The Dual Specificity JKAP Specifically Activates the c-Jun N-terminal Kinase Pathway*

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The involvement of dual specificity phosphatases (DSPs) in the mitogen-activated protein kinase (MAPK) signaling has been mostly limited to the inactivation of MAPKs by the direct dephosphorylation of the TXY motif within their activation loop. We report the cloning and characterization of a murine DSP, called JNK pathway-associated phosphatase (JKAP), which lacks the regulatory region present in most other MAP kinase phosphatases (MKPs) and is preferentially expressed in murine Lin-Scel+ stem cells. Overexpression of JKAP in human embryonic kidney 293T cells specifically activated c-Jun N-terminal kinase (JNK) but not p38 and extracellular signal-regulated kinase 2. Overexpression of a mutant JKAP, JKAP-CA885, blocked tumor necrosis factor-α-induced JNK activation. Targeted gene disruption in murine embryonic stem cells abolished JNK activation by tumor necrosis factor-α and transforming growth factor-β, but not by ultraviolet-C irradiation, indicating that JKAP is necessary for optimal JNK activation. JKAP associated with JNK and MKK7, but not SEK1, in vivo. However, JKAP did not interact with JNK in vitro, suggesting that JKAP exerts its effect on JNK in an indirect manner. Taken together, these studies identify a positive regulator for the JNK pathway and suggest a novel role for DSP in mitogen-activated protein kinase regulation.

The evolutionarily conserved mitogen-activated protein kinase (MAPK) family consists of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase. MAPKs are activated by a wide range of diverse stimuli and are essential for various cellular processes, such as stress responses, apoptosis, proliferation, differentiation, and early embryonic development (1–3). The prototypical MAPK signaling cascade is a three-kinase module, consisting of MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K), and MAPK (1, 2, 4). The upstream molecules that link the MAPK module to extracellular stimuli include small G proteins and a group of mammalian Ste20-like kinases, including hematopoietic progenitor kinase 1 (HPK1), germinal center kinase (GCK), HKP1/germinal center kinase-like kinase, germinal center kinase-like kinase (GLK), and kinase homologous to Ste20 (KHS), which have been characterized as potential MAP kinase kinase kinases (MAP4Ks) for the JNK pathway (1, 2, 4, 5). Within the three-kinase module, MAPKs are phosphorylated on both threonine and tyrosine residues within their signature sequence TXY motif by a dual specificity protein kinase MAP2K. These motifs include TEY in ERK, TYP in JNK, and TGY in p38. MAP2K is activated by phosphorylation of serine/threonine residues by MAP3Ks (1, 2, 4). In the case of ERK1/2, phosphorylation of the TEY motif also contributes to the dimerization and nuclear translocation of ERK1/2 in addition to mediating its activation (6).

The extracellular stimuli-induced activation of MAPKs is transient under many conditions, and it has been well established that protein phosphatases play an essential role in the down-regulation of MAP kinases. A variety of classes of protein phosphatases, including tyrosine-specific protein phosphatases, serine/threonine protein phosphatases, and a family of dual specificity protein phosphatases (DSPs), have been implicated in the negative regulation of MAPKs (7–9). Among them, DSPs are the major group of phosphatases that contribute to the regulated inactivation of MAP kinases by dephosphorylating both phosphotyrosine and phosphothreonine residues within the TXY motif, thus also called MAP kinase phosphatases (MKPs) (7–9). Most MKPs identified so far consist of a conserved catalytic region and an extended regulatory region. However, some MKPs lack this regulatory region, such as VH1 (10) and VH1-related (VHR) phosphatase (11). The regulatory

1. JKAP, JNK pathway-associated phosphatase; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; HEK293T, human embryonic kidney 293T cells; pNPF, p-nitrophenyl phosphate; GST, glutathione S-transferase; ATF2, activating transcription factor 2; ES, embryonic stem; MEF, mouse embryonic fibroblast; UV-C, ultraviolet-C; CFC, colony-forming cell; MKK7, MAP kinase kinase 7; JSP-1, JNK-stimulatory phosphatase-1; VHXR, VHR-related MKPX, LMW, low molecular weight.
region of MKPs is responsible for substrate binding and thus makes MKPs display varying degrees of specificity for inactivating different MAP kinases. For example, MKP-3/PYST1 completely inactivates ERK1 and ERK2 but not JNK and p38 MAP kinases (12, 13), whereas M36/6 (13) and MKP-7 (14, 15) appear to be highly specific for inactivating JNK and p38 MAP kinases.

