The MAP kinase kinase kinase MLK2 co-localizes with activated JNK along microtubules and associates with kinesin superfamily motor KIF3

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The MLK (mixed lineage) ser/thr kinases are most closely related to the MAP kinase kinase kinase family. In addition to a kinase domain, MLK1, MLK2 and MLK3 each contain an SH3 domain, a leucine zipper domain and a potential Rac/Cdc42 GTPase-binding (CRIB) motif. The C-terminal regions of the proteins are essentially unrelated. Using yeast two-hybrid analysis and *in vitro* dot-blots, we show that MLK2 and MLK3 interact with the activated (GTP-bound) forms of Rac and Cdc42, with a slight preference for Rac. Transfection of MLK2 into COS cells leads to strong and constitutive activation of the JNK (c-Jun N-terminal kinase) MAP kinase cascade, but also to activation of ERK (extracellular signal-regulated kinase) and p38. When expressed in fibroblasts, MLK2 co-localizes with active, dually phosphorylated JNK1/2 to punctate structures along microtubules. In an attempt to identify proteins that affect the activity and localization of MLK2, we have screened a yeast two-hybrid cDNA library. MLK2 and MLK3 interact with members of the KIF3 family of kinesin superfamily motor proteins and with KAP3A, the putative targeting component of KIF3 motor complexes, suggesting a potential link between stress activation and motor protein function.

Keywords: JNK/kinesin superfamily motor KIF3/
microtubules/mixed lineage kinase MLK2

Introduction

Activation of MAP kinases following ligand binding to cell surface receptors has been correlated with numerous cellular responses such as cell growth, differentiation and the regulation of metabolic pathways (reviewed in Marshall, 1995; Waskiewicz and Cooper, 1995; Kyriakis and Avruch, 1996; Robinson and Cobb, 1997). To date, three MAP kinase cascades have been well characterized in mammalian cells; ERK (extracellular signal-regulated

kinase), JNK (c-Jun N-terminal kinase or stress-activated protein kinase, SAPK) and p38 (also known as cytokine-suppressive anti-inflammatory drug-binding protein, CSBP, or reactivating kinase, RK). Activation of these MAP kinases (MAPKs) occurs through a cascade of upstream kinases; a MAP kinase kinase kinase (MAPKKK) first phosphorylates a dual-specificity protein kinase (MAPKK), which in turn phosphorylates the MAPK. In the ERK cascade, c-Raf, MEK and ERK correspond to MAPKKK, MAPKK and MAP kinase, respectively. c-Raf activation, in response to growth factor stimulation of cells, is regulated by the Ras GTPase which induces translocation of c-Raf to the plasma membrane, where it is thought to be activated (Leervers *et al.*, 1994; Stokoe *et al.*, 1994; Marais *et al.*, 1995).

JNK and p38 are strongly activated in cells exposed to extracellular stress such as UV irradiation or osmotic shock (Galcheva-Gargova *et al.*, 1994) and by proinflammatory cytokines such as tumour necrosis factor α (TNF α) and interleukin-1 (IL-1) (Kyriakis and Avruch, 1996). Accumulating evidence suggests that these MAP kinase cascades are regulated by small GTPases of the Rho family (Coso *et al.*, 1995; Minden *et al.*, 1995; Olson *et al.*, 1995). In contrast to Ras in the ERK cascade, however, the downstream targets of these GTPases and the nature of the intervening kinase cascades are far from clear. Numerous MAPKKK-like proteins have been identified that can activate the JNK and p38 pathways, including ASK1 (Ichijo *et al.*, 1997), Tpl-2 (Salmeron *et al.*, 1996), DLK (also known as MUK, Hirai *et al.*, 1996), TAK1 (Moriguchi *et al.*, 1996) and MEKK1, 2, 3 and 4 (Fanger *et al.*, 1997; Gerwins *et al.*, 1997). MEKK1 and MEKK4 can both interact directly with Cdc42 and Rac, while another Cdc42/Rac target, p65^{PAK}, belongs to the family of MAP kinase kinase kinase kinases (MAPKKKK) and has also been reported to participate in JNK pathways (Manser *et al.*, 1994; Bagrodia *et al.*, 1995; Zhang *et al.*, 1995; Brown *et al.*, 1996; Joneson *et al.*, 1996; Lamarche *et al.*, 1996). Other possible mediators of JNK activation by Rac and Cdc42 are members of the mixed lineage kinase (MLK) family (Burbelo *et al.*, 1995). The three known MLKs (MLK1, MLK2 and MLK3) each contain a potential binding site (CRIB motif) for Cdc42 and Rac, and their kinase domains most closely resemble MAPKKKKs. It was reported recently that MLK3 activates both the JNK and p38 MAP kinase pathways when transfected into cells (Rana *et al.*, 1996; Teramoto *et al.*, 1996; Tibbles *et al.*, 1996).

The three MLK kinases show ~70–75% identity in their kinase domain and in their SH3 and leucine zipper domains (Katoh *et al.*, 1995), suggesting strong functional conservation. Despite this, the C-terminal regions of the proteins are highly divergent. We show here that MLK2 and MLK3 interact strongly with Cdc42 and Rac in a

Fig. 1. Structure of MLKs. (A) Structure of MLK1, 2 and 3. The amino acid sequence identity between MLK1, MLK2 and MLK3 is shown. Structural domains of the proteins are abbreviated as follows: SH3, Src homology 3; Leu Z, leucine zipper; CRIB, Cdc42/Rac interactive binding motif. (B) Description of the MLK2 cDNA fragments constructed into expression vectors.

GTP-dependent manner. Transfection of MLK2 into COS cells leads to strong and constitutive activation of the JNK pathway, as well as activation of ERK and p38 MAP kinases, but this is unaffected by co-transfection with either constitutively active or dominant-negative Rac or Cdc42. MLK2 co-localizes with dually phosphorylated JNK to punctate structures associated with microtubules. We have identified several cellular proteins that interact with MLK2, including two members of the KIF3 family of microtubule motor proteins (Hirokawa, 1996) and KAP3, the putative targeting component of the KIF3 motor complex (Yamazaki *et al.*, 1996).

Results

MLKs are targets for Cdc42 and Rac

It has been reported previously that MLK3 contains a potential site (CRIB motif) of interaction for Cdc42 and Rac (Burbelo *et al.*, 1995). To determine whether MLKs interact with Cdc42 or Rac and to compare their interaction with that of other known GTPase targets, we first made use of a yeast two-hybrid analysis. The C-terminal region of MLK2 (amino acids 338–953) or MLK3 (amino acids 348–847) (see Figure 1) fused to a GAL4 activation domain was introduced into yeast strains containing various GTPases fused to the GAL DNA-binding domain. It can be seen in Figure 2A that MLK2 and MLK3 interact strongly with the GTP forms of both Cdc42 and Rac (L61 versions), but not with the GDP forms (N17 versions). Neither MLK interacts with Rho.

