HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway

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In mammalian cells, a specific stress-activated protein kinase (SAPK/JNK) pathway is activated in response to inflammatory cytokines, injury from heat, chemotherapeutic drugs and UV or ionizing radiation. The mechanisms that link these stimuli to activation of the SAPK/JNK pathway in different tissues remain to be identified. We have developed and applied a PCR-based subtraction strategy to identify novel genes that are differentially expressed at specific developmental points in hematopoiesis. We show that one such gene, hematopoietic progenitor kinase 1 (hpk1), encodes a serine/threonine kinase sharing similarity with the kinase domain of Ste20. HPK1 specifically activates the SAPK/JNK pathway after transfection into COS1 cells, but does not stimulate the p38/RK or mitogen-activated ERK signaling pathways. Activation of SAPK requires a functional HPK1 kinase domain and HPK1 signals via the SH3-containing mixed lineage kinase MLK-3 and the known SAPK activator SEK1. HPK1 therefore provides an example of a cell type-specific input into the SAPK/JNK pathway. The developmental specificity of its expression suggests a potential role in hematopoietic lineage decisions and growth regulation.
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Introduction

Generation of mature blood cells is a highly regulated process which responds to various environmental and physiological stimuli. Cytokines cause proliferation, differentiation or elimination of maturing or mature blood cells, each of these processes being dependent on the presence of appropriate cytokine receptors and the corresponding signal transduction elements (Cosman, 1993). In mammalian cells, distinct signaling cassettes, each containing a central cascade of 'three kinases', respond to a variety of positive and negative extracellular stimuli, leading to changes in transcription factor activity and post-translational protein modification (Cano and Mahadevan, 1995; Woodgett et al., 1996).

The mitogen-activated kinases (MAPK, ERK), whose activity depends on tyrosine and threonine phosphorylation by an upstream dual-specificity MAPK kinase (MAPKK, MEK) which in turn is activated by a MAPKK kinase (MAPKKK), were the first members of a mammalian 'three-kinase cassette' to be described. The more recently identified stress-activated protein kinases (SAPK/JNK) (Kyriakis et al., 1994; Minden et al., 1994) and the p38/RK kinase (Han et al., 1994) show strong homology to the MAPKs but differ in responding to stimuli which are associated with cellular stress rather than mitogens. Such stressors include the inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), cellular injury (heat, UV and ionizing irradiation, chemotherapeutic drugs) and osmotic shock (Bird et al., 1994; Kyriakis et al., 1994; Woodgett et al., 1996). SAPK is phosphorylated and activated directly by the SAPK/ERK kinase 1 (SEK1) (Sanchez et al., 1994) which in turn is a substrate of MEK kinase 1 (MEKK1) (Yan et al., 1994). The upstream activators and physiological targets of p38/RK presently are being defined; two dual-specificity kinases, MKK3 (Dérijard et al., 1995; Raingeaud et al., 1996) and MKK6 (Han et al., 1996; Raingeaud et al., 1996), have been shown to phosphorylate and activate p38/RK.

While a clear view is emerging of the events linking occupancy of a number of cytokine and growth factor receptors to their corresponding downstream cascades (Pawson, 1995; Taniguchi, 1995), similar upstream linkages remain to be defined precisely for the SAPK/JNK and p38/RK pathways. c-Abl has been identified as an upstream component of SAPK activation by ionizing irradiation and alkylating agents in U-937 cells and fibroblasts (Kharbanda et al., 1995a,b). Similarly, ceramide may form a potential link between TNF-α-induced apoptosis and SAPK activation (Verheij et al., 1996). Finally, the small GTP binding proteins Rac1 and Cdc42Hs stimulate the SAPK pathway most likely via additional intermediate kinases in COS1 cells, HeLa cells and fibroblasts (Coso et al., 1995; Minden et al., 1995; Zhang et al., 1995). Germinal center kinase (GCK; Pombo et al., 1995), MAPK upstream kinase (MUK; Hirai et al., 1996) and Tpl-2 (Salmerón et al., 1996) are three kinases that were shown recently to activate the SAPK pathway in transfection studies. However, their mode of action and, in particular, their regulation remain poorly understood. Interestingly, a mutant of GCK that lacks the kinase domain was still capable of SAPK activation. GCK shares homology with the kinase domain of Ste20, while MUK and Tpl-2 are more similar to Ste11. Ste20 and Ste11 both function downstream of a heterotrimeric G-protein in the prototypic yeast pheromone response pathway (Herskowitz, 1995). Following binding of mating phero-
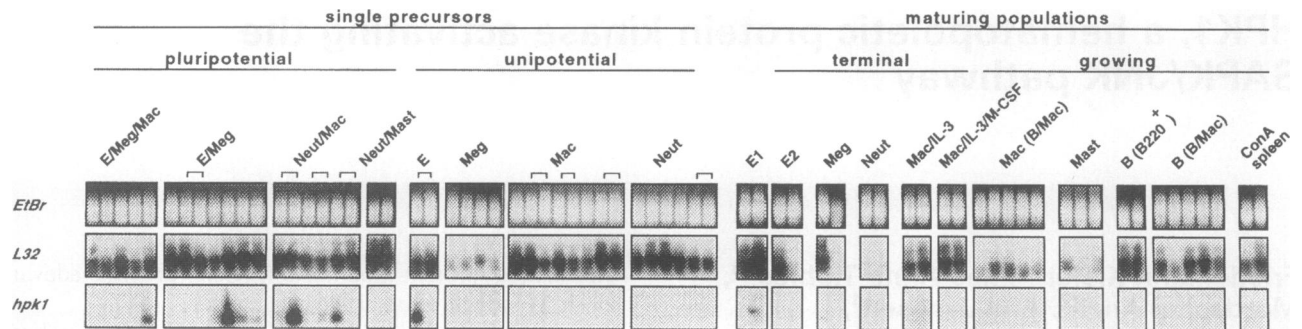
Fig. 1. Expression pattern of *mhpk1* in single hematopoietic progenitor cells. Each lane contains globally amplified cDNA from cells of the indicated biological potentials. E, erythroid cells; Meg, megakaryocytes; Mac, macrophages; Neut, neutrophils; Mast, mast cells; B, B cells; ConA spleen, splenocytes after 48 h concanavalin A stimulation. See Brady *et al.* (1995) for details on the cDNA sample set and growth conditions. Top row, ethidium bromide-stained agarose gel; middle row, hybridization with the ribosomal housekeeping gene L32; bottom row, hybridization with a cDNA probe consisting of the 3′ 1.1 kb of the *mhpk1* mRNA.

mones to their receptor, Ste20 is believed to transmit a signal to Ste11 which acts as a MAPKKK for the Ste7, FUS3/KSS1–MAPK cascade. While GCK was identified in a differential cDNA screen on the basis of its expression in germinal center B lymphocytes (Katz *et al.*, 1994), Tpl-2 was isolated from T cell lymphomas as a tumor progression locus after proviral integration (Patriotis *et al.*, 1993). Tpl-2, in contrast to other described MAPKKKs, activates the SAPK and MAPK pathways equally well upon overexpression in COS1 cells. MUK, which was cloned by a degenerate RT–PCR screen for MAPKKKs, displays strong structural homology to the family of mixed lineage kinases (MLKs), implicating these molecules as possible components of MAPK-type pathways.

Here we describe the molecular cloning of a novel serine/threonine kinase based on its expression in mouse hematopoietic progenitor cells. We named the molecule hematopoietic progenitor kinase 1 (mHPK1). Upon transfection into COS1 cells, HPK1 acts as a potent upstream activator of the SAPK/JNK pathway. We demonstrate that HPK1 activates SAPK via the mixed lineage kinase MLK-3, which in turn activates SEK1 (see accompanying paper, Tibbles *et al.*, 1996). These data establish a novel pathway to SAPK activation, demonstrating integration of multiple signals at different levels of a 'three-kinase' MAPK module.

Results

HPK1, a novel hematopoietic kinase

We recently have developed an approach that allows the identification and molecular cloning of genes that are differentially expressed in individual cells at defined stages of hematopoietic differentiation (Brady *et al.*, 1990, 1993). After amplification of representative cDNA samples from single cells of known biological potential, stage- and cell-specific 3′ sequence tags can be obtained by subtractive hybridization (Brady *et al.*, 1995). Focusing on genes expressed preferentially in pluripotent hematopoietic precursor cells, we identified a 331 bp 3′ sequence tag that defined a novel transcript in murine multi- and bipotent progenitors (Figure 1) and appeared to be down-regulated in proliferating progeny that were committed to single lineages. We found a corresponding 2.8 kb message to be expressed in a variety of hematopoietic cell lines and

Table I. Expression of *mhpk1* mRNA in cultured cells

Cells expressing *mhpk1* [a]		
Primary cells[b]		
fetal liver B220^{+} growing in IL-7		
macrophages growing in IL-3 and/or M-CSF		
CD8^{+} T cells stimulated with conA and grown in IL-2		
Hematopoietic cell lines[a,c]		
myeloid	FDC-P1	P815
	32Dcl3	DA-1
B-lymphoid	70Z/3	WEHI231
	J558	IIB4 2.1
	CB17 3A10	CB17 3I8
	SCID C6	SCID C8
T-lymphoid	CTLL-2	YAC-1
	EL-4	BW5147.3
	RBL-5	
Fibroblastic cell lines not expressing *mhpk1* mRNA[a,c]		
	NIH 3T3	95/1.7

[a]Samples were probed with a cDNA fragment encompassing 1.1 kb of *mhpk1* 3′ sequence.
[b]Expression was determined by hybridization of amplified cDNA samples from small cell populations (Cumano *et al.*, 1992).
[c]Northern blot analysis of 5 μg of poly(A)[+] RNA.

primary hematopoietic cell populations but not in fibroblastic cell lines (Table I). Using the 331 bp 3′ tag, we isolated a near full-length cDNA clone from the bipotent pre-B cell/macrophage cell line 70Z/3. Missing 5′ sequence was obtained using the 5′ RACE protocol. The deduced amino acid sequence suggested a novel 97 kDa protein kinase which we designated murine hematopoietic progenitor kinase 1 (mHPK1) (Figure 4A).