In addition to the phosphorylation that is necessary for kinase activation, the kinases within the MAPK cascade are also subject to negative regulation by phosphorylation. It has been well documented that phosphorylation of Ser-259 and Ser-261 on Raf, a MAP3K kinase for the ERK pathway, prevents the activation of Raf-1 (16–18). Apoptosis signal-regulated kinase 1 (ASK1), a MAP3K that stimulates JNK and p38 signaling pathways, is subject to negative regulation by AKT-mediated phosphorylation (19). AKT-mediated phosphorylation of stress-activated protein kinase/ERK kinase 1 (SEK1) on Ser-78 inhibits its activation and prevents its interaction with JNK, thereby suppressing the SEK1-mediated JNK signaling pathway (20). It has been recently shown that cyclin-dependent kinase 5 (cdk5) directly phosphorylates JNK3 on Thr-131 and inhibits its kinase activity and leads to reduced c-Jun phosphorylation (21). Thus, it is likely that some protein phosphatases are involved in positive regulation of MAPks by dephosphorylating those inhibitory phosphorylated residues. It has been shown that the Shp-2 tyrosine phosphatase is necessary for ERK activation by a number of growth factors including insulin growth factor-1, platelet-derived growth factor, and epidermal growth factor (22). The protein serine/threonine phosphatase PP2A acts as a positive regulator for Raf-1 (17). We have recently shown that protein phosphatase 4 (PP4), another serine/threonine phosphatase, is positively involved in tumor necrosis factor (TNF)-α-induced activation of the JNK pathway (23). Taken together, the MAPK activity is determined by the combined influences of kinases and phosphatases, and both phosphorylation and dephosphorylation play essential roles in controlling the duration and magnitude of MAPK activity depending on the cell type and stimuli.

As the relationship between DSPs and MAPK is studied, the complexity of kinase regulation increases. For example, overexpression of MKP1, which inactivates ERK, activates the ERK-upstream kinases MKK1, MKK2, and Raf-1 (24). MKP-1 itself is controlled by ERK, which induces both MKP-1 stability and activity (25, 26). In an effort to isolate transcripts differentially up-regulated in murine hematopoietic stem cells, we identified a dual specificity phosphatase, called the JNK pathway-associated phosphatase (JKAP), that specifically activates the JNK pathway and is required for the cytokine-induced JNK activation. Moreover, Jkap deficiency in murine bone marrow progenitor cells resulted in loss of sensitivity of the cells to inhibition of transforming growth factor-β (TGF-β)-β-rod colon-forming cells (CFCs). Taken together, our studies have identified a new DSP as a positive regulator for the JNK signaling pathway.

**MATERIALS AND METHODS**

**Reagents**—[γ-32P]ATP was purchased from ICN Biomedicals (Irvine, CA). An enhanced chemiluminescence system was purchased from Amersham Biosciences. TNF-α and TGF-β were purchased from R & D Systems (Minneapolis, MN). Anti-HA antibody (12CA5) was purchased from Roche Molecular Biochemicals. Anti-FLAG (M2) antibody was purchased from Sigma. Monoclonal anti-c-Jun (9E10) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-JNK1 antibody (Ab101) was described previously (27, 28). All other chemical reagents were purchased from Sigma unless otherwise noted.

**Plasmids**—The GST-Jun(1–79) was a gift from Dr. M. Karin (University of California San Diego), and GST-SEK1 was a gift from Dr. L. I. Zon (Children’s Hospital, Boston, MA). GST-ATF2(1–96) and pHA-ERK2 were provided by Dr. J. S. Gutkind (National Institutes of Health, Bethesda, MD). GST-JNK (also called GST-stress-activated protein kinase (GST-SAPK)) was a gift from Dr. D. J. Templeton (University of Virginia Medical School, Charlottesville, VA). pHA-MKK6 was provided by Dr. Z. Yao (Agen, Boulder, CO). pHA-protein kinase Cα (Cα) was a gift from Dr. M. Karin. pHA-ERK2 was a gift from Dr. W. Xiong (University of Connecticut). RAf-BXB was kindly provided by Dr. J. Bruder (GenVec, Rockville, MD). pHA-JNK1 and HA-p38 were gifts from Dr. J. Woodgett (Ontario Cancer Institute, Toronto, Canada). pMTSMYc-M36/6 was a gift from Dr. K. E. Davis (University of Oxford, Oxford, UK) (27).

**Cells and Transfection**—Human embryonic kidney 293T (HEK293T) cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 100 units/ml streptomycin/penicillin at 37 °C in a humidified atmosphere of 5% CO2. HEK293T cells were plated at a density of either 1.5 × 106 cells/35-mm plate well or 1.5 × 106 cells/100-mm dish and transfected the next day using the modified calcium phosphate precipitation protocol (Specialty Media, Inc., Lalayette, NJ). Cells were transfected with plasmids encoding β-galactosidase (0.15 μg) in combination with an empty vector or various amounts of plasmids encoding phosphatases, phosphatase mutants, kinases, or kinase mutants as indicated in the figure legends.

**Commonprecipitation, Immunocomplex Kinase Assays, Western Blot Analysis, and Phosphatase Assays—**Immunocomplex kinase and phosphatase activity assays were performed as described previously (29, 30). Western blot analysis was performed using a modified chemiluminescence detection kit according to the manufacturer’s protocols (Amersham Biosciences). The phosphatase activities of Myc-JKAP and Myc-JKAP-C88S were assayed at 30 °C using p-nitrophenyl phosphate (pNPP) as a substrate. HEK293T cells (1.5 × 105 in 100-mm dishes) were transfected with a vector control, Myc-JKAP, or Myc-JKAP-C88S. Myc-JKAP and Myc-JKAP-C88S were immunoprecipitated with an anti-Myc antibody. The immunoprecipitates were then incubated with 1 ml of 50 mM imidazole (pH 7.5) with 10 mM dithiothreitol, 20 mM pNPP for 1 h at 30 °C. The reactions were terminated by the addition of 0.1 M NaOH, and the hydrolyzed pNPP was quantified by measurement of spectrophotometric absorbance at 410 nm.

