Molecular Cloning and Functional Characterization of a Novel Mitogen-activated Protein Kinase Phosphatase, MKP-4*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y08302.

Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38/RK/CSBP (p38) mitogen-activated protein (MAP) kinases are target enzymes activated by a wide range of cell-surface stimuli. Recently, a distinct class of dual specificity phosphatase has been shown to reverse activation of MAP kinases by dephosphorylating critical tyrosine and threonine residues. By searching the expressed sequence tag data base (dbEST) for homologues of known dual specificity phosphatases, we identified a novel partial human sequence for which we isolated a full-length cDNA (termed MKP-4). The deduced amino acid sequence of MKP-4 is most similar to MKP-X/PYST2 (61% identity) and MKP-3/PYST1 (57% identity), includes two N-terminal CH2 domains homologous to the cell cycle regulator Cdc25 phosphatase, and contains the extended active site sequence motif VYVHCXGXSRRXXTX_AYM (where X is any amino acid) conserved in dual specificity phosphatases. MKP-4 produced in Escherichia coli catalyzes vanadate-sensitive breakdown of p-nitrophenyl phosphate as well as in vitro inactivation of purified ERK2. When expressed in COS-7 cells, MKP-4 blocks activation of MAP kinases with the selectivity ERK > p38 = JNK/SAPK. This cellular specificity is similar to MKP-3/PYST1, although distinct from hVH6 and orthologue of PYST1) (43–45), and MKP-X (orthologue of PYST2) (46–49). The identification and characterization of MKP-4 highlights the emergence of an expanding family of structurally homologous dual specificity phosphatases possessing distinct MAP kinase specificity and subcellular localization as well as diverse patterns of tissue expression.

Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38/RK/CSBP (p38) are distinct classes of mitogen-activated protein (MAP) kinase activated by a range of growth factors as well as pro-inflammatory cytokines and cellular stress (1–10). MAP kinases in turn phosphorylate diverse target proteins in membrane or cytosolic fractions (e.g., kinases, cytoskeletal elements, phospholipase A2, and stathmin) as well as a number of nuclear transcription factors, indicating a critical role orchestrating many short and long term changes in cell function (8, 11–16). This has been confirmed recently using specific chemical kinase inhibitors or by expressing mutant versions of different MAP kinases or their upstream activators. These studies show that ERK kinases play a pivotal role mediating neuronal differentiation in PC12 cells as well as growth factor-stimulated proliferation and oncogenic transformation in fibroblasts (17–21). Such approaches also support the view that JNK/SAPK and p38 MAP kinases are critical to processes mediating platelet aggregation and secretion, in generation of inflammatory cytokines, and in pathways leading to apoptotic death in a number of cell types (7, 10, 22–25).

Full MAP kinase activation requires phosphorylation on both tyrosine and threonine residues by selective upstream dual specificity kinases (13, 26, 27). Since MAP kinase activation is a reversible process, control of cellular protein phosphatases is likely be an important regulatory mechanism. Recently, an emerging family of dual specificity phosphatases has been shown to inactivate MAP kinase through dephosphorylating both threonine and tyrosine residues crucial for enzymatic activity (28). Currently eight distinct dual specificity phosphatases have been identified. These include MKP-1 (identical to 3CH134) (29–32), VHR (33), PAC1 (34, 35), hVH-2 (40, 41, 42), MKP-3 (identical to rVH6 and orthologue of PYST2) (43–45), and MKP-X (orthologue of PYST2) (43, 45). Among these family members, MKP-3/PYST1 and hVH-5/PYST2 appear exceptional insofar as they exhibit highly selective inactivation of either ERK or JNK/SAPK and p38 MAP kinases, respectively (45, 46). MKP-

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1 The abbreviations used are: ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; MKP, MAP kinase or ERK kinase; MKP, MAP kinase phosphatase; EGF, epidermal growth factor; MBP, myelin basic protein; MAPKAP kinase-2, MAP kinase-activated protein kinase-2; ATP2, activating transcription factor 2; EST, expressed sequence tag; CH2 domain, Cdc25 homology domain 2; GST, glutathione S-transferase; HA, hemagglutinin; pNPP, p-nitrophenyl phosphate; SPA, scintillation proximity assay; PML, promyelocytic protein; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; bp, base pair(s); MOPS, 4-morpholinepropanesulfonic acid.