Widespread expression of mHPK1 mRNA during embryonic development becomes restricted to hematopoietic organs in the adult

Northern blot analysis detected low level expression of two distinct transcripts for mHPK1 (2.8 and 3.6 kb) in all tissues tested at embryonic stage E16.5 (Figure 2A). The 2.8 kb transcript was particularly abundant in lung/heart and fetal liver. In the neonate, expression of the 2.8 kb transcript was restricted to lung, thymus, liver and kidney, while brain expressed only the larger 3.6 kb message (Figure 2B). The significance of the difference in transcript
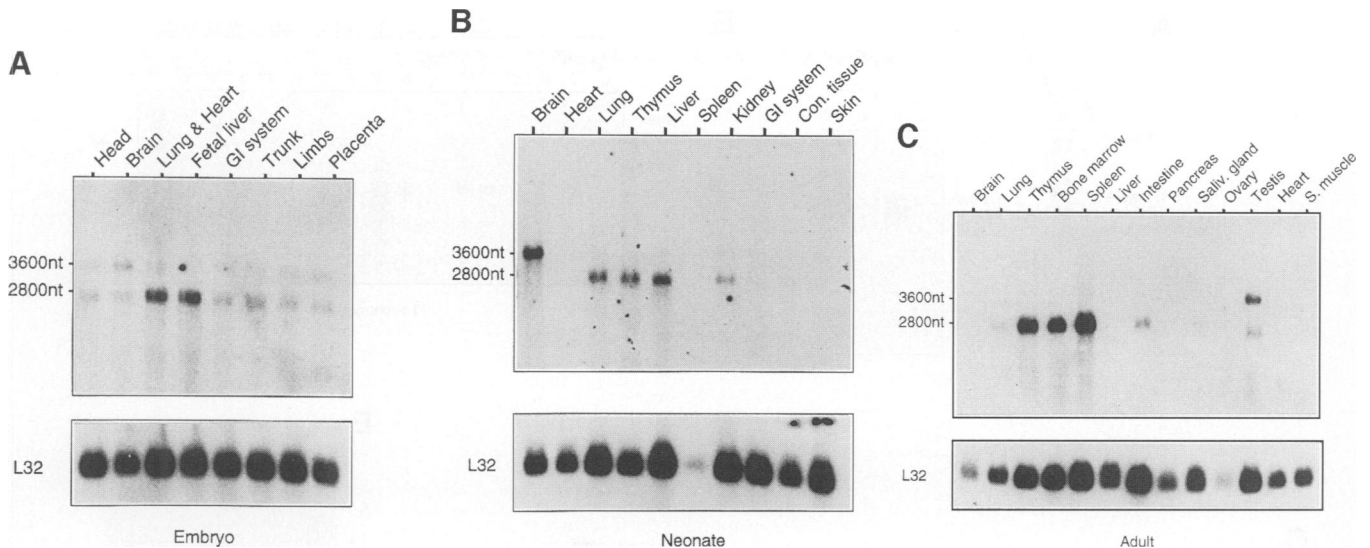
Fig. 2. Tissue-specific expression of *mhpk1* mRNA at different developmental stages. Nylon membranes containing 5 μg of poly(A)$^+$ RNA were probed with a cDNA probe corresponding to the 3' 1.1 kb of *mhpk1* mRNA. Hybridization with a probe for the ribosomal L32 gene indicated the amount and integrity of RNA. (**A**) Embryonic stage E16.5, 5 day exposure, (**B**) neonate, 5 day exposure, (**C**) adult, 12 h exposure. GI system, gastro-intestinal system; Con. tissue, connective tissue; Saliv. gland, salivary gland; S. muscle, skeletal muscle.

size is not yet known. In adult thymus, bone marrow, spleen and, at a lower level, intestine, only the 2.8 kb transcript was expressed. Both transcripts were detected in testis, and neither was detected elsewhere, including the brain (Figure 2C).

## mHPK1 kinase activity can be detected in hematopoietic cell lines and tissues

Two polyclonal rabbit antisera (designated #5 and #6) directed against a synthetic peptide deduced from the primary structure of mHPK1 immunoprecipitated a protein of the expected size of 97 kDa in *in vitro* transcription–translation experiments (Figure 3A). When incubated with cytoplasmic extracts prepared from the hematopoietic cell lines FDC-P1, 70Z/3, WEHI-3, DA-1 and 32D cl13, which all express the 2.8 kb mRNA for mHPK1 (Table I), these sera precipitated a protein of 97 kDa which was phosphorylated *in vitro* by a kinase activity present in the precipitate (Figure 3B). Phosphorylation of the 97 kDa protein *in vitro* was not influenced by the addition of protein phosphatase inhibitors during cell lysis and immunoprecipitation. The 97 kDa phosphoprotein was absent from extracts prepared from the fibroblastoid lines 95/1.7 and NIH 3T3 that lack mRNA for mHPK1 (Figure 3B). Using identical conditions, we were able to precipitate a 97 kDa protein that was phosphorylated *in vitro* from bone marrow, spleen, lymph node and thymus (Figure 3C). To investigate further the expression of mHPK1 in different primary hematopoietic cell types, we prepared T cell-depleted splenocytes by complement-mediated lysis of Thy1.2$^+$ T cells. In the resulting preparation, which consists mainly of B cells and a smaller component of myeloid cells, the kinase activity of 97 kDa was detected readily (Figure 3C; lane Spl-T), indicating that at least one of these cell populations expresses mHPK1. Similarly, when we activated T cells from peripheral lymph node by exposure to immobilized anti-CD-3 antibodies and subsequent culture for 1–3 days in the presence of IL-2, mHPK1-associated kinase activity was detected in the

resulting blast cell population (Figure 3C; lane LN+T). The same antisera #5 and #6 did not precipitate a 97 kDa kinase activity from brain and liver extracts. We conclude, on the basis of the RNA and protein expression data, that in the adult mouse mHPK1 is expressed exclusively in the myeloid and lymphoid hematopoietic lineages.

In immunoprecipitation experiments, mHPK1 appears as a 97 kDa protein which is phosphorylated in the presence of Mg^{2+} and [γ-^{32}P]ATP. To determine if phosphorylation is due to autophosphorylation or trans-phosphorylation by a tightly associated kinase, we generated a mutant protein by substituting the lysine residue at position 46 in the kinase domain (Figure 3D) with glutamic acid (K46E), a change known to destroy the activity of other protein kinases by disrupting ATP binding (Gibbs and Zoller, 1991). While the kinase activity associated with wild-type mHPK1 was detected readily in transfected COS1 cells, no mHPK1 phosphorylation was observed after transfection of the kinase-dead K46E mutant, despite comparable expression levels of both proteins (Figure 3D). These results suggest that HPK1 undergoes autophosphorylation *in vitro*. Phosphoamino acid analysis of *in vitro* autophosphorylated mHPK1 detected P-Ser and P-Thr only, suggesting that the catalytic activity of mHPK1 is specific to serine and threonine residues (Figure 3E).