**Interspecific Mouse Backcross Analysis—**Interspecific mouse backcross mapping was performed as described (31, 32) using progeny derived from matings of C57BL/6J × Mus spretusF1 × C57BL/6J mice. The presence or absence of a M. spretus-specific 12.5-kb BgII fragment, detected by a probe corresponding to nucleotide positions 2251–2566 of the Jkap cDNA, was followed in backcross progeny. A total of 205 N2 mice were used to map the Jkap locus. Recombination distances were calculated using Map Manager, version 2.6.5.

**Construction of Mammalian Expression Clone—**An 851-bp EcoRI fragment from the Jkap cDNA, consisting of the coding region and 200 bp of 3′-untranslated region, was cloned into the mammalian expression vector pCI-neo (Promega), linearized at the NotI site, generating pCI-JKAP. The mutant pCI-JKAP-C88S was generated by PCR mutagenesis with pCI-JKAP, using mutagenic primers covering the site (cysteine 88 changed to serine), a 5′ amplification primer including the vector backbone, and a 3′ amplification primer in the 3′-untranslated region.

**Construction of Targeting Clone—**To generate the plasmid backbone, pNeoUSEFUL, the 1.35-kb XhoI fragment (neo) of pPol2Sneo (provided by P. Sorianio) was blunted, cloned into the EcoRV site of pBluescript-SK+ (-), reisolated with BamHI and SalI, and finally cloned into pUSEFUL (provided by A. Bradley) cut with BamHI and Sali (releasing pGK-hprt and leaving thymidine kinase and unique cloning sites intact). The 3′ arm, a subcloned 2.8-kb SalI-XhoI fragment consisting of Jkap genomic and cDNA sequence immediately after the second targeted exon, was then blunted and cloned into the BamHI site, also blunted, of pNeoUSEFUL, generating clone B2.8-8.1. The 5′ arm, a subcloned 5.0-kb XhoI fragment consisting of genomic Jkap sequence immediately before the first targeted exon, was cloned into intermediate plasmid CXS (pSAGeo, provided by P. Sorianio; cut with HindIII and XhoI, blunted, and recircularized), cut with XhoI. The fragment was reisolated with ClaI and SalI and cloned into B2.8-8.1 cut with ClaI and SalI, generating clone BS4-1, the targeting vector.

**Generation of Jhap-targeted Mammalian Embryonic Stem (ES) Cells and Mammalian Embryonic Fibroblast (MEF) Cells—**Targeted ES cell clones were obtained by the method of Bruder et al. (33). Briefly, a targeting vector was designed that deletes the two coding exons of JKAP, which encode the catalytic domain of the phosphatase. The vector was constructed using DNA from a murine 129/SV/EV total genomic DNA library. This vector was electroporated into AB2.2 ES cells derived from 129/SV/EV mice. Resulting clones were screened by Southern blot analysis for evidence of homologous recombination. Several independent targeted
clones were obtained. Homozygous JKAP−/− ES clones were obtained by superselection in 1.5 mg/ml G418 and confirmed by Southern blotting. Jkap−/− animals were derived by breeding Jkap−/− ES cell chimera with wild-type 129SvEvBrd female animals. Because the AB2.2 ES line was derived from the same strain of the 129SvEvBrd female animal, the resulting animals were inbred with respect to all background loci.

Jkap−/− animals were born in expected Mendelian ratios (data not shown) and remained overtly healthy through adult life.