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3/PYST1 is also the first dual specificity phosphatase to display an exclusively cytosolic rather than nuclear localization (43, 45). Interestingly, several dual specificity phosphatases undergo powerful regulated expression following cell stimulation by growth factors and/or exposure to stresses, suggesting one major mechanism for control of MAP kinases (29, 32, 34–40, 42–45). One critical unanswered question is whether the existence of multiple ERK, JNK/SAPK, and p38 kinase genes
and splice variants (1–8, 13, 26, 27) demands a similarly di-
verse range of dual specificity phosphatases to allow specific
and potentially highly compartmentalized control of MAP ki-
nase signaling.

As part of an investigation of dual specificity phosphatase
diversity and cell function, we have screened the expressed
sequence tag data base (dEST) to identify new dual specificity
phosphatases. MKP-4 is one novel family member for which we
have isolated a full-length cDNA. MKP-4 is most similar to
MKP-X/PYST2 (61% identity) and MKP-3/PYST1 (57% iden-
tity) and contains two N-terminal Cdc25 homology domains as
well as an extended active site motif characteristic of this gene
family. MKP-4 inactivates MAP kinase both in vitro and when
expressed in mammalian cells, where it displays selectivity for
ERK family members. This enzymatic selectivity, together with
a distinct subcellular localization and highly restricted pattern
of tissue expression, suggests a specific regulatory role for
MKP-4.

EXPERIMENTAL PROCEDURES

Materials—Restriction and DNA modifying enzymes were purchased
from Promega Biotechnologies Inc. (Madison, WI), New England
Biolabs Inc. or Life Technologies, Inc., and Taq DNA
polymerase was from Perkin Elmer. [35S]Methionine (1000 Ci/mmol),
radiolabeled 428-bp
l
humanplacenta glutathione-Sepharose and nickel-agarose columns as described (48, 49). Generated as
Program, (Uppsala, Sweden), and murine
EGF was purchased from Dr. Glaser AG (Basel, Switzerland), an-
ti-Myc 9E10 monoclonal antibody was a kind gift from Dr. Anne Dejean (Pasteur Institute,
France), and biotinylated HA monoclonal antibody 12CA5 from Boeh-
Ringer Mannheim (Rotkreuz, Switzerland), while horseradish peroxi-
dase-conjugated goat anti-mouse second antibody were from Vector Labora-
tories, Institute of Cancer Research, London, UK). [32P]dCTP was purchased from DuPont de Nemours International S.A. (Regensdorf, Switzerland), while [32P]dCTP (3000 Ci/mmol), [35S]ATP (5000 Ci/mmol), and streptav-

Fig. 2. Amino acid homology between MKP-4 and MKP-3/ PST1. The amino acid sequences of MKP-4 and human MKP-3/PYST1
(accession number X93920) (45) are shown aligned using the GAP
routine of the Wisconsin Genetics Computer Group sequence analysis
software package 7. MKP-4 and MKP-3/PYST1 are 57% identical, and
these residues are boxed.

MKP-4 Expression in Escherichia coli—For bacterial expression the
MKP-4 open reading frame was subcloned into pGEX-T3 (Pharmacia)
as follows. A StyI 1152-bp fragment was first isolated, Klenow-treated,
and ligated to a SmaI-digested Bluescript SK(–) (Stratagene). A construct
with the correct orientation was then identified, digested with BamHI and NcoI,
Klenow-treated, and religated. The MKP-4 insert was then subcloned as a BamHI-XhoI fragment in frame with the GST coding sequence.
pGEX-MKP-4 was used to transform E. coli BLR (Novagen). Cells were grown
overnight to saturation in LB medium containing 100 μg/ml ampicillin, after which growth was resumed by diluting the culture 1:50
and incubating at 37 °C for 1 h. Following transfer to 20 °C for 1 h,
IPTG was then added to a final concentration of 100 μM and cells
were harvested, resuspended in phosphate-buffered saline containing 1% (w/v) Triton X-100, 5 mM dithio-

In Vitro ERK 2 Inactivation by MKP-4—Constitutively activated rab-
bit MAP kinase kinase (MEK1 S218,E222E) and mouse ERK2
were generated as GST fusion proteins in E. coli and purified using glutathione-Sepharose (Pharmacia, Uppsala), washed, and eluted as described by the manufacturer.

