAPK pathway. NIK interacts with MEKK1 in cells and likely signals through MEKK1 to activate JNK, suggesting that NIK directly regulates MEKK1 activity. We found that NIK contains a regulatory domain in its C-terminus that is conserved in two other members of this kinase family. This domain mediates the association of NIK with MEKK1 and is critical for NIK activation of the SAPK pathway, suggesting that the C-terminal domain of these proteins encodes a new protein domain family that couples these kinases to the SAPK pathway, possibly by interacting with MEKK1. Our finding that NIK also interacts with Nck suggests that SH2/SH3 adaptor proteins couple NIK and related kinases to activation of the SAPK/JNK pathway by different receptors.

Studies in *Saccharomyces cerevisiae* have shown that a serine/threonine kinase, Ste20, acts upstream of the *S.cerevisiae* MEKK Ste11 and downstream of the pheromone hormone receptor (Herskowitz, 1995). Two families of protein kinases that are closely related to Ste20 in the kinase domain have been identified in mammalian cells. The first family of mammalian Ste20-related kinases are activated by binding GTP-bound Cdc42 and Rac and, based on this characteristic, have been termed PAKs for

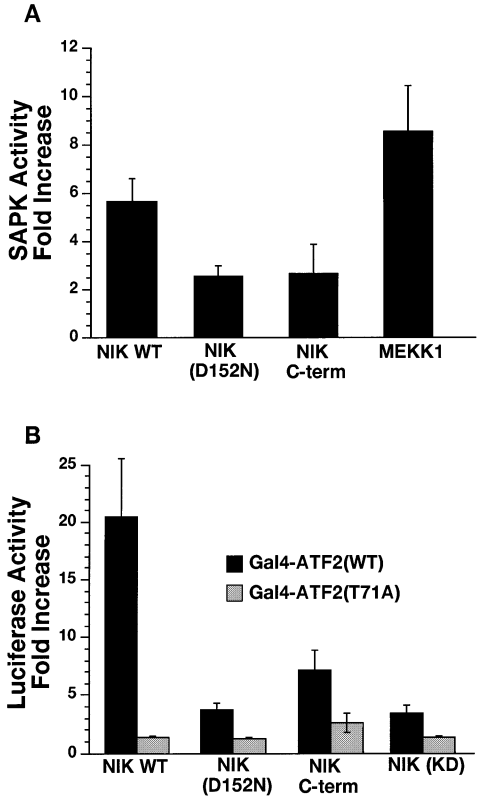

Fig. 8. NIK's C-terminal domain and kinase activity are required for full activation of the SAPK pathway. (**A**) To assess SAPK activation by the various NIK constructs, lysates from 293 cells transfected with 5 μg of the indicated plasmid were incubated with 10 μg of GST–JUN coupled to glutathione–agarose beads. After washing, SAPK activation was determined by incubating beads in kinase buffer containing [γ-^{32}P]ATP for 20 min. The reaction was stopped by boiling in sample buffer, and the reaction products were separated by SDS–PAGE (12%) and phosphorylated GST–JUN was quantitated using the phosphorimager. Mean ± SD for triplicate determinations are shown. (**B**) 0.1 μg of the various NIK constructs were co-transfected into 293 cells together with a 10 ng of a plasmid expressing a fusion protein consisting of ATF2 and the GAL4 DNA binding domain and 5 μg of a plasmid expressing a GAL4 luciferase reporter. Transfection efficiency was assessed by co-transfecting 1 μg of a plasmid expressing β-galactosidase under the control of an SV40 promoter. Mean ± SD from three experiments performed in duplicate are shown. Luciferase activity is expressed in arbitrary units after being standardized to β-galactosidase activity.

p21 Activated Kinases (Manser *et al.*, 1994; Bagrodia *et al.*, 1995; Martin *et al.*, 1995). The second family of Ste20 homologues, which includes GCK and MST1, lack Cdc42 and Rac binding domains (Katz *et al.*, 1994; Creasy and Chernoff, 1995; Pombo *et al.*, 1995). In addition, in contrast to PAKs which contain an N-terminal regulatory and a C-terminal kinase domain, members of this second family contain an N-terminal kinase domain and C-terminal regulatory region. NIK fits into this second family of Ste20 homologues. Within this family, NIK is most similar functionally and structurally to GCK and HPK1 (Pombo *et al.*, 1995). First, these kinases are constitutively active when transiently overexpressed in cells, and overexpression of all three kinases results in activation of the MKK4–SAPK MAP kinase pathway. Second, these kinases share a conserved C-terminal regulatory domain (Figure 1C).