mHPK1 and the human GCK belong to a family of Ste20-related protein kinases

A database scan (Altschul *et al.*, 1990) identified human GCK (hGCK), a serine/threonine kinase cloned from human germinal center B lymphocytes (Katz *et al.*, 1994) as the protein most closely related to HPK1 (Figure 4A). Our full length mHPK1 cDNA clone was used to identify and clone its human homolog hHPK1 (M.Hu, personal communication) with which it shares 85% amino acid identity. In contrast, both homologs share only 45% identity with hGCK. All three proteins share the overall structure of an N-terminally located kinase domain (amino acids 12–276 in mHPK1 and 9–274 in hGCK) and a long
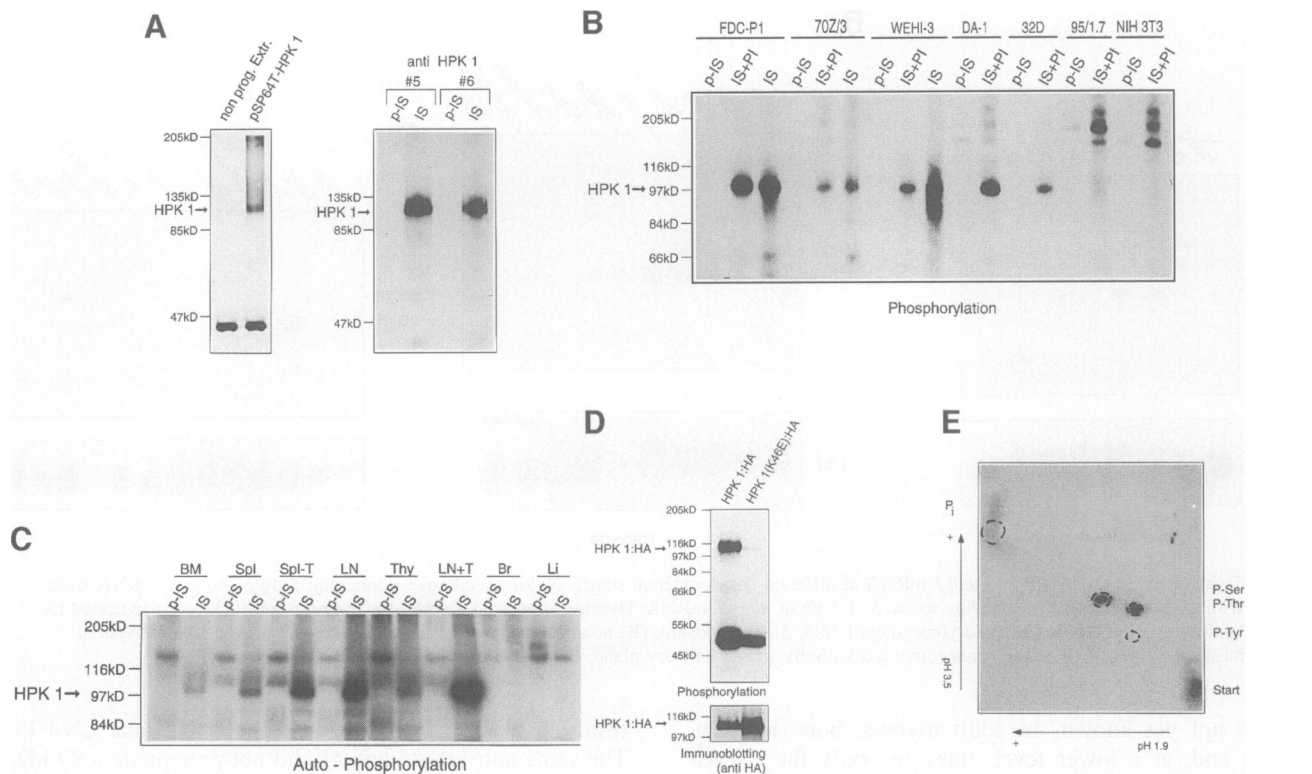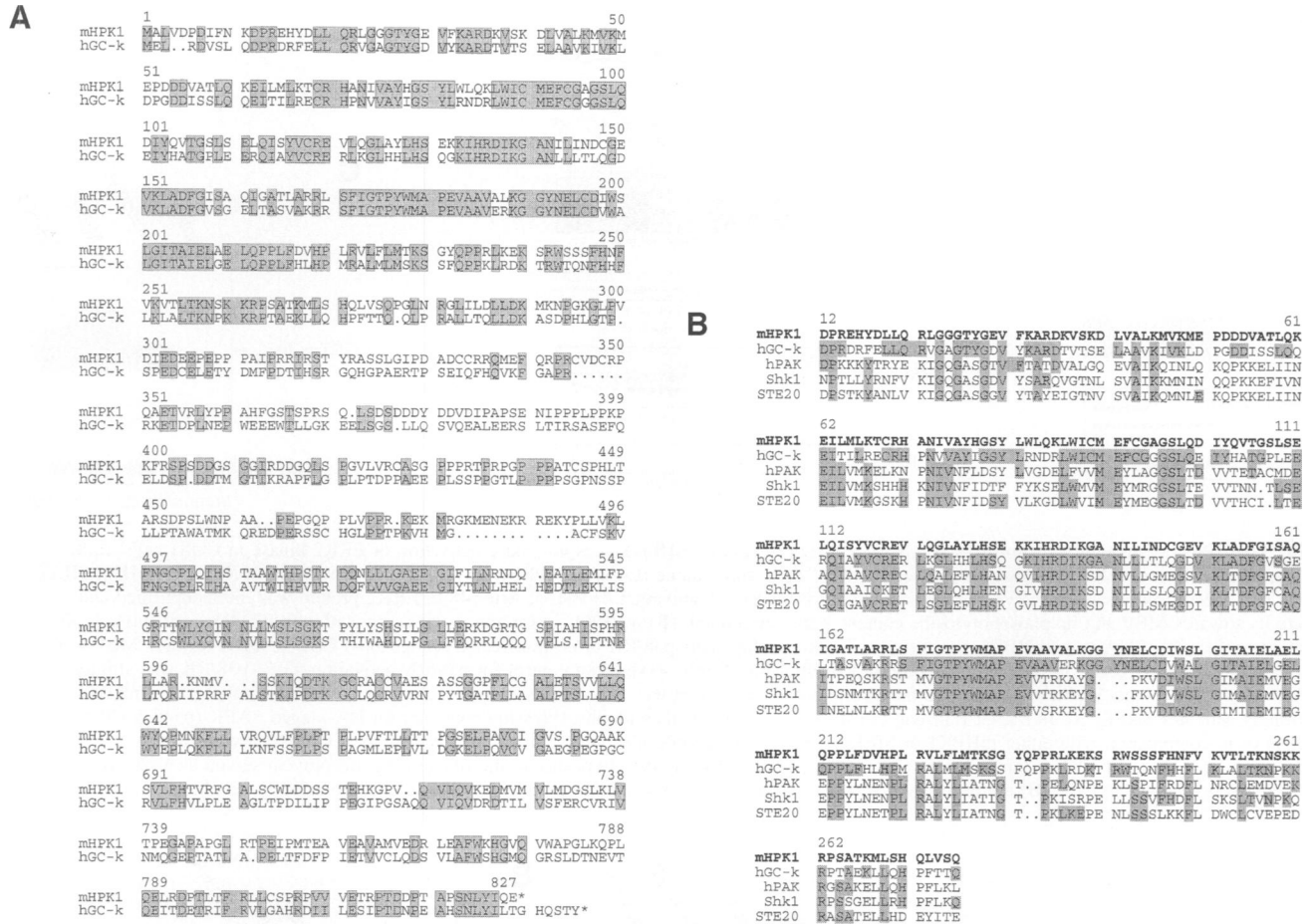
Fig. 3. Characterization of mHPK1. (**A**) *In vitro* translation of 2 μg of *in vitro* transcribed RNA from pSP64T-HPK1 in the presence of [^{35}S]methionine. An aliquot of the reaction was separated by SDS–PAGE and translation products were visualized by autoradiography. An equal aliquot was immunoprecipitated using the polyclonal anti-peptide rabbit antisera #5 and #6. After collection of immune complexes on protein A beads and washing, bound proteins were eluted and separated by SDS–PAGE. non prog. Extr., non-programed reticulocyte extract; p-IS, pre-immune serum; IS, immune serum. (**B**) Immunoprecipitation and *in vitro* kinase activity of mHPK1 in murine hematopoietic cell lines. mHPK1 was immunoprecipitated from lysates of the indicated cell lines. The experiment was performed either in the presence or absence of protein phosphatase inhibitors (PI). Immune complexes were harvested on protein A–Sepharose beads, washed and incubated in the presence of [γ-^{32}P]ATP. Reaction products were separated by SDS–PAGE and visualized by autoradiography. (**C**) Immunoprecipitation and *in vitro* kinase activity of mHPK1 prepared from primary tissues and cell populations. Bone marrow (BM), spleen (Spl), thymus (Thy), lymph node (LN), brain (Br) and liver (Li) cells were obtained from 4-week-old C57Bl/6J mice. Cleared lysates of the different tissues were analyzed as described in (B). Spl-T, spleen cells depleted of Thy1.2$^+$ T cells by complement-mediated lysis. LN+T, blast cell population from lymph node after stimulation with immobilized anti-CD3 antibodies and 3 day growth in the presence of IL-2. (**D**) mHPK1 is an active protein kinase. Upper panel: COS1 cells transiently transfected with either pMT2-HPK1:HA transducing C-terminally HA-tagged wild-type mHPK1 (HPK1:HA) or pMT2-HPK1(K46E):HA transducing the C-terminally HA-tagged kinase-deficient K46E mutation of mHPK1. After immunoprecipitation of mHPK1, the harvested immunocomplexes were split and one half was subjected to an *in vitro* kinase reaction as described in (C). The second half was used to demonstrate equal protein levels of HPK1:HA and HPK1(K46E):HA using anti-HA immunoblotting. (**E**) Phosphoamino acid analysis of *in vitro* autophosphorylated mHPK1. Ghosted circles indicate the positions of reference phosphoamino acids and P$_i$ after ninhydrin staining. All results presented in this figure are representative of two independent experiments.

C-terminal tail that has little resemblance to other known sequences and that presumably has a regulatory function. Other members of the kinase family most closely related in their kinase domains to mHPK1 include human p21-activated protein kinase (hPAK2) (Chernoff, GenBank accession No. U24153), Cdc42 binding protein of fission yeast *Schizosaccharomyces pombe* (Shk1) (Marcus *et al.*, 1995) and Ste20 of budding yeast *Saccharomyces cerevisiae* (Ramer and Davis, 1993) (Figure 4B). mHPK1 and hGCK are clearly distinct from other members of the growing family of Ste20-related protein kinases in their specific structural organization, their lack of a Cdc42/Rac1 interactive binding motif (CRIB motif; Burbelo *et al.*, 1995) and their mol. wt of ~ 97 kDa. In mammals, the CRIB motif-containing members of the PAK family of Ste20-related kinases are stimulated by the GTP-bound forms of Rac1 and Cdc42 (Bagrodia *et al.*, 1995; Knaus *et al.*, 1995; Zhang *et al.*, 1995) and have been implicated in the stimulation of MAPK pathways (Brown *et al.*, 1996). The homology between mHPK1 and these activators of

MAPK pathways suggested the possible involvement of mHPK1 in the activation of a cytoplasmic kinase cascade.

mHPK1 efficiently activates the SAPK pathway in vitro

Possible activation of mammalian MAPK pathways was tested by co-transfection of mHPK1 cDNA with either ERK2, p54β-SAPK or p38/RK into COS1 cells. In addition, we tested for activation of endogenous p38/RK by HPK1. Expression of mHPK1 neither resulted in elevated ERK2 or p38/RK kinase activity towards myelin basic protein (MBP), nor induced increased tyrosine phosphorylation of these MAPKs. Furthermore, HPK1 did not interfere with phosphorylation and activation of ERK2 and p38/RK by established agonists (Figure 5A and B). However, the kinase activity of p54β-SAPK towards its physiological target, the N-terminus of c-Jun, was on average >50-fold elevated after co-expression of mHPK1 (Figure 5C), a degree of activation comparable with the stimulation achieved by the known SAPK activator MEKK1 (Yan

A

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                1                                                           50
mHPK1    MALVDPDIFN KDPREHYDLL QRLGGGTYGE VFKARDKVSK DLVALKMVKM
hGC-k    MFL..RDVSL QDPRDRFELL QRVGAGTYGD VYKARDTVTS ELAAVKIVKL

                51                                                          100
mHPK1    EPDDDVATLQ KEILMLKTCR HANIVAYHGS YLWLQKLWIC MEFCGAGSLQ
hGC-k    DPGGDDISSLQ QEITILRECR HPNVVAYIGS YLRNDRLWIC MEFCGGGSLQ

                101                                                         150
mHPK1    DIYQVTGSLS ELQISYVCRE VLQGLAYLHS EKKIHRDIKG ANILINDCGE
hGC-k    EIYHATGPLE ERQIAYVCRE RLKGLHHLHS QGKIHRDIKG ANLLLTLQGD

                151                                                         200
mHPK1    VKLADFGISA QLGATLARRL SFIGTPYWMA PEVAAVALKG GYNELCDIWS
hGC-k    VKLADFGVSG ELTASVAKRR SFIGTPYWMA PEVAAVERKG GYNELCDVWA

                201                                                         250
mHPK1    LGITAIBLAE LQPPLFDVHP LRVLFLMTKS GYQPPRLKEK SRWSSSFHNF
hGC-k    LGITAIELGE LQPPLFHLHP MRALMLMSKS SFQPPKLRDK TRWTQNFHHF

                251                                                         300
mHPK1    VKVTLTKNSK KRESATKMLS HQLVSQPGLN RGLIILDLLDK MKNPGKGLFV
hGC-k    LKLALTKNFK KRPTAEKLLQ HPFTTQ.QLP RALLTQLLDK ASDPHLGTE.

                301                                                         350
mHPK1    DIEDEEPEPP PAIERRIRST YRASSLGIPD ADCCRRQMEF QRPRCVDCRP
hGC-k    SPEDCELETY DMFPDTLHSR GQHGPAERTP SEIQFHQVKF GAPH......