MEMPHIS—Amino acid homology between MKP-4 and MKP-3/PYST1 is shown aligned using the GAD
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were generated as GST fusion proteins in E. coli and purified using glutathione-Sepharose and nickel-agarose columns as described (48, 49). Human stathmin (50) expressed in

Phosphatase Activity—MKP-4 in vitro Transcription and Translation—Full-length MKP-4
in pBluescript SK(–) was translated in vitro using a TNT T7 RNA
polymerase-coupled reticulocyte lysate system (Promega) in 25-μl
incu-

B. Antonsson, manuscript in preparation.
0.1% Triton X-100, 5 mM EDTA, and 50 μM ATP and left to incubate for 1 h at room temperature. Plates were then centrifuged at 1800 x g for 5 min and counted in a 1450 MicrobetaPlus liquid scintillation counter (Wallac).

MKP-4 Mammalian Expression Plasmid—MKP-4 was tagged at the C terminus with the Myc epitope and subcloned into pMT-SM (43, 51) as follows. A 1152-bp Styl fragment containing the entire open reading frame of MKP-4 was blunt-ended using mung bean nuclease and ligated with two sets of double-stranded oligonucleotide adaptors, which simultaneously added SalI and EcoRI restriction sites together with sequence encoding the Myc epitope EQKLISEEDLN followed by a stop codon at the C terminus. The SalI-EcoRI fragment was then subcloned into the corresponding sites of pMT-SM.

Cell Culture, Transfection, and Stimulation—COS-7 cells were grown under 7.5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum and 2 mM glutamine. Cells were grown in 6-well plates (35 mm diameter) to 80% confluence and transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Transfections were performed using the following plasmid combinations: 1.0 μg of pcDNA1-HA-p44 ERK1, pMT2T-HA-p54-SAPKβ, or pcDNA3-HA-p38 together with 0.01, 0.05, 0.1, 0.5, or 1.0 μg of pMT-SM-Myc-MKP4. Total plasmid concentration was maintained constant by supplementing with pMT-SM vector. Following 6 h of incubation with LipofectAMINE and plasmid DNA, cells were washed and grown for 40 h before starvation by incubation in serum-free medium for 2 h. Cells were then exposed to EGF (10 nM), anisomycin (10 μg/ml), or H2O2 (0.5 mM) at 37°C for 10–30 min. Where indicated, cells were triple-transfected with 0.25 μg of pEXV3-Myc-p21ras (G12V), 1.0 μg of pcDNA1-HA-p44 ERK1, together with 0.01–1.0 μg of pMT-SM-Myc-MKP4; under these conditions, starvation was for...
the last 16 h of growth.

**Immunoprecipitation and Immune Complex Kinase Assays**—Following stimulation cells were washed twice in 2 ml of ice-cold phosphate-buffered saline and scraped into Eppendorf tubes with 300 μl of buffer TP (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% (v/v) Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM EDTA, 10 mM calyculin, and 25 mM β-glycerophosphate). Cells were then homogenized using a sonicator probe at full power for 3 s on ice. Aliquots (150 μl) of the COS-7 cell homogenate were mixed with 850 μl of buffer TP and rotary mixed for 1 h at 4°C, after which they were centrifuged at 100,000 g for 20 min at 4°C. The supernatant (800 μl) was then mixed by rotary mixing for 2 h at 4°C with 75 μl of a preformed immunoprecipitating complex

**FIG. 4.** In vitro transcription and translation of MKP-4 protein. Full-length MKP-4 subcloned in pBluescript SK(−) was translated in vitro using a RNA polymerase-coupled reticulocyte lysate in the presence of [35S]methionine. Synthesized proteins were visualized following separation in a 12% gel. MKP-4 can be seen as a major band running at ~44 kDa when using T7 polymerase (T7) but not in control incubations with T3 polymerase (T3).