Fig. 2. Interaction of Rac and Cdc42 with MLK2 and MLK3. (A) The interaction of GAL4AD–MLK2 (amino acids 338–953) or GAL4AD–MLK3 (amino acids 348–847) fusion proteins with GAL4DB–GTPase constructs in yeast. Four independent colonies from co-transformants were patched on medium lacking histidine, tryptophan and leucine, but with 3-aminotriazole, and analysed for growth. (B) Interaction of MLK2 with Rac or Cdc42 in dot-blot analysis. GST protein (20 µg), GST–p65^{PAK} (10 µg) and GST–MLK2 (Leu/CRIB/Div) (10 µg) were spotted onto a nitrocellulose filter in a volume of 10 µl and incubated with 0.5 µg of [γ -³²P]GTP-loaded L61Rac or L61Cdc42. GTPase interactions were visualized by autoradiography.

To confirm this interaction *in vitro*, we have used a dot-blot assay. Figure 2B shows that the MLK2-Leu/CRIB/Div (amino acids 338–953, lacking the kinase domain) interacts strongly with the GTP-bound form of Rac and Cdc42, with a slight preference for Rac. p65^{PAK}, on the other hand, shows a slight preference for Cdc42.

Activation of MAP kinase pathways by MLK2

Rac and Cdc42 activate both the JNK and the p38 MAP kinase pathways in transfected cells (Bagrodia *et al.*, 1995; Coso *et al.*, 1995; Minden *et al.*, 1995; Olson *et al.*, 1995; Zhang *et al.*, 1995). To determine whether MLK2 can activate MAP kinase cascades, MLK2 in the pRK5 vector was co-transfected into COS cells with either ERK, JNK or p38 constructs. The three MAP kinases were immunoprecipitated and their kinase activities determined in an *in vitro* assay. As shown in Figure 3A, MLK2 strongly activates JNK and the activation is comparable with that induced by MEKK. As with MEKK, activation of JNK by MLK2 is constitutive in transfected cells. Using various amounts of MLK2 DNA, no significant inhibitory or stimulatory effect of co-transfecting with

Fig. 3. Activation of JNK, ERK and p38 MAP kinase cascades by MLK2. (A) MLK2 activates the JNK1 pathway. FLAG-tagged JNK was expressed in COS-1 cells either alone or with Myc-tagged constructs for V12Rac, V12Cdc42, MLK2 (full-length) or MEKK (C-terminal domain) (Olson *et al.*, 1995). JNK activity was assessed by immunocomplex kinase assays using c-Jun as a substrate and revealed by autoradiography. The amount of JNK and of co-transfected proteins, as determined by Western blotting using a mixture of M2 and 9E10 antibodies, is shown in the lower panel. (B) Activation of ERK2 kinase by MLK2. COS-1 cells were transfected with vector control or pRK5-Myc-MLK2 together with pEXV-Myc-ERK2. pEXV-Myc-D12Ras was used as a positive control. MLK2 did not phosphorylate MBP directly in the conditions used. The levels of MLK2, ERK2 and Ras, visualized on Western blots using 9E10 antibody, are shown in the lower two panels. (C) Quiescent, serum-starved Swiss 3T3 cells were fixed 2–3 h after injection of Myc-tagged L61H-Ras (a and d), MLK2 (b and e) and L61Rac (c and f). Cells expressing Myc-tagged proteins were visualized by indirect immunofluorescence with 9E10 antibody (a–c). Activated dually phosphorylated ERK was visualized with anti-active ERK antibody (Promega) (d–f). Three non-injected, neighbouring cells can be seen in the bottom right corner of (b) and (e). Scale bar represents 30 μ m.

either constitutively active or dominant-negative versions of Cdc42 or Rac can be seen (data not shown). MLK2 causes moderate (11-fold; Figure 3B) activation of co-transfected ERK2 and p38 (8-fold; data not shown).

To exclude the possibility that MLK2-induced ERK activation might be indirect and caused by autocrine effects, cDNA was injected into serum-starved quiescent Swiss 3T3 cells and activation of ERK visualized using an antibody specific for dually phosphorylated ERK. As shown in Figure 3C, MLK2 activates ERK only in injected cells and not in neighbouring cells, confirming that the

effects are cell autonomous. We conclude that MLK2, expressed in COS-1 cells, is a potent activator of JNK, but also activates the p38 and ERK pathways.

Microinjection of MLK2 into Swiss 3T3 cells

In addition to their effects on MAP kinase pathways, Rac and Cdc42 are important regulators of actin polymerization and the assembly of associated integrin complexes (Ridley *et al.*, 1992; Kozma *et al.*, 1995; Nobes and Hall, 1995). Microinjection of quiescent Swiss 3T3 cells with pRK5-Myc-MLK2, however, induced no detectable changes in

Fig. 4. Localization of MLK2 in Swiss 3T3 cells. Swiss 3T3 cells were fixed 2–3 h after injection of nuclei with pRK5-Myc MLK2 (0.1 mg/ml). Myc-MLK2 (A) and microtubules (B) were visualized by indirect immunofluorescence. (C) Confocal micrograph of a cell double-labelled for microtubules (red) and Myc-MLK2 (green). Scale bar represents 30 μ m.

the actin cytoskeleton (data not shown). Rac and Cdc42 have also been shown to stimulate G_1 progression and entry into DNA synthesis when injected into quiescent Swiss cells. (Olson *et al.*, 1995). To examine whether MLK2 can stimulate G_1 progression, quiescent Swiss 3T3 cells were microinjected with pRK5-Myc-MLK2, and 6 h later ~98% of injected cells strongly expressed Myc-MLK2 (data not shown). However 20 h after injection, we were unable to assess the effects of MLK2 on DNA synthesis since most injected cells were dead (data not shown). Further analysis revealed that even when the experiment was repeated in the presence of 10% fetal calf serum (FCS), MLK2 induced cell death in >90% of injected cells by 20 h (data not shown). Time lapse video analysis revealed that cell death occurred with cytoplasmic shrinkage and nuclear condensation, characteristics of cells undergoing apoptosis. This was confirmed by incubating microinjected cells in the

presence of the protease inhibitor Z-Val-Ala-Asp(O-Me)-fluoromethylketone (100 μ M), which prevented apoptosis when added in the medium just after microinjection (data not shown).