                351                                                         399
mHPK1    QAETVRLYPP AHFGSTSPRS Q.LSDSDDDY DDVDIPAPSE NIPPPLPPKP
hGC-k    RKETDPLNEP WEEEWTLLGK EELSGS.LLQ SVQEALEERS LTIRSASEFQ

                400                                                         449
mHPK1    KFRSPSDDGS GGIRDDGQLS EGVLVRCASG PPPRTPRPGP PPATCSPHLT
hGC-k    ELDSP.DDTM GTIKRAPFLG ELPTDPPAEE PLSSPPGTLP PPPSGPNSSP

                450                                                         496
mHPK1    ARSDPSLWNP AA..PEPGQP PLVPPR.KEK MRGKMENEKR REKYPLLVKL
hGC-k    LLPTAWATMK QREDPERSSC HGLPPTPKVH MG........ ....ACFSKV

                497                                                         545
mHPK1    FNGCPLQTHS TAANTHESTK DQNLLLGAEE GIFILNRNDQ .EATLEMIFP
hGC-k    FNGCPLRTHA AVTWIHEVHR DQFLVVGAEE GIYTLNLHEL HEDTLEKLIS

                546                                                         595
mHPK1    GRTTWLYCIN NLLMSLSGKT PYLYSHSILG LLERKDGRTG SEIAHISPHR
hGC-k    HRCSWLYCVN NVLLSLSGSS THIWAHDLPG LFEQRRLQQQ VELS.IPTNR

                596                                                         641
mHPK1    LLAR.KNMV. ..SSKIQDTK GCRACCVAES ASSGGPFLCG ALETSVVLLQ
hGC-k    LTQRIIPRRF ALSTKIPDTK GCLQCRVVRN PYTGATFLLA ALPTSLLLLQ

                642                                                         690
mHPK1    WYQBMNKFLL VRQVLFPLPT BLPVFTLLTT PGSELPAVCI GVS.PGQAAK
hGC-k    WYPELQKFLL LKNFSSPLDS BAGMLEPLVL DGKELQKQVQV GAEGPEGPGC

                691                                                         738
mHPK1    SVLFHTVRFG ALSCWLDDSS TEHKGPV..Q VIQVKEDMVM VLMDGSKLKI
hGC-k    RVLFHVLPLE AGLTPDILIP PEGIPGSAQQ VIQVDRDTIL VSFERCVRIV

                739                                                         788
mHPK1    TPEGABAPGI RTPEIPMTEA VEAVAMVEDR LEAFWkHGVQ VWAPGLKQPL
hGC-k    NMQGEFTATL A.PELTFDFP IBTVVCLQDS VLAFWSHGMQ GRSLDTNEVT

                789                                         827
mHPK1    QELRDPTLTF RLICSPRPVV VETRPTDDPT APSNLYIQE*
hGC-k    QEITDETRIF RVLGAHRDII LESIPTDNEB AHSNLYILTG HQSTY*
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B

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                12                                                          61
mHPK1    DPREHYDLLQ RLGGGTYGEV FKARDKVSKD LVALKMVKME PDDDVATLQK
hGC-k    DPRDRFELLQ RVGAGTYGDV YKARDTVTSE LAAVKIVKLD PGDDISSLQQ
hPAK     DPKKKVTRYE KIGQGASGTV FTATDVALGQ EVAIKQINLQ KQPKKELIIN
Shk1     NPTLLYRNFV KIGQGASGDV YSARQVGTNL SVAIKKMNIN QQPKKEFIVN
STE20    DESTKYANLV KIGQGASGGV YTAYEIGTNV SVAIKQMNLE KQPKKELIIN

                62                                                          111
mHPK1    EILMLKTCRH ANIVAYHGSY LWLQKLWICM EFCGAGSLQD IYQVTGSLSE
hGC-k    EITILRECRH PNVVAYIGSY LRNDRLWICM EFCGGGSLQE IYHATGPLEE
hPAK     EILVMKELKN PNIVNFLDSY LVGDELFVVM EYLAGGSLTD VVTEIACMDE
Shk1     EILVMKSHHH KNIVNFIDTF FYKSELWMVM EYMRGGSLTE VVTNN.TLSE
STE20    EILVMKGSKH PNIVNFIDSY VLKGDLWVIM EYMEGGSLTD VVTHCI.LTE

                112                                                         161
mHPK1    LQISYVCREV LQGLAYLHSE KKIHRDIKGA NILINDCGEV KLADFGISAQ
hGC-k    RQIAYVCRER LKGLHHLHSQ GKIHRDIKGA NLLLTLQSDV KLADFGVSGE
hPAK     AQIAAVCREC LQALEFLHAN QVIHRDIKSD NVLLGMEGSV KLTDFGFCAQ
Shk1     GQIAAICKET LEGLQHLHSN GIVHRDIKSD NILLSLQGDI KLTDFGFCAQ
STE20    GQIGAVCRET LSGLEFLHSK GVLHRDIKSD NILLSMEGDI KLTDFGFCAQ

                162                                                         211
mHPK1    IGATLARRLS FIGTPYWMAP EVAAVALKGG YNELCDIWSL GITAIELAEL
hGC-k    LTASVAKRRS FIGTPYWMAP EVAAVERKGG YNELCDVWAL GITAIELGEL
hPAK     ITPEQSKRST MVGTPYWMAP EVVTRKAYG. ..PKVDIWSL GIMAIEMVEG
Shk1     IDSNMTKRTT MVGTPYWMAP EVVTRKEYG. ..FKVDVWSL GIMAIEMVEG
STE20    INELNLKRTT MVGTPYWMAP EVVSRKEYG. ..PKVDIWSL GIMILEMIEG

                212                                                         261
mHPK1    QPPLFDVHPL RVLFLMTKSG YQPPRLKEKS RWSSSFHNFV KVTLTKNSKK
hGC-k    QPPLFHLHPM RALMLMSKSS FQPPKLRDKT RWTQNFHHFL KLALTKNFKK
hPAK     EPPYLNENPL RALYLIATNG T..PELQNPE KLSPIFRDFL NRCLEMDVEK
Shk1     EPPYLNENPL RALYLIATIG T..PKISRPE LLSSVPHDFL SKSLITVNPKQ
STE20    EPPYLNETPL RALYLIATNG T..PKLKEPE NLSSSLKKFL DWCLCVEPED

                262
mHPK1    RPSATKMLSH QLVSQ
hGC-k    RPTAEKLLQH PFTTQ
hPAK     RGSAKELLQH PFLKL
Shk1     RPSSGELLRH PFLKQ
STE20    RASATELLHD EYITE
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Fig. 4. Primary structure of mHPK1. (**A**) Alignment of the primary sequence of mHPK1 with human germinal center kinase (hGC-k). mHPK1 and hGC-k are 64% similar and share 45% identical residues (shaded). (**B**) Alignment between the kinase domains of mHPK1 and five homologous kinases. The percentages of identity/conservation are: hGC-k 64/83; hPAK2 40/61; Shk1 38/61; Ste20 43/61.

et al., 1994). To test for possible induction of an autocrine loop, we tested supernatant media of cells transiently expressing mHPK1 and failed to detect stimulation of p54β-SAPK activity (not shown). Together, these results suggest direct and specific action of HPK1 on the SAPK pathway.

mHPK1 kinase activity is required for stimulation of the SAPK pathway

We next asked whether both the kinase domain and C-terminal portion of the HPK1 protein were required for SAPK activation. The kinase domain alone was able to activate p54β-SAPK to a degree comparable (68-fold increase in c-Jun phosphorylation) with that achieved with the full-length protein (140-fold increase in c-Jun phosphorylation, Figure 6A). The truncation product is expressed at significantly higher levels than the full-length cDNA (not shown). GST fusions lacking the kinase domain but containing the C-terminus of mHPK1, or fragments thereof, failed to stimulate SAPK to a level comparable with a full-length GST–mHPK1 fusion protein which retained activity. Furthermore, the catalytically inactive mutant mHPK1(K46E) also failed to activate SAPK significantly, directly demonstrating dependence on kinase activity (Figure 6B). The moderate increase in c-Jun phosphorylation observed after expression of N-terminal mHPK1 deletion constructs and the mHPK1(K46E) mutant

did not exceed 1/10 of the activation levels brought about by full-length HPK1 and may be within the range of experimental variation.