**FIG. 5.** In vitro measurement of MKP-4 phosphatase activity. Full-length MKP-4 was subcloned into pGEX4T3 and GST/MKP-4 purified following expression in E. coli (see “Experimental Procedures”). A, hydrolysis of pNPP was measured by incubation with purified GST/MKP-4 (8–40 μg as indicated) for 1 h at 37°C and reading optical density at 410 nm. Hydrolysis of pNPP is proportional to GST/MKP-4 added (open circles) and inhibited by addition of 1 mM sodium vanadate (closed circles). B, MKP-4 reversal of in vitro ERK2 activation. Purified ERK2 enzymatic activity was measured by SPA-detection of stathmin phosphorylation following incubation in the presence of γ-[32P]ATP. ERK2 activity was dependent upon co-incubation with purified constitutively active MEK1 (S217E,S221E), which did not itself phosphorylate stathmin. Co-incubation with increasing concentrations of GST/MKP-4 (0.02–1.6 μg, as indicated) results in dose-dependent block of stathmin phosphorylation by ERK2 (open circles). The control shows MKP-4 failure to dephosphorylate stathmin when added after incubation with activated ERK2 (closed circles). Points are the mean of three determinations and representative of three independent experiments.

**FIG. 6.** MKP-4 inhibits p44 ERK1, p54 SAPKβ, and p38 MAP kinases in intact cells. COS-7 cells were transfected with either p44 HA-ERK1, p54 HA-SAPKβ, or HA-p38 MAP kinase (1.0 μg of plasmid) together with 0.01, 0.05, 0.1, 0.5, or 1.0 μg of Myc-MKP-4 plasmid as indicated. Plasmid concentrations were maintained constant using pMT-SM vector. After culture for 40 h, cells were incubated for 2 h in serum-free medium and then stimulated with either 10 mM EGF (p44 HA-ERK1), 10 μM anisomycin (p54 HA-SAPKβ), or 0.5 mM H2O2 (HA-p38). Following incubation for 10 min (p44 HA-ERK1) or 30 min (p54 HA-SAPKβ and HA-p38), MAP kinases were immunoprecipitated using anti-HA epitope specific monoclonal antibody prebound to protein A-Sepharose beads. Immune complex assays were then performed using MBP (p44 ERK1), GST-ATF-2 (p54 SAPKβ), or GST-MAPKAP kinase 2 23 (p38) as substrates in the presence of γ-[32P]ATP. A, autoradiogram of phosphorylated substrates separated in a 15% SDS-PAGE gel. Substrate bands were excised for counting by scintillation spectrometry and calculation of kinase activity, which is indicated numerically below each lane. Activity in unstimulated cells is 1.0. B, Western blot of immunoprecipitated p44 ERK1, p54 SAPKβ, and p38 MAP kinases used for immune complex assays shown in A. Detection is with biotinylated anti-HA monoclonal antibody conjugate and chemiluminescence. C, Western blot detection of Myc-MKP-4 in cell lysates used for corresponding immunoprecipitations and immune complex assays shown on A and B. Detection is with anti-Myc monoclonal antibody and goat anti-mouse monoclonal antibody horse radish peroxidase conjugate. Myc-MKP-4 is immunodetectable as a single band of ~44 kDa when cells are transfected with 0.05 μg or more of plasmid. Data shown are representative of four separate experiments.
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(100 μl) of HA-epitope-specific HA.1 monoclonal antibody preincubated with 900 μl of 50% (v/v) protein A-Sepharose beads in 10 mM Tris-HCl, pH 7.5, for 2 h at 4°C. Beads were then sedimented by centrifugation at 10,000 x g for 3 min and washed twice in 1 ml of ice-cold buffer TP and once in 1 ml of buffer K (50 mM HEPES, pH 7.4, containing 20 mM MgCl₂, 200 mM sodium vanadate, 2 mM dithiothreitol, and 10 mM β-glycerophosphate) followed by final resuspension in 50 μl of buffer K. Immunocomplex assays were performed by mixing 10 μl of bead suspension with 10 μl of 6 μM [γ-³²P]ATP (~300,000 dpm/pmol), 10 μl of substrate protein (15 μg of MBP, 10 μg of GST-ATF-2, or 10 μg of GST-MAPKAP kinase 2 Δ3), and 30 μl of buffer K followed by incubation for 30 min at 30°C. Reactions were terminated by adding 15 μl of 10% Laemmli sample buffer (47) and heating for 5 min at 95°C. Following centrifugation at 10,000 x g for 5 min, supernatants were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described previously (43).