Immunostaining of MLK2 expressed in Swiss 3T3 cells

To determine the intracellular localization of MLK2, a Myc-tagged expression construct was microinjected into the nuclei of Swiss 3T3 cells. As seen in Figure 4A, 2–3 h later MLK2 can be seen in punctate structures that appear to be organized radially within the cells. Co-staining with an antibody to tubulin revealed that these structures were located along microtubules (Figure 4B). Further analysis using confocal laser microscopy (Figure 4C) confirmed that under these conditions the majority of

Fig. 5. Co-localization of activated JNK with MLK2. Quiescent, serum-starved Swiss 3T3 cells were fixed 2–3 h after injection of Myc-tagged MLK2 (A and B) and MLK2-Leu/CRIB/Div (C and D). The subcellular localization of expressed Myc-tagged proteins was visualized by indirect immunofluorescence with 9E10 antibody (A and C). Endogenous dually phosphorylated JNK was visualized using anti-active JNK antibody (B and D). (E) FLAG-tagged JNK was co-transfected into COS cells with pRK5 or pRK5-Myc-MLK2. Western blotting of the cell lysate was conducted with anti-active JNK antibody (top panel) or M2 antibody (bottom). Scale bar represents 30 μ m.

MLK2 is found in a punctate distribution intimately associated with microtubules.

To determine whether these structures might have functional significance, the cellular distribution of endogenous, activated JNK in the MLK2-injected cells was localized using an antibody that recognizes the dually phosphorylated active form of the JNK1 and 2 enzymes. As shown in Figure 5B, activated endogenous JNK partially co-localizes with MLK2 (Figure 5A) to punctate structures along microtubules. In addition, activated JNK can also be found in the nucleus. MLK2-Leu/CRIB/Div (lacking the kinase domain) also localized to these punctate structures (Figure 5C), but did not stimulate JNK activity (Figure 5D).

Two-hybrid screen for MLK2-interacting proteins

Since transfected MLK2 is constitutively active and is localized along microtubules, we have attempted to identify cellular proteins that might regulate this behaviour. A yeast two-hybrid, human brain, cDNA library was screened with MLK2-Leu/CRIB/Div (lacking the kinase domain, see Figure 1B). Thirteen positive clones, which showed a strong positive interaction with MLK2, were identified. Partial cDNA sequence analysis revealed that six clones corresponded to hippocalcin, five clones encoded 14-3-3 ϵ and one clone encoded the C-terminal region (starting at amino acid 551) of KAP3, a putative targeting molecule in the KIF3 microtubule motor complex (Yamazaki *et al.*, 1996). The final clone encoded a novel

cDNA which showed significant sequence similarity to the carboxy-terminus of the *Caenorhabditis elegans* kinesin superfamily member, OSM-3 (Tabish *et al.*, 1995), and mammalian kinesin superfamily proteins, KIF3A and KIF3B (Aizawa *et al.*, 1992; Yamazaki *et al.*, 1995; Hirokawa, 1996). The DNA sequence of the yeast two-hybrid clone (accession No. AF009624) corresponds to the carboxy-terminal 233 amino acids of a novel member of the KIF3 family, which we tentatively name KIF3X. The deduced partial amino acid sequence of KIF3X shows ~26% identity and 35% similarity to *C.elegans* OSM-3 and 17% identity and 23% similarity to mammalian KIF3A (Figure 6A).

To identify the binding site of hippocalcin, 14-3-3 ϵ , KIF3X and KAP3 on MLK2, fragments of MLK2 were used in the two-hybrid assay. KIF3X, KAP3 (Figure 6B), 14-3-3 ϵ and hippocalcin (data not shown) interact only with the C-terminal divergent region of MLK2. MLK3 also interacts with these four proteins (Figure 6B and data not shown). Since KIF3X appears to belong to the KIF3 subfamily of the kinesin superfamily of motor proteins, we wished to determine whether other members of the family could also interact with MLKs. The C-terminal fragments of KIF3A (amino acids 531–701) and KIF3B (amino acids 524–747) were obtained by PCR from a mouse brain cDNA library. Figure 6B shows that MLK2 and MLK3 interact with KIF3A (more strongly in fact than with KIF3X), whereas KIF3B interacts weakly with MLK3 and not with MLK2.

Complex formation between MLK2 and hippocalcin, 14-3-3 ϵ , KIF3A and KAP3A

To identify whether MLK2 can form complexes in mammalian cells with the proteins identified in the two-hybrid screen, expression vectors containing FLAG-hippocalcin, FLAG-14-3-3 ϵ , FLAG-KAP3A and FLAG-KIF3A were co-transfected with Myc-tagged MLK2 into COS cells. For these experiments, we have used cDNAs encoding full-length versions of KIF3A and KAP3A (Aizawa *et al.*, 1992; Yamazaki *et al.*, 1995, 1996). Figure 7 shows that full-length KAP3A, KIF3A, 14-3-3 ϵ and hippocalcin can be co-precipitated with MLK2 under these conditions. To determine whether any of the interacting proteins can affect MLK2 kinase activity, they were each co-transfected into COS cells with MLK2 and JNK. In each case, there was no detectable inhibition or enhancement of MLK2-induced activation of JNK (data not shown).

To determine the intracellular location of MLK2-interacting proteins, the corresponding expression vectors were injected into the nuclei of Swiss 3T3 cells. 14-3-3 ϵ and hippocalcin were found diffusely distributed throughout the cytoplasm (data not shown). KAP3A also appeared diffusely distributed (Figure 8A) as previously reported (Yamazaki *et al.*, 1996), but KIF3A localized to punctate structures along microtubules (Figure 8B) indistinguishably from MLK2 (Figure 5A) and activated JNK (Figure 5B).