mHPK1 associates with the mixed lineage kinase MLK-3

To determine at which level mHPK1 activates the SAPK cascade, we first tested the effect of blockade of the pathway by SEK-AL, a dominant-negative mutant of SEK1 which is the pathway component immediately upstream of SAPK (Sanchez et al., 1994). Co-expression of SEK-AL and mHPK1 in COS1 cells substantially reduced p54β-SAPK activation by mHPK1 (Figure 7A), suggesting that mHPK1 exerts its action upstream of SEK1. When we probed for physical interaction between mHPK1 and MEKK1, co-precipitation was not detected (not shown). Using yeast two-hybrid analysis, we tested for direct interaction partners of mHPK1. Several proline-rich motifs located C-terminally of the mHPK1 kinase domain (Figure 7B) suggested that mHPK1 might be recognized by the SH3 domains of other signaling molecules. We therefore probed the ability of fusions between these proline-rich motifs and the yeast Gal4 transactivation domain to interact with fusions between the Gal4 DNA binding domain and several SH3 domains. While the C-terminal SH3 domain of Crk showed no affinity, the SH3 domain of Abl bound to the HPK1–Gal4 bait (Figure
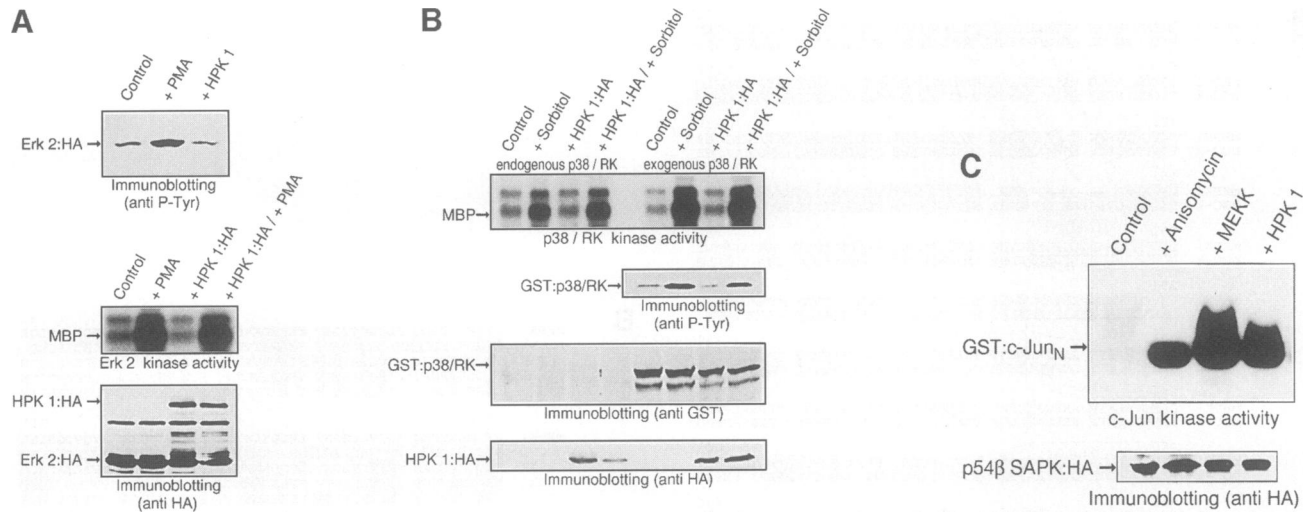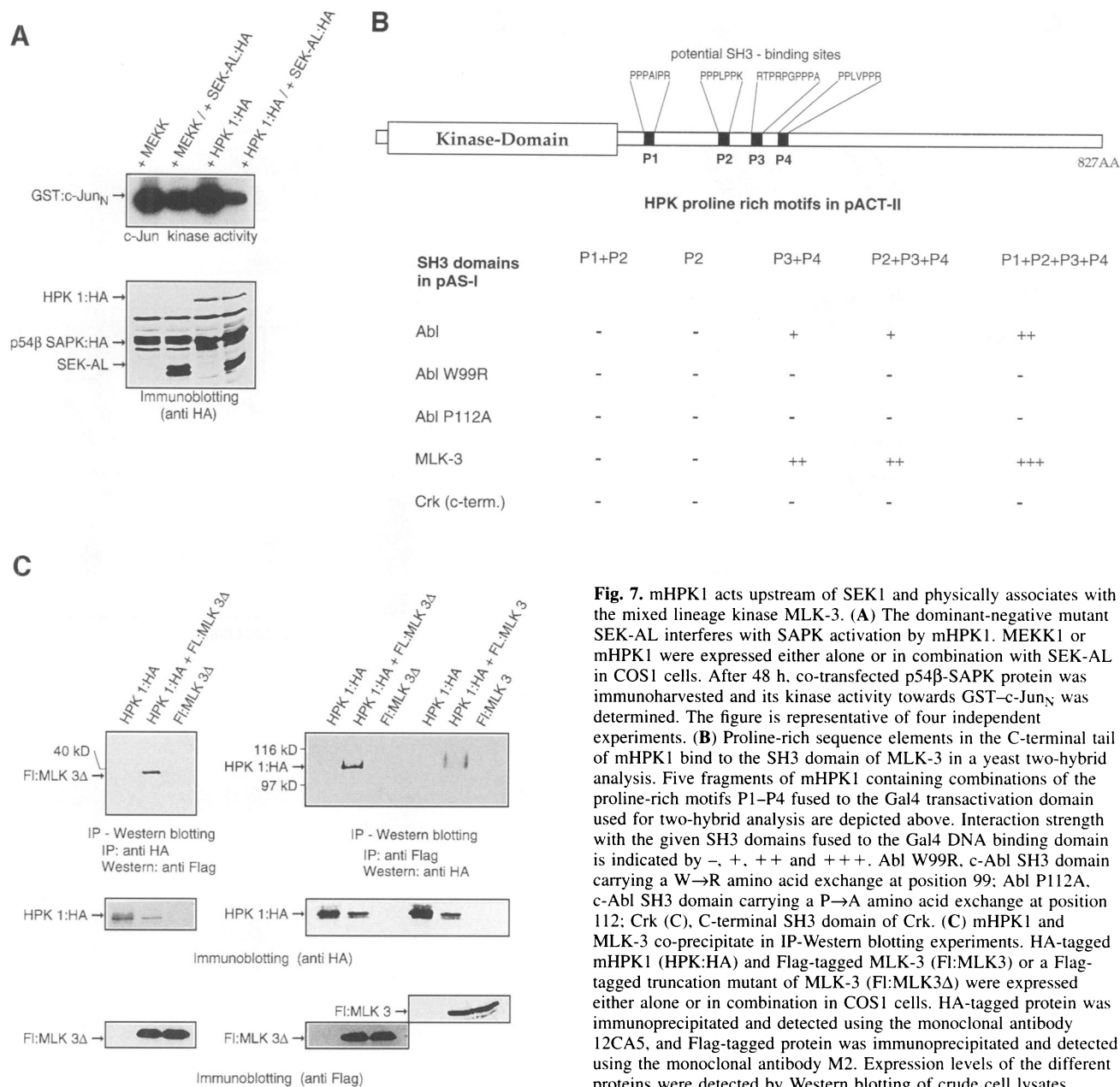
Fig. 5. mHPK1 is a potent activator of SAP kinase. (**A**) Expression of mHPK1 does not cause activation of ERK2 kinase in COS1 cells. Epitope-tagged ERK2 kinase (ERK2:HA) was expressed in COS1 cells either alone (Control, +PMA) or together with mHPK1 (+HPK1 or +HPK1:HA). The non-specific agonist PMA served as a positive control for ERK2 activation (+PMA). After 48 h, ERK2 protein was immunopurified and its activity towards MBP and its phosphotyrosine content were determined. (**B**) mHPK1 expression is not associated with p38/RK activation. At 48 h after transfection of HA-tagged HPK1, the activity of either endogenous p38/RK or co-transfected (exogenous) GST–p38/RK towards MBP was determined in COS1 cells. Stimulation with 300 mM sorbitol for 40 min served as a control for p38/RK activation. GST–p38/RK was affinity purified on glutathione–Sepharose beads and the phosphotyrosine content was analyzed by anti-phosphotyrosine immunoblotting. (**C**) mHPK1 strongly activates SAPK. At 48 h after transient transfection of COS1 cells with an expression construct for HA-tagged SAPK (p54β-SAPK:HA) alone or in combination with either mHPK1 or MEKK1, p54β-SAPK molecules were affinity purified and their kinase activity towards a recombinant N-terminal GST–c-Jun fragment (GST:c-Jun$_N$) was tested *in vitro*. Exposure to the fungal drug anisomycin served as a positive control for SAPK activation. (A) and (B) are representative of two independent experiments and (C) was repeated independently in a large number of transfection experiments (>20).
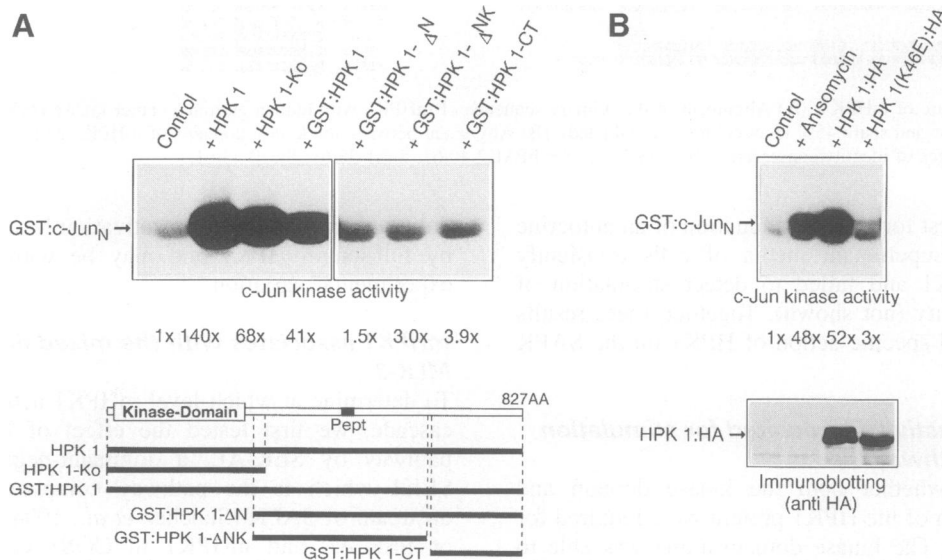
Fig. 6. Activation of SAPK by mHPK1 is dependent on its catalytic activity. (**A**) The kinase domain of mHPK1 but not the C-terminal part of the molecule activates SAPK. p54β-SAPK:HA was expressed alone or with either full-length mHPK1 (HPK1), the kinase domain of mHPK1 (HPK1-Ko), a GST fusion of mHPK1 (GST:HPK1), a GST fusion of the complete C-terminal part of mHPK1 (GST:HPK1-$_\Delta$N) or fragments thereof (GST:HPK1-$_\Delta$NK, GST:HPK1-CT). The regions of mHPK1 included in the various constructs are shown underneath. Expression of the GST fusion proteins was confirmed by Western blotting with polyclonal anti-GST rabbit antiserum (not shown). p54β-SAPK kinase activity towards recombinant GST–c-Jun$_N$ was determined 48 h after transfection. Numbers represent the fold increase over control in ^{32}P incorporation as determined by volume integration using a PhosphorImager (Molecular Dynamics). (**B**) Activation of SAPK by mHPK1 is dependent on mHPK1 kinase activity. p54β-SAPK was expressed in COS1 cells alone (Control) or in combination with either HA-tagged mHPK1 (HPK1:HA) or the HA-tagged K46E mutant of mHPK1 [HPK1(K46E):HA]. After 48 h, p54β-SAPK protein was harvested and its kinase activity towards GST–c-Jun$_N$ was determined. The fold increase in ^{32}P incorporation is given underneath. Equivalent protein expression of HPK1:HA and HPK1(K46E):HA was demonstrated by immunoblotting. The data are representative of three independent experiments.