Western Blotting—For immunodetection of MAP kinases precipitated for immune complex assay, 10 μl of immunocomplex beads (see above) were diluted with 20 μl of 10 X Laemmli sample buffer and heated for 5 min at 95°C, followed by centrifugation at 10,000 x g for 5 min. Western analysis was then performed using supernatant fractions (20 μl) by SDS-polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose membranes as described (43). Immunoprecipitated HA-ERK1, HA-p54-SAPK6, and HA-p38 MAP kinases were detected using biotinylated HA-epitope-specific monoclonal antibody 12CA5 (Boehringer Mannheim) together with avidin-horseradish peroxidase conjugate and enhanced chemiluminescence. To detect levels of Myc-MKP-4 and Myc-p21ras (G12V) expression in transfected COS-7 cells, Western analysis was performed on cell homogenates (20 μg of protein) using anti-Myc epitope monoclonal antibody 9E10 and goat anti-mouse IgG horseradish peroxidase conjugate with chemiluminescence.

Tissue Northern Analysis—Northern analysis was performed using ~2 μg of human tissue poly(A)⁺ RNA separated on formaldehyde-agarose (1.2%) gels, transferred to nylon membranes, and fixed by ultraviolet irradiation (Clontech Multiple Tissue Northern Blots, catalog nos. 7750-1, 7754-1, 7755-1, 7759-1, and 7760-1). A random [³²P]dCTP-radioabeled 428-bp Styl fragment corresponding to the 3'-untranslated sequence of MKP-4 was hybridized to membranes and exposed as described (43).

Subcellular Localization of MKP-4—Rat sympathetic neurons from superior cervical ganglia of newborn rats were prepared, cultured, and microinjected with pMT-SM-Myc-MKP4 or pMT-SM-Myc-MKP3 as described (43). COS-7 or NIH 3T3 cells were transfected with the same MKP-4 plasmid using LipofectAMINE as described above. Immunocytochemistry was performed using monoclonal antibody 9E10 with FITC-conjugated goat anti-mouse secondary antibody. In double labeling experiments, nuclear bodies were labeled with a rabbit polyclonal antibody detecting promyelocytic protein (PML), which was detected using a combination of biotinylated anti-rabbit antibody and avidin-Cy3 conjugate. Microscopic analysis of cells was performed using ultraviolet fluoroscein.

Somatic Cell Hybrid Analysis—PCR primers corresponding to the sequences 5'-CAACGATGCTATGACTCG-3' (sense; nucleotides 1052–1071, Fig. 1) and 5'-GAAGGGCCATCAGGTTG-3' (antisense; nucleotides 1231–1250, Fig. 1) were used to amplify a 200-bp fragment of the 3' coding sequence of MKP-4 from a panel of human/rodent somatic cell hybrid DNAs (obtained from the UK Human Genome Mapping Project Resource Center). DNA amplification was performed with 22 cycles of denaturation (94°C, 30 s), annealing (52°C, 30 s), and extension (72°C, 1 min), with a final extension (72°C, 5 min). The products were analyzed by electrophoresis in 4% Metaphor (FMC Bioproducts) agarose gels. Chromosomes retained by the hybrids are summarized schematically in Fig. 11. The same primers and PCR conditions were used to amplify MKP-4 from a panel of radiation hybrid DNAs (52).

Southern Blot Analysis—Human male and female genomic DNAs (10 μg) were digested overnight with EcoRI or HindIII and subjected to agarose gel electrophoresis followed by transfer to Hybond N+ (Amer sham). Membranes were hybridized with a random [³²P]dCTP-radioabeled 428-bp Styl fragment corresponding to part of the 3'-untranslated region of MKP-4. Hybridization was performed as described (53).