**FIG. 1. JKAP sequence analysis.** A, JKAP is a dual specificity phosphatase. Amino acid sequences corresponding to the catalytic domains of JKAP and selected MKPs were aligned in ClustalW version 1.8. The catalytic signature motifs are indicated by a shaded box. GenBank™ sequence accession numbers are as follows: DUSP9 (MKP-4), Q99956; DUSP6 (MKP-3), XP038308; DUSP7 (MKP-X), XP037430; DUSP3 (human VHR), P51452; SKRP1, AB063186; DUSP10 (MKP-5), XP039628; DUSP5 (hVH3), Q16690; DUSP12 (YVH-1), XP001951; DUSP14 (MKP-6), NP068957; DUSP4 (MKP-2), XP027545; DUSP1 (MKP-1), XP001951; DUSP2 (PAC-1), Q05923; DUSP8 (hVH-5, M3/6), DUSP8 (hVH-5, M3/6/36594), Q13202; SKRP1, AB063186; DUSP10 (MKP-5), XP039628; DUSP5 (hVH3), Q16690; DUSP12 (YVH-1), XP001951; DUSP14 (MKP-6), Q13202; MKP-7, XP039106. B, Jkap is a mouse orthologue of human JSP-1. Full-length amino acid sequences of mouse JKAP, human JKAP, and selected MKPs were aligned in ClustalW version 1.8. The catalytic signature motifs are indicated by a shaded box. The conserved amino acids are indicated by asterisks on the consensus line. C, schematic representation of JKAP amino acid identity with other MKPs. Cdc25 homology domain 2 (CH2) domains and phosphatase catalytic domains are indicated by black and open boxes, respectively. The numbers represent the percentage of JKAP amino acid identity with other MKPs within the phosphatase catalytic domain. D, phylogenetic comparison of the catalytic domains of MKPs. Aligned sequences encompassing the entire catalytic domain of each MKP were prepared in ClustalW and manually edited. Sequence affinities were then estimated by Bayesian phylogenetic inference using Metropolis-coupled Markov chain Monte Carlo methods implemented in MRBAYES version 2.01. The consensus tree resulted from an estimate of the posterior probabilities after 500,000 replicates with burn in of 50,000 replicates (27).
resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and then subjected to Western blotting with an anti-Myc antibody. The membrane was then stripped with stripping buffer (62.5 mM Tris-HCl (pH 6.7), 100 mM 2-mercaptoethanol, 2% SDS) and reprobed with an anti-GST antibody.

RESULTS

Isolation of a Putative MAPK Phosphatase Gene, jkap, Preferentially Expressed in Murine Lin–Sca-1+ Stem Cells—In an effort to isolate transcripts differentially up-regulated in murine hematopoietic stem cells by employing differential display PCR, we identified the Jkap transcript, among others, as being expressed preferentially in the stem cell-enriched Lin–Sca-1+ population of murine bone marrow cells obtained by fluorescence-activated cell sorting, compared with the Lin–Sca-1– population, which is deficient in pluripotent stem cell activity. Lin+ (i.e. lineage negative) refers to the absence of cell surface expression of CD4, CD8, B220, Mac-1, GR-1, and Ter115, which are expressed upon commitment to helper T cell, killer T cell, B cell, macrophage, granulocyte, and erythrocyte lineages, respectively. Sca-1+ or Sca-1– refers the presence or absence of cell surface expression of stem cell antigen 1 encoded by Ly6A2.

A full-length murine Jkap cDNA consisting of 3012 bp was obtained by screening an adult muscle cDNA library. This cDNA contained a 700-bp open reading frame with similarity to cDNA contained a 700-bp open reading frame with similarity to RFK phosphatase 1 (JSP-1) (36), and that JKAP/JSP-1 is distinctive in its catalytic motif, with the relationship to human VHR SRS 36595 1.3-kb transcript was detected in the spleen. The broad expression of JKAP mRNA in mouse tissues was examined by Northern blot analysis using mouse JKAP cDNA probe. As shown in Fig. 2, two mRNA species of 3.0 and 1.3 kb were detected. The longer 3.0-kb transcript was abundantly expressed in the heart, brain, liver, and kidney but expressed at low levels in the testis and skeletal muscle. The shorter 1.3-kb transcript was abundantly expressed in the testis and liver and, to a lesser extent, in the kidney and heart. Neither 3.0-kb transcript nor 1.3-kb transcript was detected in the spleen. The broad expres-
sion of JKAP implies that JKAP functions in a wide range of tissues in the adult mouse. The differential expression patterns of the 3.0- and 1.3-kb transcripts (e.g. only the 3.0-kb transcript in the brain and the 1.3-kb transcript in the testis) indicate a tissue-specific splicing or processing of JKAP mRNA. JKap Is Expressed in Adult, but Not Embryonic, Murine Hematopoietic Stem Cells—To further characterize the expression pattern of JKap in murine tissues, we isolated Lin−Sca-1− and Lin−Sca-1+ cells from adult murine bone marrow by fluorescence-activated cell sorting and performed in situ hybridization analysis with a JKap antisense riboprobe. As expected, we confirmed that JKap is preferentially expressed in Lin−Sca-1+ cells, as compared with Lin−Sca-1− cells (Fig. 3, A and B), suggesting a role for JKAP in hematopoietic precursor cells. Whole-mount in situ hybridization of embryonic day 10.5 mouse embryos detected the highest levels of JKap transcripts in the somites and branchial arches (Fig. 3, C–E). No expression was observed in the embryonic dorsal aorta, a region identified as a site of definitive hematopoiesis in chick, Xenopus, mouse, and human embryos at similar developmental stages (37, 38), suggesting that the earliest intraembryonic hematopoietic cells do not express JKap at a high level (Fig. 3F).