7B). This interaction was abrogated by substitutions of residues in the Abl SH3 domain known to be important for binding proline-rich ligands, suggesting a specific association of the Abl SH3 domain with the HPK1 C-terminal region. While the biological significance of this interaction remains to be demonstrated, a qualitatively strong binding of MLK-3 (Ezoe *et al.*, 1994; Gallo *et al.*, 1994; Ing *et al.*, 1994) SH3 domain was highly suggestive

A

c-Jun kinase activity

GST:c-Jun_N

Immunoblotting (anti HA)

HPK 1:HA →
p54β SAPK:HA →
SEK-AL →

B

potential SH3 - binding sites

PPPAIPR PPPLPPK RTPRPGPPPA PPLVPPR

Kinase-Domain

P1   P2   P3 P4

827AA

HPK proline rich motifs in pACT-II

SH3 domains in pAS-I	P1+P2	P2	P3+P4	P2+P3+P4	P1+P2+P3+P4
Abl	-	-	+	+	++
Abl W99R	-	-	-	-	-
Abl P112A	-	-	-	-	-
MLK-3	-	-	++	++	+++
Crk (c-term.)	-	-	-	-	-

C

40 kD
Fl:MLK 3Δ →

IP - Western blotting
IP: anti HA
Western: anti Flag

116 kD
HPK 1:HA →
97 kD

IP - Western blotting
IP: anti Flag
Western: anti HA

HPK 1:HA →

Immunoblotting (anti HA)

HPK 1:HA →

Fl:MLK 3Δ

Immunoblotting (anti HA)

Fl:MLK 3Δ

Fl:MLK 3 →

Fl:MLK 3Δ →

Immunoblotting (anti Flag)

Fig. 7. mHPK1 acts upstream of SEK1 and physically associates with the mixed lineage kinase MLK-3. (**A**) The dominant-negative mutant SEK-AL interferes with SAPK activation by mHPK1. MEKK1 or mHPK1 were expressed either alone or in combination with SEK-AL in COS1 cells. After 48 h, co-transfected p54β-SAPK protein was immunoharvested and its kinase activity towards GST–c-Jun_N was determined. The figure is representative of four independent experiments. (**B**) Proline-rich sequence elements in the C-terminal tail of mHPK1 bind to the SH3 domain of MLK-3 in a yeast two-hybrid analysis. Five fragments of mHPK1 containing combinations of the proline-rich motifs P1–P4 fused to the Gal4 transactivation domain used for two-hybrid analysis are depicted above. Interaction strength with the given SH3 domains fused to the Gal4 DNA binding domain is indicated by –, +, ++ and +++. Abl W99R, c-Abl SH3 domain carrying a W→R amino acid exchange at position 99; Abl P112A, c-Abl SH3 domain carrying a P→A amino acid exchange at position 112; Crk (C), C-terminal SH3 domain of Crk. (**C**) mHPK1 and MLK-3 co-precipitate in IP-Western blotting experiments. HA-tagged mHPK1 (HPK:HA) and Flag-tagged MLK-3 (Fl:MLK3) or a Flag-tagged truncation mutant of MLK-3 (Fl:MLK3Δ) were expressed either alone or in combination in COS1 cells. HA-tagged protein was immunoprecipitated and detected using the monoclonal antibody 12CA5, and Flag-tagged protein was immunoprecipitated and detected using the monoclonal antibody M2. Expression levels of the different proteins were detected by Western blotting of crude cell lysates.

of a possible involvement of this molecule in HPK1 signaling. The interaction was mediated mainly by the polyproline motifs P3 and P4 (Figure 7B), while the additional presence of P1 led to a further affinity enhancement.

To substantiate further a possible interaction between HPK1 and MLK-3, we co-expressed epitope-tagged versions of both molecules in COS1 cells. When we co-transfected hemagglutinin (HA)-tagged HPK1 (HPK1:HA) and a Flag-tagged C-terminal truncation mutant of MLK-3 (Fl:MLK3Δ), we detected Fl:MLK3Δ in anti-HA immuno-precipitates by anti-Flag Western blotting (Figure 7C). The truncation mutant Fl:MLK3Δ consists of the N-terminal glycine-rich, SH3 and kinase domains of MLK-3. FL:MLK3Δ lacks the entire C-terminus of MLK-3 and, due to the absence of the 26 C-terminal amino acids of the kinase domain, this mutant does not retain catalytic activity. Conversely, after co-expression of HA-tagged

HPK1 and either Flag-tagged MLK-3 or MLK-3Δ followed by immunoprecipitation using a monoclonal anti-Flag antibody, we detected HPK1 in both anti-Flag precipitates. These data suggest association of HPK1 and MLK-3 at least *in vitro* (Figure 7C).

mHPK1 phosphorylates and activates the SAPK pathway via MLK-3

To test the possibility that MLK-3 is a substrate of HPK1, we co-precipitated mHPK1 and a kinase-deficient version of MLK-3 [Fl:MLK3(K144E)]. After incubation of the precipitate in the presence of Mg^{2+} and $[\gamma-^{32}P]ATP$, we observed both autophosphorylation of mHPK1 and transphosphorylation of the kinase-dead MLK-3 mutant (Figure 8A). This result indicates that MLK-3 can be phosphorylated by HPK1 *in vitro*. We performed a mixed kinase assay containing the reverse combination, namely the kinase-dead mHPK1(K46E) variant and kinase active

A

Phosphorylation

Immunoblotting
(anti HA)

Immunoblotting
(anti Flag)

B

Reprecipitation

C

c-Jun kinase activity

Immunoblotting (anti HA)

Immunoblotting (anti Flag)
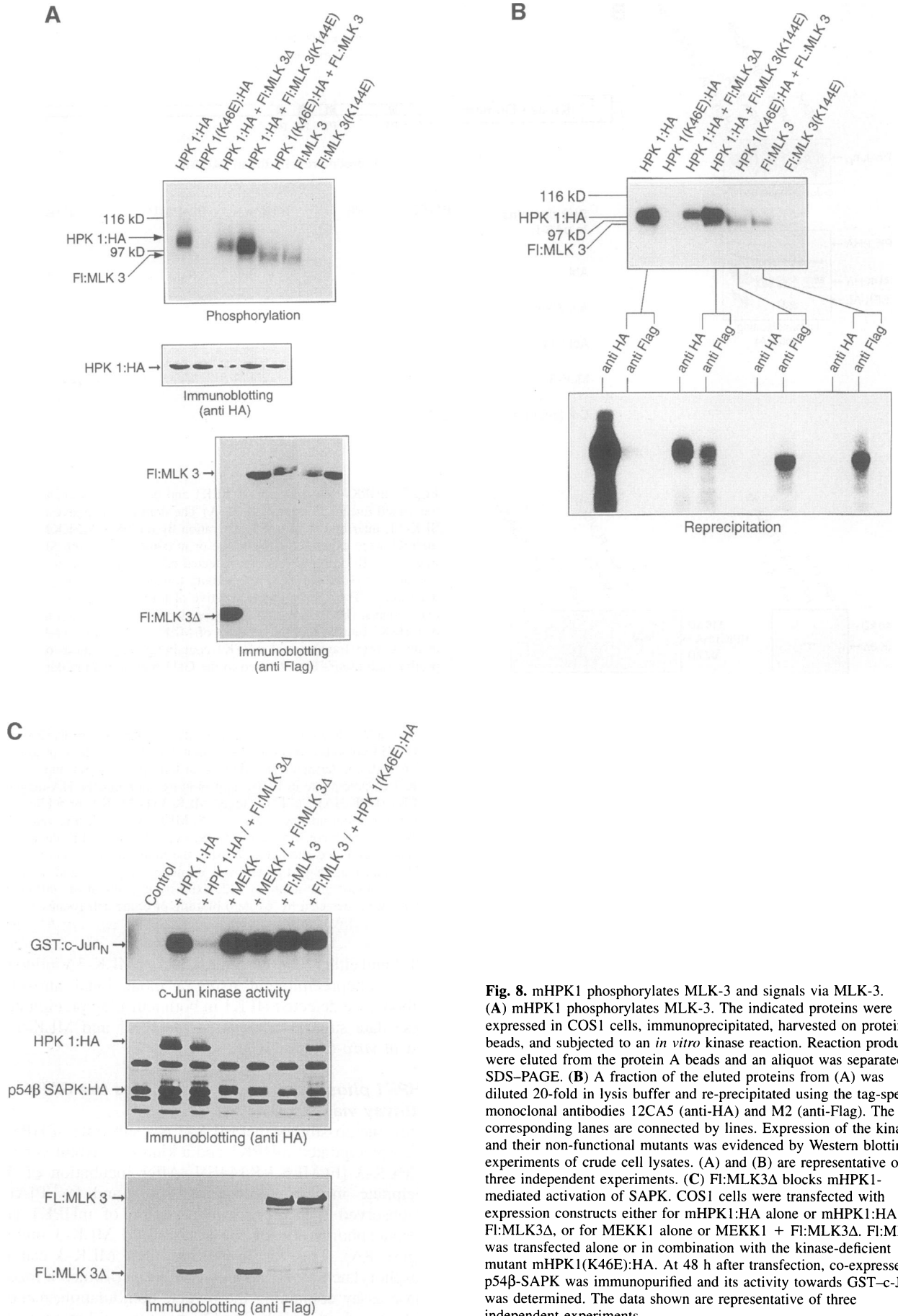
Fig. 8. mHPK1 phosphorylates MLK-3 and signals via MLK-3.
(**A**) mHPK1 phosphorylates MLK-3. The indicated proteins were
expressed in COS1 cells, immunoprecipitated, harvested on protein A
beads, and subjected to an *in vitro* kinase reaction. Reaction products
were eluted from the protein A beads and an aliquot was separated by
SDS–PAGE. (**B**) A fraction of the eluted proteins from (A) was
diluted 20-fold in lysis buffer and re-precipitated using the tag-specific
monoclonal antibodies 12CA5 (anti-HA) and M2 (anti-Flag). The
corresponding lanes are connected by lines. Expression of the kinases
and their non-functional mutants was evidenced by Western blotting
experiments of crude cell lysates. (A) and (B) are representative of
three independent experiments. (**C**) Fl:MLK3Δ blocks mHPK1-
mediated activation of SAPK. COS1 cells were transfected with
expression constructs either for mHPK1:HA alone or mHPK1:HA +
Fl:MLK3Δ, or for MEKK1 alone or MEKK1 + Fl:MLK3Δ. Fl:MLK3
was transfected alone or in combination with the kinase-deficient
mutant mHPK1(K46E):HA. At 48 h after transfection, co-expressed
p54β-SAPK was immunopurified and its activity towards GST–c-Jun$_N$
was determined. The data shown are representative of three
independent experiments.