Discussion

In addition to their effects on the actin cytoskeleton, Rac and Cdc42 activate the JNK and p38 MAP kinase cascades when transfected into cells (Coso *et al.*, 1995; Minden

A

KIF3X		L L N V Y D S Q E E V R A K S K L L E K M Q R K L R A A E V E I K D L Q S E F
OSM-3	457	L L Q V Y S T Q E K I D A V T S Q L E K E V K K S K G Y E R E I E D L H G E F
KIF3A	530	L E E K Y T S L Q E E A Q G K T K K L K K W T M L M A A K S E M A D L Q Q E H
KIF3X		Q L E K I D Y L A T I R R Q E R S M L L Q Q L L E G V Q P L I R R D C N Y S N
OSM-3		E L D R L D Y L D T I R K Q D Q L K L L M Q I M P K I Q P I I K K D T N Y S N
KIF3A		Q R E I E G L L E N I R Q L S R L R L Q M L I I L N F I P Q D Y Q E M I E N Y
KIF3X		L E K I L R E S C W D E D N G F W K I P H P V T K T S L P V A V S T G P Q N K
OSM-3		V D R I K K E A V W N E D E S R W I L P E M S M S R T I L P L A N N G Y M Q E P
KIF3A		V H W N E D I G E W Q K C V A Y T G N N M R K Q T P V P D K K E
KIF3X		P A R K T S A A Y N G E P N M E F D R Y R L M L S R S N S E N I A S N Y F R S K
OSM-3		A R Q E N T L L R S N F D D K L R E R L A K S D S E N I A N S Y F K P V
KIF3A		R D P F E V D L S H V Y L A Y T E E S L R Q S L M K L
KIF3X		R A S Q I L S T D A R K S L H T S Q L A T R P Q L P T Q Q Q L C H P P T Q A P E
OSM-3		K Q I N V I N K Y K S D Q K L S T S K S L E P S K T P T F D G L V N G V V Y T D
KIF3A		E R P R T S K G K A R P K T G R R K R S A K P E T V I D S
KIF3X		M P Q P R P F R L E S L D I P F T K A K R K K S K S N F G S E P L *
OSM-3		A L Y E R A Q S A K R P P R L A S L N P K * 670
KIF3A		L Q *701

B

	pYTH	pACTII
MLK2-Div	-	
MLK2-Div KIF3X		
MLK2-Div KIF3A		
MLK2-Div KIF3B		
MLK2-Div KAP3		
MLK3-Div	-	
MLK3-Div KIF3X		
MLK3-Div KIF3A		
MLK3-Div KIF3B		
MLK3-Div KAP3

Fig. 6. MLK2 interacts with the KIF3 family of kinesin superfamily proteins. (A) Comparison of the KIF3X partial amino acid sequence with those of KIF3A and OSM-3. The predicted rod α -helix domain is underlined. Identical and conserved amino acids are denoted by boxes and shaded boxes, respectively. (B) The interaction of GAL4DB–MLK2 (Div) and GAL4DB–MLK3 (amino acids 348–847) with GAL4AD–KIF3X, KIF3A (amino acids 531–701), KIF3B (amino acids 524–747) and KAP3 (C-terminal region from amino acid 511 obtained by yeast two-hybrid screening).

et al., 1995; Olson *et al.*, 1995). Several groups have reported that p65^{PAK}, a ser/thr kinase which is a target for both Rac and Cdc42, might mediate JNK activation, while others have reported that it does not (Bagrodia *et al.*, 1995; Zhang *et al.*, 1995; Brown *et al.*, 1996; Lamarche *et al.*, 1996; Joneson *et al.*, 1996; Westwick *et al.*, 1997). In a database search for additional Cdc42/Rac target proteins, the MLK family of ser/thr kinases was identified as having a potential GTPase-interacting (CRIB) motif (Burbelo *et al.*, 1995). The kinase domain of this family is most closely related to the MAP kinase kinase kinase family, and it has been reported recently that MLK3 can activate the JNK MAP kinase pathway, raising the possibility that MLKs mediate Cdc42/Rac activation of MAP kinases (Rana *et al.*, 1996; Teramoto *et al.*, 1996; Tibbles *et al.*, 1996).

We show here that MLK2 and MLK3 (through their CRIB motifs) interact strongly with both Rac and Cdc42 in a GTP-dependent manner, suggesting that they are

cellular targets of these GTPases. As previously reported for MLK3 (Tibbles *et al.*, 1996), we could not observe complex formation of MLK2 with active Cdc42 or Rac, perhaps due to the transient nature of the interaction between these molecules. When transfected into COS cells with reporter plasmids, MLK2 is a potent activator of the JNK kinase cascade, though it also activates the ERK and p38 pathways. It appears, therefore, that MLK2 belongs to a growing number of MAPKKKs that can activate the JNK pathway, including MLK3, ASK1, Tpl-2, DLK, TAK1 and MEKK1–4 (Hirai *et al.*, 1996; Moriguchi *et al.*, 1996; Salmeron *et al.*, 1996; Fanger *et al.*, 1997; Gerwins *et al.*, 1997; Ichijo *et al.*, 1997).

The mechanism by which MAPKKKs are activated is unclear. In addition to MLKs, MEKKs 1 and 4 (Fanger *et al.*, 1997; Gerwins *et al.*, 1997) have also been shown to contain a Cdc42/Rac interaction site. It has been reported by one group that MLK3 activity can be modified by co-expression of Cdc42 (Teramoto *et al.*, 1996), but

Fig. 7. Interaction of MLK2 with KAP3A, KIF3A, 14-3-3 ϵ and hippocalcin in COS cells. Co-immunoprecipitation of Myc-MLK2 with: (A) FLAG-tagged, full-length KAP3A; (B) FLAG-tagged full-length KIF3A; (C) FLAG-tagged 14-3-3 ϵ ; and (D) FLAG-tagged hippocalcin. Lysates (~90 μ g of protein) from transfected COS-1 cells were immunoprecipitated (IP) with M2 (FLAG) or 9E10 (MYC) antibody as indicated. Western blotting (WB) was carried out with a mixture of 9E10 and M2 antibodies. In (A) and (B), the first panel is a Western blot showing total protein levels in transfected cells and the second panel is a Western blot of immunoprecipitated material.

in our hands MLK2 transfected into COS-1 (this study), NIH-3T3 or PC12 cells (data not shown) is constitutively active, and co-transfection with activated or dominant-negative versions of Cdc42 or Rac has no effect on its activity. The significance of the GTPase interaction site on MLKs is, therefore, unclear. Cdc42 has been reported to play a role in the regulation of MAP kinase pathways in yeast, though recent work has suggested that its role may not be in activation *per se*, but in localizing target kinases to distinct intracellular locations (Peter *et al.*, 1996). Perhaps the role of mammalian Cdc42 and Rac is to localize MLKs to discrete cellular compartments or molecular complexes.