While the upstream signals that regulate MAP kinase cascades are diverse, each module is activated by sequential phosphorylation of highly conserved components (Davis, 1994; Cobb and Goldsmith, 1995; Herskowitz, 1995). The MAP kinase modules are composed of three protein kinases that act sequentially to stimulate a kinase cascade and are composed of a MAP kinase, an enzyme that activates MAP kinase (known as a MAP kinase kinase or MEK) and a kinase that activates MEK (known as MAP kinase kinase kinase or MEKK). At least six distinct MAP kinase modules have been identified in yeast and three distinct MAP kinase modules have been found in mammalian cells (Herskowitz, 1995). While this system allows for crosstalk between the different pathways, for the most part these pathways function independently of one another in both yeast and mammals. The finding that NIK activates the JNK/SAPK MAP kinase pathway, but fails to activate the ERK MAP kinase cascade, indicates that NIK also maintains specificity by activating only one of the several MAP kinase cascades.

The mechanism whereby specificity is imparted to different MAP kinase pathways is not known. It has been proposed that scaffolding proteins impart specificity and regulate the activation of only a single MAP kinase cascade by binding and therefore juxtaposing all members of a particular cascade. For example, the yeast protein STE5 functions as a scaffolding protein in *S.cerevisiae* and binds STE20, STE11 and STE7 via non-overlapping regions (Choi *et al.*, 1994; Marcus *et al.*, 1994). NIK may selectively activate a single MAP kinase module either by functioning directly as a scaffolding protein or by interacting with a scaffolding protein to juxtapose NIK specifically with members of the MEKK1–MKK4 pathway. Alternatively, by regulating MEKK1 specifically, NIK may activate a single pathway via MEKK1. This possibility is supported by studies showing that overexpression of Raf or MEKK1 activates distinct MAP kinase modules; overexpression of MEKK1 predominantly activates JNK, whereas overexpression of Raf predominantly activates ERK1 and ERK2 (Minden *et al.*, 1994).

While MEKK1 has been shown to function upstream of MKK4 (also known as SEK1 and JNKK) (Minden *et al.*, 1994; Yan *et al.*, 1994), regulatory elements upstream of MEKK1 have been poorly characterized. In some instances, Ras has been shown to function upstream of MEKK1 in mammalian cells (Lange-Carter and Johnson, 1994). However, it is clear that MEKK1 is also regulated by pathways that do not involve Ras. Based upon studies in yeast, it has been proposed that a mammalian homologue of Ste20/PAK regulates MEKK1 in mammalian cells (Herskowitz, 1995). However, direct evidence for a PAK family member activating an MEKK in mammalian cells has not yet been published. While we have been unable to prove definitively that NIK directly regulates MEKK1, our finding that NIK and MEKK1 co-immunoprecipitate, coupled with the finding that dominant-negative MEKK1 blocks JNK/SAPK activation by NIK, suggests that NIK directly regulates MEKK1 activity *in vivo*. Therefore, NIK is a good candidate for upstream regulation of MEKK1 by a PAK/Ste20 family member.

Since the kinase domain of MEKK1 is constitutively active when isolated from cells transiently transfected with the full-length MEKK1 cDNA, we have been unable to assess whether NIK activates MEKK1′s kinase activity.

In contrast to previous studies that overexpressed truncated versions of MEKK1, we have overexpressed the full-length MEKK1 clone (Minden *et al.*, 1994; Yan *et al.*, 1994; Xu *et al.*, 1996). Thus, it is unlikely that the MEKK1 clone we used is constitutively active based upon the lack of a critical negative regulatory domain. A previous report has suggested that the endogenous MEKK1 is inactive (Lange-Carter and Johnson, 1994). It is possible that under physiological levels of expression, MEKK1 is kept inactive by interacting with a negative regulator. Overexpression of MEKK1 may titrate out a negative regulator and allow MEKK1 activation. The finding that NIK binds MEKK1 raises the possibility that overexpression of NIK activates MEKK1 by displacing this putative negative regulator from MEKK1. Alternatively, although not mutually exclusive, phosphorylation of MEKK1 by NIK, possibly facilitated by the interaction of the C-terminus of NIK with MEKK1, may induce a conformational change that contributes to MEKK1 activation; this would account for our finding that NIK's kinase activity is required for full activation of the SAPK pathway.