MLK-3, to ask if MLK-3 would phosphorylate HPK1 *in vitro*. In this experiment, we only observed autophosphorylation of MLK-3 (Figure 8A), indicating that HPK1 is not a substrate for MLK-3. Because both kinases are of a similar molecular weight and consequently difficult to separate by SDS–PAGE, we eluted the phosphorylated reaction products from protein A–Sepharose beads and reprecipitated them using the tag-specific monoclonal antibodies M2 and 12CA5 (Figure 8B). From a reaction mixture of kinase-active mHPK1 and kinase-dead MLK-3, we were able to precipitate phosphorylated species of both proteins. From a mixture of kinase-dead HPK1 and kinase-active MLK-3 we only precipitated phospholabeled MLK-3. We thereby confirmed our observation that MLK-3 is a potential substrate of HPK1 *in vitro* but not vice versa. Similarly, when we eluted the kinase reaction products shown in Figure 8A and prepared two-dimensional tryptic phosphopeptide maps, the maps supported our suggestion that MLK-3 may serve as a substrate for HPK1, but HPK1 seems not to be phosphorylated by MLK-3 (not shown).

To define further the role of MLK-3 in HPK1 signaling, we co-expressed HPK1 with either the kinase-dead C-terminally truncated version of MLK-3 (FL:MLK3Δ) (Figure 8C) or the kinase-dead MLK-3 mutant Fl:MLK3(K144E) (not shown) both of which led to a pronounced inhibition of HPK1-induced SAPK activation. When we co-expressed the kinase-dead mHPK1(K46E) mutant and MLK-3 in COS1 cells, activation of the SAPK pathway by MLK-3 was not impaired, suggesting that MLK-3 might act downstream of HPK1 to regulate SAPK activation. Taken together, these data identify mHPK1 as a signaling element most likely functioning upstream of the mixed lineage kinase MLK-3 or a related MAPKKK. We show that *in vitro* HPK1 is able to phosphorylate MLK-3, and a kinase-deficient MLK-3 mutant efficiently blocks HPK1-induced stimulation of the SAPK pathway.

Discussion

Using a subtractive approach, we have cloned from single cell-derived libraries a novel signaling protein whose expression is developmentally regulated. mHPK1 is a serine/threonine kinase that is expressed widely in embryonic tissues and becomes restricted to tissues or organs that undergo lineage decisions in the neonate. In the adult mouse, HPK1 expression is restricted to hematopoietic organs and testis. Within cultured murine hematopoietic cells it is expressed mainly during a window of development that is associated with regulated growth and differentiative decisions.

Based on its homology to Ste20, we probed for HPK function as an upstream activator of a mammalian MAPK cascade and found it to be a potent and specific activator of the SAPK pathway using a transfection model. We furthermore demonstrated complex formation between HPK1 and the mixed lineage kinase MLK-3, a molecule of hitherto unknown function, after co-expression in COS1 cells. HPK1-mediated stimulation of SAPK is blocked efficiently by a kinase-dead truncation mutant of MLK-3, an observation which is in agreement with HPK1 acting upstream of MLK-3 and signaling via MLK-3. Alternatively, the dominant-negative truncation mutant MLK-3Δ
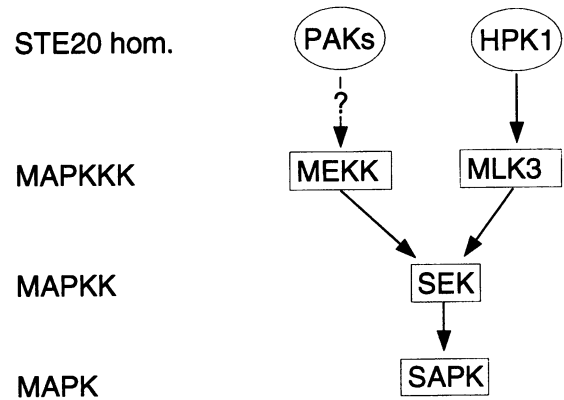

Fig. 9. Model of mHPK1 action.

may block binding of HPK1 to an unrelated kinase which is the physiological downstream element of HPK1. However, our results, in combination with the observation that MLK-3 binds and phosphorylates the SAPK activator SEK1 (see accompanying paper, Tibbles *et al.*, 1996), make MLK-3 or another MLK-3-related kinase an attractive target for HPK1 action, and suggest a novel pathway leading to SAPK activation. The interaction between HPK1 and MLK-3 most likely involves the MLK-3 SH3 domain and a stretch of proline-rich motifs downstream of the HPK1 kinase domain. Formal proof of an involvement of SH3-mediated interactions in SAPK signaling will, however, have to await the generation of mutations in either of these domains. Recently, it was shown in yeast that the transmembrane osmosensor Sho1p activates the MAPKK Pbs2p by SH3 domain-mediated interaction (Maeda *et al.*, 1995). However, in contrast to mHPK1, Sho1p is not a protein kinase and Pbs2p activation probably only requires Sho1p binding.

HPK1 is a novel member of a growing family of proteins that activate MAPK cascades. For several of these activators, their position in the hierarchy of interacting kinases and their physiological substrates have not yet been established. Based on homologies within their kinase domains, these molecules can be classified roughly into two separate groups. One group which act as MAPKK kinases and include Raf, MEKK1, MUK, Tpl-2 and now MLK-3 display, at least distantly, homology to the yeast pheromone pathway element Ste11. The second group includes the Ste20 homologs HPK1, GCK and the PAKs. While some of the MAPKKKs preferentially activate one MAPK module (Raf, MEKK1), others may stimulate more than one pathway (Tpl-2, MLK-3) (see also accompanying paper, Tibbles *et al.*, 1996). The highly divergent structure of these proteins outside their kinase domains suggests that they probably respond to and are regulated by very different cellular elements.

This report is the first to identify a continuous cascade of interacting kinases from the level of Ste20 homologs leading to SAPK activation (Figure 9). If the surprisingly faithful homology to the archetypic yeast pheromone pathway is conserved in mammalian cells beyond the level of Ste20-related proteins, one might expect HPK1 and GCK to be immediately downstream of membrane-proximal signaling elements such as transmembrane receptors or small G-proteins. The PAK family of Ste20-related

kinases has indeed been shown to be activated by small GTP binding proteins including Rac1 and Cdc42 (Bagrodia *et al.*, 1995; Knaus *et al.*, 1995; Zhang *et al.*, 1995), and hPAK1 recently has been suggested to act upstream of MEKK (Brown *et al.*, 1996). It will be interesting to see if direct interaction of MAPKKKs and PAKs can be demonstrated.

Presently, the upstream mechanisms that lead to mHPK1 activation remain to be identified and are under investigation. Our results demonstrate that enzymatic activity of mHPK1 is a prerequisite for its SAPK activator function. All kinase-deficient HPK1 mutants tested led to <10% of the SAPK stimulation caused by either kinase-active full-length HPK1 or the HPK1 kinase domain. In contrast, the homologous GCK activates SAPK in the absence of its catalytic domain up to 30% of the level achieved by full-length GCK (Pombo *et al.*, 1995). Presently, we cannot exclude the possibility that truncated HPK1, consisting of the kinase domain and a few adjacent amino acids (HPK1-Ko, Figure 6A) including the proline-rich region P1 (Figure 7B), activates SAPK upon overexpression via a non-specific mechanism different from the route utilized by full-length HPK1. Interestingly the proline-rich motifs P1–P4 are not conserved between HPK1 and GCK, providing some basis for the finding that these two kinases seem to function via different mechanisms. Still, HPK1 and GCK are likely to share at least a subset of their downstream targets, since in our transfection model MLK-3Δ efficiently blocks SAPK activation by both kinases (Tibbles *et al.*, accompanying paper). Although the ubiquitously expressed MLK-3 is a good candidate for a physiological downstream element of HPK1, in the relevant hematopoietic cell types other MLK-3-like kinases may function downstream of HPK1. Interestingly, both HPK1 and GCK contain the GxGTYG motif in their kinase domains, a hallmark of cyclin-associated kinases whose phosphorylation leads to down-regulation of their catalytic activity (Mueller *et al.*, 1995). We have not yet detected any influence of the phosphorylation state of mHPK1 on its autophosphorylation activity *in vitro*. Upon transfection into COS1 cells, HPK1 appears to be constitutively active, which may reflect a lack of appropriate regulatory elements in this cell type. Similarly MLK-3 appears to be active in several transfection models (Tibbles *et al.*, accompanying paper) and, likewise, no regulation has been defined so far for the MAPKKK MEKK. While we were able to show phosphorylation of MLK-3 by HPK1 *in vitro*, the high basal activity of recombinant MLK-3 has so far precluded the detection of enzymatic activation by HPK1. Alternative mechanisms of regulation of MLK-3 or related kinases through HPK1 may exist.

While the 'three-kinase' MAPK cassettes were viewed initially as linear signal transduction chains, primarily serving the purpose of signal amplification, it is becoming increasingly clear that there may be extensive cross-talk between pathways leading to the integration of multiple stimulatory and inhibitory signals at the level of MAPKKKs and MAPKKs. Identification of novel MAPK activators like mHPK1 now provides a biochemical handle for identifying their upstream elements, and will lead ultimately to a better understanding of the regulatory network that determines activation of the MAPK cascades. Collectively, the limited expression pattern of HPK1 and

its implied role as an input into a stress signaling pathway suggest an intriguing potential involvement in the regulation of hematopoietic differentiation and growth control.

Materials and methods

RNA preparation and Northern blot analysis
Poly(A)+ RNA was prepared as described by Wang *et al.* (1991). RNA (5 μg) was separated in an agarose gel and transferred to a nylon membrane. Integrity of the transferred material was demonstrated by probing with the ribosomal housekeeping gene L32. *hpk1* probing was performed using a cDNA probe consisting of the 3' 1.1 kb of the m*hpk1* mRNA.

Cloning of hpk1
Poly(A)+ RNA of various hematopoietic cell lines was tested for hybridization to a 331 bp *mhpk1* fragment obtained by subtractive hybridization between two single cell cDNA samples derived from a granulocyte/macrophage and an erythrocyte/megacaryocyte progenitor (for details, see Brady *et al.*, 1995; the 331 bp fragment is designated 'm13'). *mhpk1* was found to be expressed in the pre-B cell line 70Z/3 (Paige *et al.*, 1978) and subsequently cloned from a 70Z/3-derived λ-ZapII library kindly provided by Drs Z.Yu and C.Paige, The Wellesley Research Institute, Toronto. The two longest λ clones isolated contained an open reading frame that extended upstream beyond the putative start codon to the 5' border of the cDNA clone (position –35). A 5' RACE reaction (5'-AmpliFINDER RACE KIT, Clonetch) extended the cDNA sequence for an additional 91 nucleotides and identified an upstream in-frame stop codon (position –69), thereby validating the start codon.