Since MLK2 and MLK3 are constitutively active after transfection, it seems likely that the endogenous MLKs are sequestered by cellular proteins. It was interesting to find, therefore, that MLK2 localizes to punctate structures along microtubules. The observation that dually phos-

Fig. 8. Localization of KAP3A and KIF3A in Swiss 3T3 cells. Swiss 3T3 cells were fixed 2–3 h after injection of nuclei with 0.1 mg/ml each of (A) pRK5-FLAG-KAP3A (full-length) and (B) pRK5-FLAG-KIF3A (full-length). Expressed proteins were visualized by indirect immunofluorescence. Scale bar represents 30 μ m.

phorylated JNK co-localizes to the same structures, as well as in the nucleus, suggests that they are functionally significant. Interestingly, it has been shown recently that three components of the hedgehog signalling pathway, fused (a ser/thr kinase), cubitus interruptus (a transcription factor) and costal2 are sequestered in a complex along microtubules and released upon activation of the pathway (Robbins *et al.*, 1997). The mechanism of microtubule attachment is through costal2, which is a kinesin-related protein, though there is no evidence that any motor-like activity is required.

To find proteins that might regulate the activity or localization of MLK2, we performed a yeast two-hybrid screen and identified four proteins that interact with the divergent, C-terminal region of the molecule. Two of these are 14-3-3 ϵ and hippocalcin. The 14-3-3 family of proteins have received much attention since they associate with numerous signalling proteins including c-Raf-1 and the phosphatase cdc25 (reviewed in Aitken, 1996). The functional significance of these interactions is still unclear, though recent genetic analysis of the Ras/Raf pathway in *Drosophila* suggests that 14-3-3 is an essential component (Chang and Rubin, 1997; Kockel *et al.*, 1997). It has been shown that 14-3-3 proteins interact with a phosphorylated consensus sequence, RGLpSPP; this motif is found in MLK2 (which interacts with 14-3-3), but not in MLK3 (which we find does not interact with 14-3-3, data not shown). However, we were unable to find any effect of 14-3-3 expression on MLK2 activity and, as expected since it interacts with a wide range of proteins, 14-3-3 showed a diffuse cytoplasmic localization pattern.

Hippocalcin is a 23 kDa Ca^{2+} -binding protein with three EF-hand structures and is found predominantly in hippocampus (Kobayashi *et al.*, 1992). It has a striking sequence homology to the recoverin family, members of which serve to regulate photo-signal transduction systems (Dizhoor *et al.*, 1991). It is possible that hippocalcin serves as an anchor for the specific intracellular localization of MLK2, since hippocalcin translocates to membranes in Ca^{2+} -dependent manner (Kobayashi *et al.*, 1993). However, as with 14-3-3, hippocalcin showed a diffuse cytoplasmic location and co-expression had no effect on MLK2 activity.

Two additional MLK2-interacting clones were isolated in the two-hybrid screen. The first, KAP3, associates with the non-motor, tail domain of the kinesin superfamily microtubule motor protein, KIF3 (Yamazaki *et al.*, 1996).

The other interacting clone encodes a novel member of the KIF3 family of kinesin superfamily of motor proteins, tentatively referred to as KIF3X. Further analysis revealed that KIF3 itself also interacts with MLK2 and MLK3. Kinesin is a motor protein that transports vesicles along microtubules towards their plus end (Brady, 1995). It is composed of two heavy chains and two light chains that form a globular (ATP/microtubule-binding) head domain, a stalk domain and a tail globular domain, which is thought to associate with membranous organelles via an associated protein, kinectin (Burkhardt, 1996). Interestingly, Rho, Rac and Cdc42 have each been found to associate with kinectin in a yeast two-hybrid analysis, suggesting that these GTPases might influence the kinesin motor (Hotta *et al.*, 1996). It is now clear that kinesin is one member of a large superfamily of microtubule motor proteins, referred to as KIFs (Hirokawa, 1996). KIF3A and KIF3B are two members of the kinesin superfamily that form a heterodimeric plus end-directed motor. They are highly expressed in neurons and testis, but are expressed ubiquitously at lower levels (Aizawa *et al.*, 1992; Yamazaki *et al.*, 1995). Although the proteins contained in vesicles transported by KIF3A/KIF3B motors have not been identified, vesicular recognition is thought to be modulated by a KIF3 tail-interacting protein, KAP3 (Yamazaki *et al.*, 1996).

It appears, therefore, that MLK2 potentially can associate with at least two components of KIF motor complexes; the motor molecule itself (KIF3A and KIF3X) and the putative cargo recognition molecule (KAP3). When KAP3 was expressed in Swiss cells it localized in a diffuse cytoplasmic pattern, as previously reported (Yamakazi *et al.*, 1996). KIF3A, however, localized predominantly in a similar punctate pattern to MLK2 and activated JNK, along microtubules. The nature of these structures is unclear at the moment; we have no evidence that they are active motor complexes.

The data reported here indicate that MLKs can associate with components of KIF3 motors and that they can activate JNK and to a lesser extent ERK/p38 cascades. This raises the intriguing possibility that microtubule motors and MAP kinase cascades are co-ordinately controlled. One possibility is that motor components can be phosphorylated by active MLKs or JNKs in response to stress, and it is interesting that the closest known homologue to KIF3X (the new kinesin family member identified in our two-hybrid screen) is the *C.elegans* protein OSM-3. Mutants in this locus fail to avoid high osmotic concentrations of salts and sugar, conditions that are a classic stimulus for the JNK/p38 MAP kinase pathways in mammalian cells as well as in yeast (Kyriakis and Avruch, 1996). We would like to suggest the possibility that MAP kinase pathways and the activity of microtubule motor complexes can be co-ordinately regulated; whether MLKs are delivered by the motor complex to a site of JNK MAP kinase activation, or whether the activity of the motor complex is regulated by a MLK/Cdc42/Rac interaction is now open to investigation.

Materials and methods

Materials

The following constructs were kind gifts from colleagues; Dr M.Terada (National Cancer Center Research Institute, Tokyo), human MLK2; Dr

C.J.Marshall (Chester Beatty Laboratories, London), pEXV-Myc-ERK2 and pEXV-Myc-D12Ras; Drs M.Karin (University of California) and J.Ham (Eisai London Research Laboratories), pCMV-FLAG-JNK1 and pGEX-c-Jun; Dr L.Zon (Dana Farber Cancer Institute, Boston), dominant-negative pMT2-HA-SEK-1AL(S220A, T224L); Dr M.H.Cobb (University of Texas), pCEF-HA-p38; Dr B.Shamoon (Chiron Corp., CA), human brain cDNA library fused to the GAL4 activation domain (GAL4AD) in pACTII; Dr S.Moss (University College London), anti-Myc antibody (9E10). Anti-FLAG-tag antibody (M2) and anti-HA-tag antibody (12CA5) were purchased from Kodak Inc. and Boehringer Inc., respectively. Anti-tubulin antibody (rat) was from Dr J.Kilmartin (MRC Laboratory of Molecular Biology, Cambridge). Anti-active ERK antibody, recognizing dual-phosphorylated ERK1 and ERK2 (corresponding to T183 and Y185 of ERK2), and anti-active JNK antibody, recognizing dual-phosphorylated JNK1 and JNK2 (corresponding to T183 and Y185), are multistep affinity-purified reagents from Promega Corp. (Madison, WI) (Khokhlatchev *et al.*, 1997). 14-3-3e, hippocalcin, mouse KAP3A, mouse KIF3A and KIF3X (C-terminal region obtained in two-hybrid screen) were subcloned into the pRK5 vector containing the FLAG-tag. All constructs were verified by DNA sequencing. Human MLK3 (amino acids 348–847) was obtained by yeast two-hybrid screening with an Epstein–Barr virus-transformed B-cell library and L61Cdc42 as bait.