The C-terminus of NIK is highly conserved between mouse NIK, its *C.elegans* homologue (contained in cosmid Zc504) and two related kinases, GCK and HPK1 (Pombo *et al.*, 1995; Hu *et al.*, 1996). These findings, coupled with the demonstration that this domain mediates the association of NIK with MEKK1, suggest that the C-terminal regions in these proteins may encode a new protein domain family that mediates protein–protein interaction. Moreover, the finding that overexpression of the C-terminus of NIK by itself activates the SAPK pathway, together with the finding that a truncated NIK lacking its C-terminus fails to fully activate this pathway, is consistent with the idea that this domain couples NIK to the SAPK pathway *in vivo*, possibly via the interaction with MEKK1. NIK's kinase activity, however, is required for maximal stimulation; activation of JNK/SAPK signaling by overexpressing the C-terminal domain or a kinase-defective NIK was less than that observed for the wild-type NIK. Only one previous study has demonstrated interaction of MEKK1 with a Ste20 family member, HPK1 (Hu *et al.*, 1996). However, in contrast to the association of NIK with MEKK1, the kinase domain of HPK1 bound the kinase domain of MEKK1. Although HPK1 contains a domain in its C-terminus that is homologous to the C-terminus of NIK (Figure 1C), these investigators were unable to demonstrate that HPK1's C-terminus either activates SAPK or associates with MEKK1; however, they only tested binding of HPK1 to a truncated MEKK1 lacking the N-terminal residues that we identified as being critical for associating with NIK. It is intriguing that, of the kinases related to NIK, only those containing a domain that is homologous to the C-terminus of NIK, HPK1 and GCK, activate the SAPK pathway when overexpressed (Creasy and Chernoff, 1995; Pombo *et al.*, 1995; Hu *et al.*, 1996). For example, while SOK1 is the kinase most related to NIK in its kinase domain, SOK1 lacks a similar C-terminal regulatory domain and also does not activate SAPK (Pombo *et al.*, 1996). Many different PAKs and MEKKs have now been identified (Lange-Carter and Johnson, 1994; Blank *et al.*, 1996). Based on these findings, we suggest that specific PAK family members regulate specific MEKKs, and that this regulation is mediated by direct protein–protein interaction.

The role of NIK binding to Nck in NIK regulation and NIK activation of the JNK/SAPK pathway is most intriguing. One possibility is that subcellular targeting of NIK by Nck in growth factor-stimulated cells localizes NIK adjacent to MEKK1, thereby enabling the two proteins to interact. The finding that MEKK1 is found predominantly in the particulate fraction (Xu *et al.*, 1996) suggests that binding of the SH2 domains of Nck to autophosphorylated receptors, or to other tyrosine-phosphorylated proteins in growth factor-stimulated cells, localizes NIK to a similar subcellular compartment as MEKK1. In support of this idea, NIK is recruited to the platelet-derived growth factor receptor (PDGFR) in cells transiently transfected with epitope-tagged NIK (data not shown). Thus far, we have been unable to assess subcellular localization of endogenous NIK or whether external factors regulate NIK's kinase activity due to the fact that endogenous NIK protein is present in low abundance.

Several lines of evidence now place Nck as an important adaptor molecule coupling receptors to reorganization of the cytoskeleton. The finding that Nck binds WASP, a protein responsible for mediating the effect of CDC42Hs on reorganization of the actin cytoskeleton, together with the finding that a *Drosophila* protein related to Nck, DOCK, also couples receptors to cytoskeletal changes, raises the possibility that NIK participates in regulating this pathway (Rivero-Lezcano *et al.*, 1995; Aspenstrom *et al.*, 1996; Garrity *et al.*, 1996; Symons *et al.*, 1996). The potential ability of Nck's three SH3 domains simultaneously to bind several different effector molecules raises the possibility that crosstalk among the different molecules bound to Nck's SH3 domains determines biological response. Future studies will focus on the upstream signals that regulate NIK, how NIK regulates the JNK/SAPK pathway, and on determining whether NIK is involved in mediating cytoskeletal changes by Nck.