JKAP Selectively Activates the JNK Pathway—Given the close sequence similarity of JKAP to MKPs, we sought to determine the effects of JKAP on JNK, p38, and ERK2. To our surprise, overexpressed JKAP had no inhibitory effect on the activity of either TGF-β-activated kinase 1-activated JNK1, MKK6-activated p38, or protein kinase C-activated ERK2 (data not shown). Instead, we found that JKAP had the ability to activate JNK1 (Fig. 4). We cotransfected HA-JNK1 into HEK293T cells with wild-type JKAP or a catalytic site mutant JKAP-C88S. In JKAP-C88S, the Cys88Ser substitution abolished the phosphatase activity of JKAP. To determine the specificity of this effect, we examined the effects of JKAP on JNK, p38, and ERK2. We found that cotransfection of JKAP resulted in activation of JNK1, whereas JKAP-C88S did not (Fig. 4A, upper panel). These data indicate that the stimulatory effect of JKAP on JNK was dependent on the phosphatase activity of JKAP. To examine the effects of JKAP on p38 and ERK2, we cotransfected HA-p38 (Fig. 4B) or HA-ERK2 (Fig. 4C) with JKAP into HEK293T cells. As a positive control, MKK6 was cotransfected with p38, and Raf-BXB was cotransfected with ERK2. HA-p38 and HA-ERK2 were immunoprecipitated with an anti-HA antibody, and

FIG. 4. JKAP activates JNK but not p38 or ERK in HEK293T cells. A, JKAP, but not JKAP-C88S, activates JNK1. HEK293T cells were transfected with 0.1 μg of HA-JNK1 alone, or HA-JNK1 plus 2 μg of either JKAP or JKAP-C88S. Empty vectors were used to normalize the amount of transfected DNA. At 44 h post-transfection, cells were collected, and cell lysates were prepared. HA-JNK1 was immunoprecipitated with an anti-HA antibody, and immunocomplex kinase assays were performed using GST-c-Jun-(1–79). Equivalent levels of HA-JNK1 expression were verified by immunoblot analysis using an anti-HA antibody. Lower panel, in vitro phosphatase activity of JKAP. 293T cells were transfected with a vector control, Myc-JKAP, or Myc-JKAP-C88S. Myc-JKAP and Myc-JKAP-C88S were immunoprecipitated with anti-Myc antibody and then incubated with pNPP. Cleavage of pNPP was measured by absorbance at 410 nm. B, JKAP cannot activate JNK. HEK293T cells were transfected with 2 μg of HA-p38 alone or plus 2 μg of JKAP. As a control, HA-p38 was cotransfected with 2 μg of MKK6. Empty vectors were used to normalize the amount of transfected DNA. HA-p38 was immunoprecipitated with an anti-HA antibody, and immunocomplex kinase assays were performed using GST-ATF2 as a substrate. Equivalent levels of HA-p38 and HA-ERK2 expression were verified by immunoblot analysis using an anti-HA antibody. C, JKAP cannot activate ERK2. HEK293T cells were transfected with 2 μg of HA-ERK2 either alone or plus 2 μg of JKAP. HA-ERK2 was cotransfected with 0.5 μg of Raf-BXB as a control. Empty vectors were used to normalize the amount of transfected DNA. HA-ERK2 kinase assays were performed as described above except that myelin basic protein (MBP) was used as a substrate. Equivalent levels of HA-p38 and HA-ERK2 expression were verified by immunoblot analysis using an anti-HA antibody. D, JKAP mutant blocks TNFα-induced JNK activation. HEK293T cells transfected with 0.1 μg of HA-JNK1 alone or HA-JNK1 plus 2 μg of JKAP-C88S were treated with TNF-α (10 ng/ml) for 10 min. The cells were then collected, and cell lysates were prepared. HA-JNK1 was immunoprecipitated with an anti-HA antibody (12CA5), and immunocomplex kinase assays were performed using GST-c-Jun-(1–79) as a substrate. Equivalent levels of HA-JNK1 expression were verified by immunoblot analysis using an anti-HA antibody.
their activities were determined in vitro by immunocomplex kinase assays using GST-ATF2 as a substrate for p38 and myelin basic protein as a substrate for ERK2. As shown in Fig. 4, B and C, neither p38 nor ERK2 was activated by overexpression of JKAP. Therefore, JKAP acts as a specific positive regulator of JNK.

To confirm the functional relevance of JKAP to the JNK signaling pathway, we examined the contribution of JKAP to JNK activation by TNF-α, a known JNK stimulus. HEK293T cells were transfected with HA-JNK1 alone or HA-JNK1 plus JKAP-C88S. The transfected cells were treated with TNF-α (10 ng/ml) for 10 min. We found that TNF-α-induced JNK activation was blocked by JKAP-C88S (Fig. 4D), suggesting that intact phosphatase activity of JKAP is necessary for JNK activation in TNF-α signaling.