Yeast two-hybrid analysis

cDNAs encoding various GTPases were fused to the GAL4 DNA-binding domain (GAL4DB) in the pYTH6 vector, and constructs were integrated into the genome of *Saccharomyces cerevisiae*, strain Y190. MLK2 (amino acids 338–953), MLK3 (amino acids 348–847) and RhoGAP were fused to the GAL4AD in the pACTII vector (Aspenstrom and Olson, 1995). After transformation of pACTII constructs into yeast strains containing integrated pYTH6-GTPase constructs, cells were spread on medium lacking tryptophan, leucine and histidine but supplemented with 25 mM 3-aminotriazole. Cells containing interacting fusion proteins appeared on this plate after 3 days. LacZ expression was assayed by the membrane transfer method (Aspenstrom and Olson, 1995).

To screen for MLK2-interacting proteins, Y190:pYTH6-MLK2 (amino acids 338–953) was transformed with a human brain cDNA library in pACTII and 1.2×10^6 transformants were screened essentially as described before (Aspenstrom and Olson, 1995). Plasmid DNA recovered from 13 positive clones retained the ability to induce the reporter gene when transformed into Y190:pYTH6-MLK2. The obtained partial cDNA of KIF3X was sequenced by dideoxynucleotide chain termination methods.

Expression and purification of recombinant proteins

GTPases were expressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins and purified on glutathione–Sepharose beads. The recombinant proteins were released from the beads by cleavage with human thrombin as described (Lamarche *et al.*, 1996). MLK2 [amino acids 338–953 for Leu-zipper, CRIB motif and divergent region (MLK2-Leu/CRIB/Div); amino acids 341–451 for Leu-zipper (MLK2-Leu); amino acids 338–539 for Leu-zipper and CRIB motif (MLK2-Leu/CRIB) and amino acids 497–953 for divergent region (MLK2-Div)], p50rhoGAP (amino acids 198–439), full-length p65^{PAK}, c-Jun and ATF-2 were also expressed as GST fusion proteins in *E.coli*. These fusion proteins were eluted from the beads with 5 mM reduced glutathione (Sigma Inc.). Purified proteins were dialysed against 15 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.1 mM dithiothreitol (DTT) and concentrated by ultrafiltration using Centricon-10 (Amicon Inc.). Protein concentration was determined by the method of Bradford, and the purity of protein preparations was visualized on Coomassie blue-stained SDS–polyacrylamide gels.

Dot-blot assay

The interaction of target proteins with Rac and Cdc42 was assessed using a dot-blot assay as previously described (Diekmann *et al.*, 1995). Briefly, the indicated amounts of various GST–MLK2 fusion protein fragments (MLK2-Leu/CRIB/Div, MLK2-Leu/CRIB, MLK2-Leu and MLK2-Div) or p65^{PAK} were spotted onto nitrocellulose membranes and the filter air dried and incubated with blocking buffer (1 M glycine, 5% milk powder, 1% ovalbumin and 5% FCS) for 2 h at room temperature. The membrane was washed in buffer A (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM MgCl₂ and 1 mM DTT) and incubated for 5 min at 4°C with [γ -³²P]GTP-bound L61Rac or L61Cdc42 in buffer A. The filters were washed quickly three times with cold buffer A containing 0.1% Tween-20, and interacting GTPases were visualized by autoradiography.

COS-1 cell transfection

COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics and were transfected by the DEAE-dextran method (Olson *et al.*, 1995). Plasmid amounts per 10 cm Petri dish were as follows: 5 µg of pCMV-FLAG-JNK1 with or without 3 µg each of pRK5-Myc, pRK5-Myc-L61Rac, pRK5-Myc-L61Cdc42, pMT-Myc-MEKK-C terminal domain or pRK5-Myc-MLK2; 5 µg of pEXV-Myc-ERK2 with or without 3 µg each of pEXV-Myc, pEXV-Myc-D12Ras or pRK5-Myc-MLK2; and 5 µg of pCEF-HA-p38 with or without 5 µg each of pRK5-Myc, pRK5-Myc-L61Cdc42 or pRK5-Myc-MLK2. After 24 h, transfected cells were serum starved for 16 h and then harvested. For assessing complex formation in COS cells, 5 µg of pRK5-Myc-MLK2 were transfected with or without 2.5 µg each of pRK5-FLAG-hippocalcin, pRK5-FLAG-14-3-3e, pRK5-FLAG-KAP3A or pRK5-FLAG-KIF3A.

Immunoprecipitation

The plasmids pRK5-Myc-MLK2, pRK5-FLAG-14-3-3e, pRK5-FLAG-hippocalcin, pRK5-FLAG-KIF3A and pRK5-FLAG-KAP3A were transfected into COS-1 cells in various combinations. The cells were harvested with 400 µl of lysis buffer C containing 40 mM Tris-HCl pH 7.5, 50 mM NaCl, 50 mM NaF, 100 µM Na₃VO₄ 0.2% NP-40, 10 µg/ml aprotinin and 10 µg/ml leupeptin. For experiments involving KAP3A and KIF3A, cells were lysed in buffer D containing 50 mM HEPES pH 6.9, 1 mM MgCl₂, 5 mM EGTA, 10% glycerol, 0.2% NP-40, 10 µg/ml aprotinin and 10 µg/ml leupeptin. Insoluble material was removed by centrifugation at 4°C for 10 min at 10 000 g, and 50 µl of lysate (50–90 µg of protein) were used for each assay. Myc-MLK2, FLAG-14-3-3e, FLAG-hippocalcin, FLAG-KIF3A and FLAG-KAP3A were immunoprecipitated using 9E10 or M2 antibodies. After washing the precipitates three times with the lysis buffer, the precipitates were subjected to SDS-PAGE (12 or 7.5% gel) and proteins were transferred to nitrocellulose filters. Western blotting was carried out with 9E10 and M2 antibodies for detecting Myc- and FLAG-tagged proteins, respectively.