Materials and methods

Yeast two-hybrid assay

Full-length Nck was amplified by PCR and expressed as a fusion with the LexA DNA binding domain using the vector BTM116 (LexA–Nck) (Voyjtek *et al.*, 1993; Isakoff *et al.*, 1996). The cDNA library used to screen for interactions was made from TA1 cells that had been partially differentiated into adipocytes. The cDNA library was cloned into the vector, pVP16, and expressed as a fusion protein with the activation domain of VP16 (pVP16-TA1) (Voyjtek *et al.*, 1993; Isakoff *et al.*, 1996). To identify novel proteins that interact with Nck, L40 yeast cells stably expressing LexA–Nck were transformed with pV16-TA1. cDNA clones that interacted with Nck were identified by selecting for growth on medium lacking histidine in the presence of 3 mM 3-aminotriazole. Yeast transformations and routine care of yeast were as described previously (Guthrie and Fink, 1992; Voyjtek *et al.*, 1993).

Isolation of full-length cDNA and sequencing

The clone identified in the yeast two-hybrid system contained a region that interacted with Nck but did not contain the 3′ or 5′ ends of the gene. To obtain the full-length cDNA, the clone identified in the yeast two-hybrid system was labeled using random primers and [α-^{32}P]dCTP and used to screen a λ-Zap 70 murine brain cDNA library (Stratagene) as previously described (Skolnik *et al.*, 1991). Positive clones were plaque-purified and the excised cDNAs were sequenced using an automated sequencer (Applied Biosystems). While the initial screening identified clones containing the 3′ end of the gene, it did not identify the 5′ end. To obtain more 5′ sequence information, the 5′-most 250

nucleotides of the largest clone (cl. 16) was amplified by PCR and, after labeling with [α-^{32}P]dCTP, used to screen a λ zap 70 murine embyro cDNA library (Stratagene). Positive clones were analyzed as described above.

Northern blot analysis

An adult tissue Northern blot (Clontech) was hybridized with the [α-^{32}P]dCTP-labeled clone identified in the yeast two-hybrid system (corresponding to amino acids 443–618 of the full-length clone) according to the manufacturer's specifications.

Constructs, epitope tagging, mutagenesis and transient cell expression

All NIK constructs were expressed in the cytomegalovirus-based expression vector pRK5 (Li *et al.*, 1992). The NIK kinase domain (amino acids 1–310) and C-terminus (amino acids 908–1233) of NIK were obtained by PCR using full-length NIK as a template. Both constructs were Myc-epitope-tagged at their C-terminus as described below and a Kozak sequence was incorporated into the 5′ oligonucleotide of the C-terminal construct to enable expression in mammalian cells (Kozak *et al.*, 1989). GST-tagged JNK/SAPK and ΔMKK4(K-R) were expressed using the vector pEBG (Sanchez *et al.*, 1994; Yan *et al.*, 1994). Hemagglutinin (HA)-tagged MKK4 was expressed using the vector pCDNA3 (Han *et al.*, 1996). The C-terminal 320 amino acids of EE-epitope-tagged ΔMEKK1 and ΔMEKK1(K-R) were expressed in the vector pRK5 (Yan *et al.*, 1994). Dominant-negative MKK4 and dominant-negative ΔMEKK1 contained a K→R mutation in the ATP binding site of the kinase domain (Yan *et al.*, 1994; Han *et al.*, 1996). Full-length HA-tagged MEKK1 and MEKK1(1–719) have been described previously (Xu *et al.*, 1996). The GAL4-ATF2 and GAL4-ATF2(T71A) are in the vector pSG424 as described previously (Sadowski and Ptashne, 1989)

NIK was Myc-epitope-tagged (DQKLISEEDL) at the C-terminus using PCR and cloned into pRK5. Briefly, an oligonucleotide was synthesized containing the 3′ end of NIK followed by the Myc epitope sequence, a stop codon and a convenient restriction site. This 3′ oligonucleotide was used in a PCR reaction to generate a C-terminal fragment of NIK which was then used to replace an *Xba*I–*Hin*dIII fragment in the 3′ end of full-length NIK.

For site-directed mutagenesis, NIK was subcloned into the vector pALTER (Promega), and mutagenesis was performed according to the manufacturer's specification. To facilitate screening of mutants, novel restriction sites were incorporated into the oligonucleotides used for mutagenesis, and mutant NIK constructs were identified by restriction digest. The NIK mutants were subcloned back into pRK5 for expression in 293 cells. The following oligonucleotides were used:

NIK(D152N)	5′-CGTTATTCACCGAAATATTAAGGGCCA-AAAT
NIK(P574A,P577A)	5′-CCGCTCTCAGGATGCATGTCCAGCTTC-CCGCAGT
NIK(P611A,P614A)	5′-GACGAGGTGCCTGCAAGGGTTGCCGT-GAGAACGA

293 cells were transfected with $Ca_2(PO_4)_3$ as previously described (Chen and Okiyama, 1987).