**JKAP Is Necessary for Full Induction of the JNK Pathway**—To further confirm the functional involvement of JKAP in JNK signaling, we investigated the response of cells deficient in JKAP to various known JNK stimuli. We generated murine ES cells heterozygous for the deletion of Jkap through homologous recombination and derived homozygous clones by secondary selection (Fig. 5A). The inferred gene structure of Jkap based on the current human genome assembly indicates that the locus is composed of seven exons and spans about 60 kb (Fig. 5A). The targeted mutation (Fig. 5B) deleted all of exons 5 and 6 (cDNA positions 249–495 and codon positions 64–145) and resulted in a null for mRNA as assessed by Northern blot (Fig. 5C). Jkap+/− and Jkap−/− ES cells were exposed to TNF-α, TGF-β, and ultraviolet-C (UVC) irradiation, which are known stimuli for the JNK pathway. TNF-α- and TGF-β-induced JNK activation was significantly reduced in Jkap−/− cells in comparison with Jkap+/− cells (Fig. 6A, top and middle panels, respectively). In contrast, the fold induction of JNK activity by UV-C was comparable in Jkap+/− and Jkap−/− cells (Fig. 6A, bottom panel). These data indicate that JKAP is necessary for full activation of JNK in response to cytokines but not UV-C irradiation. Comparable JNK1 protein was expressed in Jkap+/− and Jkap−/− cells (Fig. 6B), indicating that JKAP deficiency does not cause a obvious secondary disturbance in JNK expression or stability. The specific involvement of JKAP in the JNK signaling pathway was further confirmed by the comparable responses of p38 to UV-C irradiation and of ERK2 to phorbol 12-myristate 13-acetate in Jkap+/− and Jkap−/− MEF cells (data not shown). Taken together, these data further

**FIG. 5.** Generation of Jkap−/− ES cells. A, the Jkap genomic locus, targeting vector, and mutated locus are schematically represented. Restriction enzyme sites (BamHI, XhoI, and Sall) and the probe used to detect targeting events are indicated. B, genomic DNA was isolated from Jkap+/−, Jkap−/−, and Jkap−/− ES cells, which were derived through selection of Jkap−/− ES cells lines in 2 μg/ml G418. The DNA was digested with BamHI, transferred for Southern analysis, and hybridized with a probe flanking the 5′ insertion site of the targeting vector. Molecular weights in kilobase pairs are indicated on the left. C, total RNA from Jkap+/+ Jkap−/−, and Jkap−/− ES cells was isolated and hybridized with a Jkap cDNA probe. RNA integrity and quantity were evaluated by ethidium blue staining after Northern transfer. Molecular size in kilobase pairs is indicated on the left.

**FIG. 6.** JKAP deficiency abolishes JNK activation by TNF-α and TGF-β but not by UV-C. A, Jkap+/+ and Jkap−/− murine ES cells grown to ~80% confluence in 60-mm dishes were treated with TNF-α (10 ng/ml) for 10 min (top panel), TGF-β (10 ng/ml) for 10 min (middle panel), or UV-C (300 J/m2) for 30 min (bottom panel). Endogenous JNK1 was immunoprecipitated (IP) with an anti-JNK1 antibody (Ab101), and immunocomplex kinase assays were performed using GST-c-Jun(1–79) as a substrate. B, JNK1 expression in Jkap+/+ and Jkap−/− ES cells. The expression levels of JNK1 in Jkap+/+ and Jkap−/− ES cells were monitored by immunoblot analysis using an anti-JNK1 antibody (Ab101). WB, Western blot.
suggest that JKAP is a critical signaling component of the JNK pathway in response to TNF-α and TGF-β.

**JKAP Plays a Role in TGF-β1-induced Inhibition of CFCs**

The original finding that Jkap is differentially expressed in cell populations with the more primitive Lin-“Sca-1” cell surface phenotype suggested that a functional role for JKAP might be discerned in the hematopoietic lineage. Extensive studies have demonstrated a particular role for TGF-β1 in the inhibition of hematopoietic precursors (39). Primitive hematopoietic precursors are exquisitely sensitive to inhibition by TGF-β1 (40, 41). We were then prompted to investigate the responses of MEF cells and bone marrow progenitors derived from either wild-type or Jkap mutant embryos to TGF-β1 stimulation, which results in prompt growth arrest in a variety of cell types. MEFs derived from either wild-type or Jkap mutant embryos responded equally to TGF-β1, with both showing a dose-dependent decrease in proliferation (Fig. 7A). Jkap−/− bone marrow progenitors, in contrast, show less inhibition by TGF-β1 compared with wild type (Fig. 7B). These progenitors represent predominantly cells committed to the myeloid lineage. The reduced inhibition of Jkap−/− cells was TGF-β1 dose-dependent and could be partially overcome at the highest doses of the cytokine. The results demonstrate a functional requirement for JKAP in regulating the quantitative response of hematopoietic precursors to TGF-β1. This is the first evidence of any functional requirement for a DSP in a hematopoietic cell type.

**JKAP Associates with JNK in Cells, but Not in Vitro**—We next investigated whether JKAP interacts with JNK. We co-transfected Myc-JKAP into HEK293T cells with HA-JNK1 and examined the JKAP-JNK1 association by immunoprecipitation and Western blotting analysis. HA-JNK1 was coimmunoprecipitated with Myc-JKAP when an anti-Myc antibody was used to immunoprecipitate JKAP (Fig. 8A, top panel). Conversely, Myc-JKAP was coimmunoprecipitated with HA-JNK1 when HA-JNK1 was immunoprecipitated with an anti-HA antibody (12CA5). Therefore, a complex of JKAP and JNK1 was formed between JKAP and JNK1 in transfected HEK293T cells. To further determine whether JKAP and JNK1 interact directly with each other in vitro, we incubated GST-JNK fusion protein with cell lysates from HEK293T cells overexpressing Myc-JKAP. The JKAP-JNK interaction was analyzed by SDS-PAGE and Western blotting using an anti-GST antibody (middle panel). IP, immunoprecipitation; WB, Western blot.