JNK, ERK and p38 MAP kinase assay

For JNK assays, transfected COS-1 cells were lysed and harvested in buffer B (25 mM HEPES pH 7.6, 0.3 M NaCl, 50 mM NaF, 0.1 mM vanadate, 5 mM EGTA, 40 mM sodium pyrophosphate, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 3 mM phenylmethylsulfonyl fluoride) containing 1% Triton X-100. JNK1 kinase activity in cell extracts was measured after immunoprecipitation with anti-FLAG M2 antibody, using 2 µg of GST-c-Jun as a substrate (Olson *et al.*, 1995). For ERK2 activity, the cells were harvested with buffer B containing 1% Triton X-100 and 0.5% deoxycholic acid, and kinase activity in cell extracts was measured after immunoprecipitation with anti-Myc 9E10 antibody, using 5 µg of myelin basic protein (MBP) as a substrate (Olson *et al.*, 1995). p38 kinase activity was assayed following a protocol almost identical to that of JNK assay. Briefly, the cells were harvested with buffer B containing 1% Triton X-100 and 2 µM okadaic acid. p38 kinase activity in cell extracts was measured after immunoprecipitation with anti-HA 12CA5 antibody, using 1 µg of bacterially expressed ATF-2 as a substrate as described (Teramoto *et al.*, 1996).

Immunocomplex kinase reactions were fractionated by SDS-PAGE (12 or 13.5% gel) followed by blotting onto nitrocellulose membrane and autoradiography. The relative phosphorylation levels of c-Jun, MBP or ATF-2 were determined by Phospho-Imager (BioRad Inc.) analysis. The amount of immunoprecipitated JNK1, ERK2 and p38 was evaluated on Western blots using M2, 9E10 and 12CA5 antibodies, respectively as revealed by chemiluminescence detection using an ECL kit (Amersham Inc.).

Swiss 3T3 cell culture, microinjection and immunofluorescence

Swiss 3T3 cells were maintained in DMEM containing 10% FCS. Quiescent, serum-starved Swiss 3T3 cells were prepared for microinjection as follows: cells were seeded at a density of 5×10^4 cells into 15 mm diameter wells containing 13 mm glass coverslips. Six to ten days after seeding, cells were serum starved overnight (16 h) in DMEM containing 2 g/l NaHCO₃. For detection of Myc-tagged-MLK2 and microtubules, cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton-X 100 for 5 min and incubated with mouse monoclonal 9E10 and rat anti-tubulin antibodies for 1 h followed by incubation with fluorescein isothiocyanate-labelled anti-mouse IgG and rhodamine-labelled anti-rat IgG. For the detection of activated ERK or activated JNK, rabbit anti-active ERK or JNK antibody was used as the primary

antibody, and rhodamine-labelled anti-rabbit IgG as the secondary antibody. Cells were viewed on a Zeiss Axioplan fluorescence microscope or a laser scanning confocal microscope (MRC-1024, BioRad).

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References

- Aitken, A. (1996) 14-3-3 and its possible role in co-ordinating multiple signalling pathways. *Trends Cell Biol.*, **6**, 341–347.
- Aspenstrom, P. and Olson, M.F. (1995) Yeast two-hybrid system to detect protein-protein interaction with rho GTPases. *Methods Enzymol.*, **256**, 228–241.
- Aizawa, H., Sekine, Y., Takemura, R., Zhang, Z., Nangaku, M. and Hirokawa, N. (1992) Kinesin family in murine central nervous system. *J. Cell Biol.*, **119**, 1287–1296.
- Bagrodia, S., Derijard, B., Davis, R.J. and Cerione, R.A. (1995) Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. *J. Biol. Chem.*, **270**, 27995–27998.
- Brady, S.T. (1995) A kinesin medley: biochemical and functional heterogeneity. *Trends Cell Biol.*, **5**, 159–164.
- Brown, J.L., Stowers, L., Baer, M., Trejo, J., Coughlin, S. and Chant, J. (1996) Human Ste20 homologue hPAK1 links GTPases to JNK MAP kinase pathway. *Curr. Biol.*, **6**, 598–605.
- Burbelo, P.D., Drechsel, D. and Hall, A. (1995) A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J. Biol. Chem.*, **270**, 29071–29074.
- Burkhardt, J.K. (1996) In search of membrane receptors for microtubule-based motors—is kinectin a kinesin receptor? *Trends Cell Biol.*, **6**, 127–131.
- Chang, H.C. and Rubin, G.M. (1997) 14-3-3e positively regulates Ras-mediated signaling in *Drosophila*. *Genes Dev.*, **11**, 1132–1139.
- Coso, O.A., Chiariello, M., Yu, J.C., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J.S. (1995) The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell*, **81**, 1137–1146.
- Diekmann, D., Nobes, C.D., Burbelo, P.D., Abo, A. and Hall, A. (1995) Rac GTPase interacts with GAPs and target proteins through multiple effector sites. *EMBO J.*, **14**, 5297–5305.
- Dizhoor, A.M. *et al.* (1991) Recoverin: a calcium sensitive activator of retinal rod guanylate cyclase. *Science*, **251**, 915–918.
- Fanger, G.R., Johnson, N.L. and Johnson, G.L. (1997) MEK kinases are regulated by EGF and selectively interact with Rac/Cdc42. *EMBO J.*, **16**, 4961–4972.
- Galcheva-Gargova, Z., Derijard, B., Wu, I.H. and Davis, R.J. (1994) An osmosensing signal transduction pathway in mammalian cells. *Science*, **265**, 806–808.
- Gerwins, P., Blank, J. and Johnson, G.L. (1997) Cloning of a novel mitogen-activated protein kinase kinase kinase, MEKK4, that selectively regulates the c-jun amino terminal kinase pathway. *J. Biol. Chem.*, **272**, 8288–8295.
- Hirai, S., Izawa, M., Osada, S., Spyrou, G. and Ohno, S. (1996) Activation of the JNK pathway by distantly related protein kinases, MEKK and MUK. *Oncogene*, **12**, 641–650.
- Hirokawa, N. (1996) Organelle transport along microtubules—the role of KIFs. *Trends Cell Biol.*, **6**, 135–141.
- Hotta, K., Tanaka, K., Mino, A., Kohno, H. and Takai, Y. (1996) Interaction of the Rho family small G proteins with kinectin, an anchoring protein of kinesin motor. *Biochem. Biophys. Res. Commun.*, **225**, 69–74.
- Ichijo, H. *et al.* (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science*, **275**, 90–94.
- Joneson, T., McDonough, M., Bar-Sagi, D. and Van-Aelst, L. (1996) RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase. *Science*, **274**, 1374–1376.
- Katoh, M., Hirai, M., Sugimura, T. and Terada, M. (1995) Cloning and characterization of MST, a novel (putative) serine/threonine kinase with SH3 domain. *Oncogene*, **10**, 1447–1451.