RESULTS AND DISCUSSION

Identification and Cloning of MKP-4—To identify novel dual specificity phosphatases, we performed BLAST computer searches of the expressed sequence tag data base (dbEST) for partial cDNAs showing similarity with MKP-3 (43). One human sequence (accession number R51175) was found to be highly homologous to the C terminus of MKP-3 and, moreover, encoded the extended active site sequence motif VXVHXCGX-SRSXTTXXAYLM conserved in dual specificity phosphatases (28, 42, 43). This clone (ID 38872) was obtained from Research Genetics, Inc. (Huntsville, AL) I.M.A.G.E. Consortium (LLNL)
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MKP-4 Expression, Phosphatase Activity, and MAP Kinase Inactivation—MKP-4 is predicted to encode a protein of 41.8 kDa, and this is in agreement with the size of a single 35S-labeled protein band generated upon in vitro transcription and translation using a T7 RNA-polymerase-coupled reticulocyte lysate system (Fig. 4). To establish whether MKP-4 possesses endogenous catalytic activity, we overexpressed full-length MKP-4 as a GST fusion protein in *E. coli* and tested the purified protein using pNPP as substrate. Increasing concentrations of GST/MKP-4 resulted in a linear rise in pNPP hydrolysis (up to 40 \mu g of protein), and this catalytic activity was effectively inhibited by the protein-tyrosine phosphatase inhibitor sodium vanadate (Fig. 5A).

MKP-4 open reading frame encodes a protein of 384 amino acids, and comparison of this sequence with the GenBank™/EMBL Data Bank revealed greatest homology with other members of the dual specificity phosphatase family (see Introduction), with identities ranging from 61% for MKP-X/PYST2 down to 35% for hVH-5/M3–6. Direct comparison between MKP-4 and its closest full-length homologue, MKP-3/PYST1 (57% identity), indicates that regions of greatest similarity include, and fall either side of, the extended active site sequence motif V\_X\_VHC\_X\_AG\_X\_SRS\_X\_T\_X\_3AY(L/I)M (where X is any amino acid) (Fig. 2, solid boxes). This is also true for all other members of the dual specificity phosphatase family, within which MKP-4 displays significantly greater sequence identity within the catalytic C-terminal half of the molecule (Fig. 3).

Importantly, this region in MKP-4 includes Asp-259, Cys-290, and Ser-300 (Fig. 1), which are cognate to Asp-92, Cys-124, and Ser-131 of the dual specificity phosphatase VHR and are likely to represent critical residues underlying enzymatic activity (54, 55). According to a model of VHR catalysis, Cys-290 of MKP-4 may function as an active-site nucleophile forming a covalent thiol-phosphate intermediate, while Asp-259 acts as a general acid to donate a proton to the leaving group (54, 55). Despite lower homology within the N-terminal half of the dual specificity phosphatases (Figs. 2 and 3), this region of MKP-4 contains two stretches containing residues conserved with two segments flanking the active site of the Cdc25 phosphatase (Fig. 1, shaded boxes). These regions of homology are termed CH2 domains and are also found in other dual specificity phosphatases, although their functional significance is currently unknown (56, 57).
Given the close homology between MKP-4 and MKP-3/PYST1, which is highly selective for inactivating ERK family MAP kinases (45, 46), we next established whether GST/MKP-4 is able to inactivate ERK2 in vitro. Using a combination of purified recombinantly expressed proteins, ERK2 enzymatic activity was assessed by measuring phosphorylation of the MAP kinase substrate stathmin (50). Phosphorylation of stathmin is dependent upon ERK2 co-incubation with the constitutively active MAP kinase MEK1 (S217E,S221E) (19). This phosphorylation reflects ERK2 activation as MEK1 (S217E,S221E) is alone unable to phosphorylate stathmin directly (data not shown). Inclusion of GST/MKP-4 in the reaction mix results in efficient blocking of ERK2 enzymatic activity (Fig. 5B). Importantly, GST/MKP-4 is unable to dephosphorylate stathmin directly (control, Fig. 5B), indicating highly effective ERK2 inactivation under these assay conditions.