**Fig. 8.** JKAP associates with JNK in vivo but not in vitro. A, JKAP associates with JNK1 in HEK293T cells. HEK293T cells (1.5 × 10⁶ cells in a 100-mm dish) were transfected with Myc-JKAP (10 μg) alone, HA-JNK1 (10 μg) alone, or Myc-JKAP (10 μg) plus HA-JNK1 (10 μg). Empty vector was used to normalize the amount of transfected DNA. 42 h post-transfection, cell lysates were prepared. JKAP was immunoprecipitated with an anti-Myc antibody. The immunoprecipitants were then immunoblotted with an anti-HA antibody (12CA5). Conversely, JNK1 was immunoprecipitated with an anti-HA antibody (12CA5), and the immunoprecipitants were immunoblotted with an anti-Myc antibody. The expression levels of JKAP and HA-JNK1 were monitored by immunoblotting using anti-Myc and anti-HA antibodies, respectively. B, JKAP does not interact with JNK1 in vitro. HEK293T cells (1.5 × 10⁶ cells in 100-mm dishes) were transfected with 10 μg of either Myc-JKAP or Myc-M3/6. 42 h post-transfection, the cell lysates were prepared. 600 μg of lysate was incubated with GST or GST-JNK fusion protein immobilized onto glutathione-agarose beads for 2 h at 4°C. The JKAP-JNK1 and M3/6-JNK interactions were analyzed by immunoblotting with an anti-Myc antibody to detect Myc-JKAP (upper panel) or Myc-M3/6 (lower panel) bound to GST-JNK after SDS-PAGE. The GST and GST-JNK were monitored by immunoblotting with an anti-GST antibody (middle panel). IP, immunoprecipitation; WB, Western blot.
JKAP Phosphatase

Fig. 9. JKAP associates with MKK7 but not with SEK1. A, JKAP associates with MKK7. HEK293T cells (1.5 x 10⁶ cells in a 100-mm dish) were transfected with Myc-JKAP (10 μg) alone, FLAG-MKK7 (10 μg) alone, or Myc-JKAP (10 μg) plus FLAG-MKK7 (10 μg). Empty vector was used to normalize the amount of transfected DNA. 42 h post-transfection, cell lysates were prepared. JKAP was immunoprecipitated with an anti-Myc antibody. The immunoprecipitants were then immunoblotted with an anti-FLAG antibody (M2). The expression levels of JKAP and FLAG-MKK7 were monitored by immunoblotting using anti-Myc and anti-FLAG (M2) antibodies, respectively. B, JKAP does not associate with SEK1. HEK293T cells (1.5 x 10⁶ cells in a 100-mm dish) were transfected with Myc-JKAP (10 μg) alone, GST-SEK1 (10 μg) alone, or Myc-JKAP (10 μg) plus GST-SEK1 (10 μg). Empty vector was used to normalize the amount of transfected DNA. 42 h post-transfection, cell lysates were prepared. JKAP was immunoprecipitated with an anti-Myc antibody. The immunoprecipitants were then immunoblotted with an anti-GST antibody. The expression levels of JKAP and GST-SEK1 were monitored by immunoblotting using anti-Myc and anti-GST antibodies, respectively. IP, immunoprecipitation; WB, Western blot.

immunoprecipitation does not prove that JKAP exerts its effect on the JNK pathway indirectly, however. It may not interact with JNK in vitro because an additional protein is missing or the correct modification (such as phosphorylation by another kinase) of one or more of the proteins is not present.

JKAP Associates with MKK7—The absence of a direct interaction between JNK and JKAP could suggest that JKAP exerts its effect on JNK by regulating molecules that regulate JNK, in particular the molecule(s) that are subject to negative regulation by phosphorylation. It has been recently shown that SEK1 (also called MKK4), an immediate JNK upstream activating kinase, is subject to phosphorylation that interferes with the SEK1-JNK interaction and prevents JNK from activation (20). It is reasonable to expect that a phosphatase that is capable of dephosphorylating the inhibitory phosphoresidue should finally exert a positive effect on the JNK pathway. We were then prompted to examine whether JKAP could interact with SEK1 and MKK7, the other immediate JNK upstream activating kinase. We cotransfected Myc-JKAP into HEK293T cells with FLAG-MKK7 or GST-SEK1 and examined the association of MKK7 with JKAP (Fig. 9A). We cotransfected Myc-JKAP into HEK293T cells with FLAG-MKK7 or GST-SEK1 and examined the association of MKK7 with JKAP (Fig. 9A). We further confirmed these results using Myc-JKAP with an anti-Myc antibody. The immunoprecipitants were then subjected to Western blotting using an anti-FLAG antibody for MKK7 or an anti-GST antibody for SEK1. We found that JKAP associated with MKK7 (Fig. 9A). However, no JKAP-SEK1 association was detected (Fig. 9B). These data suggest that MKK7 may be a specific interacting target for JKAP.