- Khokhlatchev, A. *et al.* (1997) Reconstitution of mitogen-activated protein kinase phosphorylation cascades in bacteria. *J. Biol. Chem.*, **272**, 11057–11062.
- Kobayashi, M., Takamatsu, K., Saitoh, S., Miura, M. and Noguchi, T. (1992) Molecular cloning of hippocalcin, a novel calcium-binding protein of the recoverin family exclusively expressed in hippocampus. *Biochem. Biophys. Res. Commun.*, **189**, 511–517.
- Kobayashi, M., Takamatsu, K., Saitoh, S. and Noguchi, T. (1993) Myristoylation of hippocalcin is linked to its calcium-dependent membrane association properties. *J. Biol. Chem.*, **268**, 18898–18904.
- Kockel, L., Vorbruggen, G., Jackle, H., Mlodzik, M. and Bohmann, D. (1997) Requirement for *Drosophila* 14-3-3 ζ in Raf-dependent photoreceptor development. *Genes Dev.*, **11**, 1140–1147.
- Kozma, R., Ahmed, S., Best, A. and Lim, L. (1995) The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol. Cell. Biol.*, **15**, 1942–1952.
- Kyriakis, J.M. and Avruch, J. (1996) Protein kinase cascades activated by stress and inflammatory cytokines. *BioEssays*, **18**, 567–577.
- Lamarche, N., Tapon, N., Stowers, L., Burbelo, P.D., Aspenstrom, P., Bridges, T., Chant, J. and Hall, A. (1996) Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p5PAK and the JNK/SAPK MAP kinase cascade. *Cell*, **87**, 519–529.
- Leevers, S.J., Paterson, H.F. and Marshall, C.J. (1994) Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature*, **369**, 411–414.
- Manser, E., Leung, T., Salihuddin, H., Zhao, Z.S. and Lim, L. (1994) A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature*, **367**, 40–46.
- Marais, R., Light, Y., Paterson, H.F. and Marshall, C.J. (1995) Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *EMBO J.*, **14**, 3136–3145.
- Marshall, C.J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, **80**, 179–185.
- Minden, A., Lin, A., Claret, F.X., Abo, A. and Karin, M. (1995) Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell*, **81**, 1147–1157.
- Moriguchi, T. *et al.* (1996) A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J. Biol. Chem.*, **271**, 13675–13679.
- Nobes, C.D. and Hall, A. (1995) Rho, Rac and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia and filopodia. *Cell*, **81**, 53–62.
- Olson, M.F., Ashworth, A. and Hall, A. (1995) An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science*, **269**, 1270–1272.
- Peter, M., Neiman, A.M., Park, H.O., van Lohuizen, M. and Herskowitz, I. (1996) Functional analysis of the interaction between the small GTP binding protein Cdc42 and the Ste20 protein kinase in yeast. *EMBO J.*, **15**, 7046–7059.
- Rana, A., Gallo, K., Godowski, P., Hirai, S., Ohno, S., Zon, L., Kyriakis, J.M. and Avruch, J. (1996) The mixed lineage kinase SPRK phosphorylates and activates the stress-activated protein kinase activator, SEK-1. *J. Biol. Chem.*, **271**, 19025–19028.
- Ridley, A.J., Paterson, H.F., Johnston, C.J., Diekmann, D. and Hall, A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*, **70**, 401–410.
- Robbins, D.J., Nybakken, K.E., Kobayashi, R., Sisson, J.C., Bishop, M. J. and Therond, P.P. (1997) Hedgehog elicits signal transduction by means of a large complex containing the kinesin-related protein Costal2. *Cell*, **90**, 225–234.
- Robinson, M.J. and Cobb, M. H. (1997) Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.*, **9**, 180–186.
- Salmeron, A., Ahmad, T.B., Carlile, G.W., Pappin, D., Narsimhan, R.P. and Ley, S.C. (1996) Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. *EMBO J.*, **15**, 817–826.
- Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M. and Hancock, J.F. (1994) Activation of Raf as a result of recruitment to the plasma membrane. *Science*, **264**, 1463–1467.
- Tabish, M., Siddiqui, Z.K., Nishikawa, K. and Siddiqui, S.S. (1995) Exclusive expression of *C.elegans* osm-3 kinesin gene in chemosensory neurons open to the external environment. *J. Mol. Biol.*, **247**, 377–389.
- Teramoto, H., Coso, O.A., Miyata, H., Igishi, T., Miki, T. and Gutkind, J.S. (1996) Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. *J. Biol. Chem.*, **271**, 27225–27228.
- Tibbles, L.A., Ing, Y.L., Kiefer, F., Chan, J., Iscove, N., Woodgett, J.R. and Lassam, N.J. (1996) MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. *EMBO J.*, **15**, 7026–7035.
- Waskiewicz, A.J. and Cooper, J.A. (1995) Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Opin. Cell Biol.*, **7**, 798–805.
- Westwick, J.K., Lambert, Q.T., Clark, G.J., Symons, M., van Aelst, L., Pestell, R.G. and Der, C.J. (1997) Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. *Mol. Cell. Biol.*, **17**, 1324–1335.
- Yamazaki, H., Nakata, H., Okada, Y. and Hirokawa, N. (1995) KIF3A/B: a heterodimeric kinesin superfamily protein that works as a microtubule plus end-directed motor for membrane organelle transport. *J. Cell Biol.*, **130**, 1387–1399.
- Yamazaki, H., Nakata, T., Okada, Y. and Hirokawa, N. (1996) Cloning and characterization of KAP3: a novel kinesin superfamily-associated protein of KIF3A/3B. *Proc. Natl Acad. Sci. USA*, **93**, 8443–8448.
- Zhang, S., Han, J., Sells, M.A., Chernoff, J., Knaus, U.G., Ulevitch, R.J. and Bokoch, G.M. (1995) Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J. Biol. Chem.*, **270**, 23934–23936.

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