GST fusions and in vitro binding studies

Oligonucleotides flanking the regions of interest and containing appropriate restriction sites were synthesized and PCR was used to amplify the DNA fragments. The products obtained from PCR were sublconed into pGEX3X and the various GST fusions were isolated as described previously (Lee *et al.*, 1993). The following fusion proteins were produced: full-length Nck, the three SH3 domains of Nck (amino acids 2–257), the SH3 domain of p85, ITK (amino acids 185–231) and PLCγ, full-length Grb2 and full-length p38 (Skolnik *et al.*, 1991, 1993; Lee *et al.*, 1993; Han *et al.*, 1994).

To assay binding of NIK to GST fusion proteins, myc-tagged NIK were overexpressed in 293 cells. 500 μg of lysates containing myc-tagged NIK were incubated for 90 min with 4 μg of the various GST fusion proteins coupled to glutathione–agarose beads. After washing the beads four times with lysis buffer (Skolnik *et al.*, 1993), proteins were separated by SDS–PAGE (8%) and immunoblotted with the anti-myc antibody 9E10 (Evan *et al.*, 1985). Binding of NIK mutants containing alanine for proline substitutions in putative SH3 binding motifs to GST–Nck were performed as for wild-type NIK.

Cell lysis, immunoprecipitation and immunoblotting

Cell lysis, immunoprecipitation and immunoblotting were as previously described by Skolnik *et al.* (1993).

Antibodies

The anti-NIK antibody is a rabbit polyclonal antibody raised against a GST fusion protein corresponding to amino acids 443–619 of full-length NIK. Antibodies against the FLAG-epitope (M2) and against the HA-epitope (12CA5) were purchased from IBI (Kodak) and Boehringer Mannheim respectively. The antibody 9E10 was used for immunoprecipitation and immunoblotting the Myc-tagged constructs (Evan *et al.*, 1985).

Kinase assays

To assay for NIK kinase activity, 293 cells were transfected with NIK. After 48 h, cells were lysed and NIK was immunoprecipitated using either the anti-myc antibody 9E10 or the anti-NIK antibody described above. The immune complex was then washed three times with lysis buffer and twice with kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 10 mM NaF, 0.2 mM orthovanadate and 1 mM dithiothreitol). After a 30 min incubation at 30°C in kinase buffer containing 10 μCi [γ-^{32}P]ATP and 5 μg MBP, the kinase reaction was terminated by adding an equal volume of 2× sample buffer and the reaction products were separated by SDS–PAGE (12.5%).

Immune complex kinase reactions using JNK/SAPK or MKK4 were performed as described previously (Han *et al.*, 1996). Briefly, epitope-tagged JNK/SAPK or MKK4 were co-transfected into 293 cells with NIK or with vector control. At 24 h after transfection, cells were serum-starved overnight in DMEM containing 0.2% FBS, lysed and precipitated using glutathione–agarose. An *in vitro* kinase assay was then performed on the precipitates as described above. GST-c-jun (containing amino acids 1–223) coupled to glutathione–agarose was used as a substrate for JNK/SAPK and GST-p38 coupled to glutathione–agarose was used as a substrate for MKK4 (Kallunki *et al.*, 1994; Minden *et al.*, 1994; Han *et al.*, 1996).

SAPK kinase activity using GST–c-Jun (containing amino acids 1–223 of c-Jun) coupled to glutathione–agarose beads was determined by incubating 500 μg of lysates from 293 cells transfected with the various cDNAs with 10 μg of GST–c-Jun for 2 h at 4°C. After washing the beads three times with lysis buffer and twice with kinase buffer, the kinase reaction was started by incubating beads in 25 μl kinase buffer containing 20 μM non-radiolabelled ATP and [γ-^{32}P]ATP (15 μCi per sample). After incubation at 30°C for 20 min, the reaction was terminated by boiling the mixtures in sample buffer for 5 min and the reaction products were separated by SDS–PAGE (12%).

Reporter assays

A fusion protein consisting of ATF2 (amino acids 1–505) and the GAL4 DNA binding domain was expressed in 293 cells either alone or together with NIK (Gupta *et al.*, 1995). Transcriptional activation of ATF2 was measured by co-transfecting a luciferase reporter plasmid containing 5× GAL4 DNA binding domains. All transfections were standardized by co-transfecting a control plasmid expressing β-galactosidase (Promega).

Accession number

The NIK sequence has been submitted to the DDBJ/EMBL/GenBank; accession number U88984.

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