DISCUSSION

A well accepted model is that a phosphorylating kinase is coupled with a dephosphorylating phosphatase to balance the response of a signaling pathway to a stimulus (42). Previous functional studies of DSPs with activity in the MAPK pathways have primarily highlighted their roles as down-regulators, inactivating the target MAPK through direct dephosphorylation of the phosphothreonine and phosphotyrosine residues in the TXY motif of the catalytic domain of the kinase (7, 9). We reported here that a dual specificity phosphatase is preferentially expressed in the stem cell-enriched Lin-Sca-1 population of murine bone marrow cells, referred to as JKAP. Unlike most other DSPs/MKPs identified so far, JKAP is unique in two aspects. First, JKAP protein lacks an extended noncatalytic domain and is consequently domain and is consequently absent and shorter than most other MAPK phosphatases. Second, JKAP acts as a specific positive regulator for the JNK signaling pathway. It appeared that activation of JNK requires phosphatase activity, since the catalytically inactive C88S mutant of JKAP could not activate JNK. The requirement of JKAP for the JNK signaling pathway was further demonstrated by the genetic studies. In Jkap-deficient murine ES cells, cytokine-induced JNK activation was significantly reduced. To our knowledge, this is the first report that has combined both biochemical and genetic studies to indicate that a DSP acts as a specific positive regulator for the JNK signaling pathway.

During the preparation of this manuscript, we found by sequence analysis that JKAP is a murine orthologue of human JNK-stimulatory phosphatase (JSP)-1 (36) or VHR-related MKPX (VHX) (43). All of these phosphatases lack the regulatory region containing the Cdc25 homology domain, which is conserved in most known MKPs. However, the effects of these lower molecular weight DSPs on MAPKs are controversial in the literature. Whereas human VHX possesses a higher capacity than VHR phosphatase, which is thought to be specific for ERK1/2, to dephosphorylate ERK2 in vitro (43), JSP-1 acts as a specific positive regulator for the JNK pathway (36). Moreover, low molecular weight (LMW) DSP2, another identified murine orthologue of human JSP-1/VHX, is able to dephosphorylate and inactivate p38 and JNK both in vitro and in cells (44). Overexpression of JKAP or JSP-1 resulted in activation of JNK, but these results do not demonstrate a direct regulatory role in the JNK pathway, since JNK responds to a variety of cell stresses, and overexpression may have acted in one of those pathways indirectly activating JNK. The targeted mutation of Jkap demonstrated definitively that it both acts as a positive regulator for the JNK pathway and is required for the cytokine-induced activation of the JNK pathway. This result provides the first demonstration of a functional requirement for a DSP in MAPK signaling. It is not clear at this point what causes the significant difference for these low molecular weight DSPs in both their substrate specificity and the nature of their effect on MAPKs. JKAP is 20 amino acids longer than JSP-1, VHX, and

A

Myc-JKAP Flag-MKK7

IP: anti-Myc

WB: anti-Flag

WB: anti-Myc

B

Myc-JKAP

Flag-MKK7

IP: anti-Myc

WB: anti-Flag

WB: anti-Myc

WB: anti-GST

WB: anti-Myc

Fig. 9A

Fig. 9B

1

2

3

4

1

2

3

4

- - + +

- - + +

- + + +

- + + +
JKAP Phosphatase

LMW DSP2. We, in fact, detected two transcripts of 3.0 and 1.3 kb in mouse tissues (Fig. 2). It is possible that JKAP functions differently from LMW DSP2 simply due to the fact that they are different isoforms of the same gene through differential splicing.

It is unique for JKAP and JSP-1 isoforms to be able to activate JNK. It is not clear how these low molecular weight DSPs control their substrate specificity without the regulatory region, which is conserved in most other MKPs containing an essential domain for its substrate binding and substrate specificity. It was reported that the conserved docking motifs of p38 and JNK are not essential for their interaction with LMW DSP2 (44). Since the physiological substrate for JKAP remains to be determined, the pathway specificity might be achieved in one of the following ways. First, if JKAP, like other MKPs, acts directly on JNK, a distinct mechanism must exist to determine its substrate specificity. The apparent specificity of JKAP for JNK could be inherent in the catalytic pocket or could be mediated through an interacting protein complex. Second, if JKAP loses its ability to bind MAPK/JNK due to its lack of the essential domain for its substrate binding and substrate specificity, it could exert its positive effect on the pathway specificity. It was reported that the conserved docking motifs of p38 and JNK activation distinguishes JKAP among the DSPs. Specific regulators of JNK activation, like JKAP, could be reasonable targets for therapeutic drug development, given the broad roles of the JNK pathway in response to stress, growth, and apoptosis. The effects of this unique phosphatase in the JNK pathway support a more complex role for phosphatases in MAP kinase signaling.

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The Dual Specificity JKAP Specifically Activates the c-Jun N-terminal Kinase Pathway

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