In vitro transcription–translation of hpk1
In vitro translation of RNA transcribed from the longest isolated *hpk1* cDNA clone in pBluescript (Stratagene) failed to produce a detectable protein product. To remove a potentially interfering GC-rich sequence preceding the initiating ATG, an *Nde*I site was generated by site-directed mutagenesis of the three nucleotides (GGA→CAT) immediately preceding the initiation site (U.S.E. Mutagenesis Kit, Pharmacia Biotech). An additional *Nde*I site was generated 13 nucleotides downstream of the stop codon (position +2498: CATGGG→CATATG), resulting in an *Nde*I fragment containing the complete *mhpk1* coding sequence which was blunt-inserted into the *Bgl*II site of pSP64T (Krieg and Melton, 1984). To obtain the epitope-tagged version, pSP64T-HPK1:HA, the stop codon and the immediately following nucleotides were mutated into a *Not*I site (position +2482: TGAGCCATTG→TAGCGGCCGC) allowing in-frame fusion of a three-tandem HA tag. *In vitro* transcription–translation reactions were carried out using T7 or SP6 transcription *in vitro* systems (Promega) and rabbit reticulocyte lysate (Promega) following the manufacturer's specifications.

Cell lines, tissue culture and transfection
The murine hematopoietic cell lines DA-1 (Ihle *et al.*, 1984), 32D cl13 (Greenberger *et al.*, 1983), FDC-P1 (Dexter *et al.*, 1980), WEHI-3 (Warner *et al.*, 1969) and 70Z/3 (Paige *et al.*, 1978) were shown to express mHPK1 mRNA by Northern blot analysis of 5 μg of poly(A)+ RNA. mHPK1 mRNA was not detected in NIH 3T3 fibroblasts and the bone marrow fibroblast cell line 95/1.7 (Iscove *et al.*, 1988).

All cell lines were grown in IMDM + 5% fetal calf serum. Where necessary, murine rIL-3 was added as the conditioned medium of the X63 Ag8-653 IL-3 myeloma cell line (Karasuyama and Melchers, 1988). A total of 10⁶ COS1 cells were seeded into a 10 cm dish and transfected the following day by calcium phosphate co-precipitation.

Generation of polyclonal anti-peptide antisera
A synthetic peptide (RKEKMRGKMENEKRREKY) representing amino acids 473–492 of the primary structure of mHPK1 coupled to keyhole limpet hemocyanin via a C-terminally added cysteine residue was selected for immunization of two rabbits. Typically, 5 μl of the resulting sera designated #5 and #6 were used for immunoprecipitation of mHPK1 from lysates of 10⁶ cells. The position of the peptide within the HPK1 primary structure is indicated in Figure 6A.

mHPK1 expression constructs
To enhance the efficiency of *mhpk1* translation in COS1 cells, the sequence preceding the initiating ATG (position –10: GCCTCCAGGA) was changed by site-directed mutagenesis into GATATCACC to resemble

more closely the Kozak consensus sequence (Kozak, 1986). In addition, an *Nde*I site was generated 13 nucleotides downstream of the stop codon (position +2498: CATGGG→CATATG), utilizing the newly generated *Eco*RV and 3' *Nde*I sites and *mhpk1* was blunt-inserted into the *Eco*RI site of pMT2 (Kaufman *et al.*, 1989), resulting in pMT2-HPK1. By mutagenesis of the stop codon and the immediately following nucleotides of *mhpk1* (position +2482: TGAGCCATTG→TAGCGGCCGC), a *Not*I site was generated allowing the in-frame fusion of a three-tandem HA epitope tag, recognized by the monoclonal antibody 12CA5 (Field *et al.*, 1988). The resulting vector is referred to as pMT2-HPK1:HA. An identical vector carrying the (K46E) mutation is called pMT2-HPK1(K46E):HA.

Generation of a kinase-deficient variant of mHPK1

The nucleotides TTG AAG at position +131 encoding the amino acids LK were replaced by CTC GAG encoding LE. This (K46E) mutation introduces a diagnostic *Ava*I–*Xho*I site.

RT–PCR cloning of hmlk3

Poly(A)$^+$ RNA (5 µg) from the human leukemia cell line AML-2 (Ma *et al.*, 1994) was primed using random hexamers and reverse transcribed at 50°C using superscript II (Gibco/BRL) following the manufacturer's protocol. Due to the high GC content of the MLK-3 coding region, the full-length MLK-3 coding sequence could not be amplified as a single fragment. The following primer pairs were designed on the basis of the published MLK-3 sequence (Ing *et al.*, 1994) and used to amplify smaller fragments of the MLK-3 coding region: 1A, 5'-CACCCAGA-GAAGGTCCTCCACAC-3'; 1B, 5'-AGCTCACCTCGCCAGCTGCC-CCTGTACACCTTGC-3'; 2A, 5'-GCAAGGTGTACAGGGGCAGCTG-GCGAGGTGAGCT-3'; 2B, 5'-GTTGTTGGACTTGAGATCACGGT-GGATGACGG-3'; 3A, 5'-CCGTCATCCACCGTGATCTCAAGTCCA-ACAAC-3'; 3B, 5'-GCTAGGCCGCGGGGGGGTTACCATTGAGTGC-3'; 4A, 5'-CGTCTGGAGGACTCAAGCAATGGAGAGCG-3'; 4B, 5'-GGTCTGTGCCCTGCAGTCCTGGG-3'.

Using the corresponding PCR fragments, the MLK-3 coding sequence between the *Nco*I site at the initiation codon and an *Apa*I site 66 bp upstream of the stop codon was reconstructed in pBluescript. A double-stranded Flag-linker consisting of the annealed and phosphorylated oligonucleotides 5'-pCATGGCCTTGTCGTCGTCGTCCTTGTAGTC-CATGGTGGCG-3' and 5'-pAATTCGCCACCATGGACTACAAGGA-CGACGACGACAAGGC-3' was added to the 5' end of MLK-3.

Cloning of MLK-3 expression constructs and a kinase-dead version

An *Eco*RI–*Apa*I fragment corresponding to an N-terminal in-frame fusion of the Flag peptide (MDYKDDDDKA) and MLK-3 lacking its 22 C-terminal amino acids were then cloned into the *Eco*RI site of pcDNA3 (Invitrogen).

A kinase-dead version of MLK-3 was generated by introduction of an A→G point mutation at position 911 (codon AAG→GAG), resulting in a K→E amino acid exchange of amino acid 144 in the ATP binding loop of the MLK-3 kinase domain.

A kinase-deficient deletion construct containing the Flag-tagged MLK-3 sequence up to amino acid 336 inserted into the *Eco*RI site of pMT2 is referred to as pMT2-MLK3Δ.

Yeast two-hybrid analysis

Fragments of HPK1 were cloned into the *Bam*HI–*Eco*RI sites of the pACTII vector (Durfee *et al.*, 1993). SH3 domains from different sources were cloned into the pASI vector (Ausubel *et al.*, 1990). Two-hybrid analysis was performed as described (Durfee *et al.*, 1993). Briefly, pASI vectors encoding fusions between the DNA binding domain of GAL4 and different SH3 domains and pACTII vectors encoding the transcriptional activation domain of Gal4 fused to different fragments of the mHPK1 proline-rich regions were transformed into *S.cerevisiae* strains Y153 and Y187, respectively. Yeast transformation was performed by the lithium acetate method (Ausubel *et al.*, 1990) except that 10% dimethylsulfoxide (DMSO) was included during a 42°C heat shock. Co-expression of pASI and pACTII vectors was achieved by mating Y153 and Y187 yeast strains, each containing the appropriate vector. β-Galactosidase activity was detected on X-gal plates after permeabilization of the yeast by liquid nitrogen treatment.

Immune precipitations, kinase assays and IP-Western blot experiments

A total of 10×10^6 cells of the indicated cell lines or one 10 cm dish of 80% confluent COS1 cells were lysed on ice in lysis buffer A [120 mM

NaCl, 50 mM Tris pH 7.5, 1% NP-40, 5 mM dithiothreitol (DTT), 200 µM vanadate, 10 mM pyrophosphate, 25 mM NaF, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Cleared lysates were normalized for equal protein concentration and pre-absorbed with either protein A– or protein G–Sepharose. One ml of pre-absorbed lysates was incubated with 5 µl each of sera #5 and #6 (IS) or their respective pre-immune sera (p-IS), 5 µg of anti-mouse MAP kinase rabbit polyclonal IgG (Upstate Biotechnology Incorporated), 5 µl of anti-p38 polyclonal rabbit serum, 75 µl of crude culture supernatant of the hybridoma cell line 12CA5 (Field *et al.*, 1988) or 6 µg of purified antibody M2 (Eastman, Kodak). Immune complexes were harvested on protein A– or protein G–Sepharose beads. For kinase assays, immune complexes were washed three times with TNET (140 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 1% NP-40), twice with TNE (140 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA) and once with KB (50 mM Tris pH 7.5, 8 mM MgCl$_2$, 2 mM MnCl$_2$ and 1 mM DTT). Autophosphorylation kinase reactions were performed in 100 µl of KB at 30°C for 20 min in the presence of 10 µCi of [γ-^{32}P]ATP. SAPK, ERK2 and p38/RK kinase assays were performed in 30 µl of SKB (50 mM Tris pH 7.4, 10 mM MgCl$_2$, 1 mM EDTA, 40 µM ATP) in the presence of 1.2 µCi of [γ-^{32}P]ATP and 2–5 µg of GST–c-Jun$_N$ or 5 µg of MBP. The reaction was stopped by addition of 6× SDS sample buffer and phosphoproteins were separated by SDS–PAGE and visualized by autoradiography/phosphorimaging (Molecular Dynamics). For IP-Western experiments, immunocomplexes were washed three times with phosphate-buffered saline containing 10% glycerol, 1% Triton X-100, 1.5 mM MgCl$_2$, 10 mM pyrophosphate, and 25 mM NaF prior to SDS–PAGE. Pre-treatment of cells with 1 µM PMA, 300 mM sorbitol or 50 µg/ml of the fungal drug anisomycin for 20–40 min prior to cell lysis served as positive controls for ERK2, p38/RK and SAPK activation. Phosphotyrosine moieties were detected in Western blotting experiments using the anti-P-Tyr antibody RC20 (Transduction Laboratories).

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