To establish whether MKP-4 displays similar enzymatic activity within intact cells, we next co-transfected the HA-tagged MAP kinases p44 ERK1, p54-SAPKβ, or p38 together with Myc-tagged MKP-4 in COS-7 cells. To obtain a clear impression of the relative effectiveness of MKP-4 to inactivate each MAP kinase, cells were transfected using a range of plasmid concentrations (0.01–1.0 μg/well). These concentrations were chosen to give a reproducible dose-dependent increase in the levels of immunodetectable MKP-4 protein (at ~4 kDa), while expression of each MAP kinase is unaltered (Fig. 6). Thus, although with higher plasmid concentrations levels of MKP-4 protein are likely to represent a considerable overexpression, these conditions allow a direct comparison of MKP-4-dependent inactivation of different MAP kinase family members. Using this approach MKP-4 displays moderate selectivity for p44 ERK1 when compared with p54 SAPKβ and p38 MAP kinases. Hence, EGF-stimulated p44 ERK1 activation is inhibited by ~50% when cells were transfected with only 0.01 μg of plasmid, conditions under which MKP-4 protein was not detected by Western analysis (Fig. 6). This level of MKP-4 expression had little effect on anisomycin-stimulated p54 SAPKβ or H2O2-dependent activation of p38 MAP kinase (Fig. 6). Inhibition of p44 ERK1 activation increased to ~80% following transfection with 0.05 μg of plasmid, while under identical conditions stress-induced activation of p54 SAPKβ and p38 MAP kinases was suppressed by ~50% (Fig. 6). Full blockade of p54 SAPKβ and p38 MAP kinases required maximal expression of MKP-4 using 1.0 μg of plasmid (Fig. 6). Together, these studies confirm that as observed in vitro (Fig. 5B), MKP-4 is able to inactivate ERK family MAP kinases when expressed in mammalian cells. Importantly, these results also indicate that MKP-4 selectivity (ERK > p38 = JNK/SAPK) is similar to its close homologue MKP-3/PYST1, which is significantly more effective against ERK family isoforms (ERK > JNK/SAPK > p38) (45, 46). In contrast to this, MKP-4 enzymatic specificity is quite different from hVH-5/M3–6 (JNK/SAPK − p38 >> ERK) (46), MKP-2/hVH-2/TYP-1 (ERK = JNK/SAPK > p38) (58), and PAC-1 (ERK = p38 > JNK/SAPK) (58). It is of note that, despite these observations, we cannot exclude the possibility that additional cellular proteins, possibly unrecognized MAP kinases, also represent targets for MKP-4 action particularly in more physiological levels of expression. Notwithstanding this caveat, the existence of multiple dual specificity phosphatases with clear selectivity for inactivation of known MAP kinases strongly suggests specific functional roles for different family members.

Mutated constitutively activated p21ras (G12V) stimulates ERK MAP kinases, and this may underlie mitogenesis and cellular transformation induced by this oncogene (19, 59, 60). To test whether MKP-4 is able to block oncogenic p21ras-dependent MAP kinase activation, COS-7 cells were triple-transfected with constitutively active Myc-tagged p21ras (G12V), p44 HA-ERK1, and varying concentrations of Myc-MKP-4 plasmid. This experiment reveals that, as with acute exposure to EGF (Fig. 6), MKP-4 blocked completely p44 ERK1 activation by p21ras (G12V), although this required higher concentrations of plasmid and expressed protein (Fig. 7). MKP-4-dependent blockade of growth factor and oncogene-stimulated MAP kinase activation as observed here appears functionally equivalent to previous observations of ERK inhibition following exposure of a dominant negative mutant form of MAP kinase MEK1 (19), or in cells treated with the MEK1 inhibitor PD098059 (61). Since inhibition of ERK activity using these approaches also blocks growth factor-stimulated proliferation and even reverts oncogene-driven transformation (19, 61), one important function for MKP-4 could be to inhibit cellular proliferation and possibly act as a tumor suppressor.

**MKP-4 Tissue Distribution**—To establish which cells and tissues may be major sites for MKP-4 action, we performed Northern blot analysis on poly(A)+ RNA isolated from a range of human cell and tissue types. Using a probe from the 3'-untranslated sequence of MKP-4, we detected a single band at 2.5 kilobases expressed only in placenta and kidney (Fig. 8) and fetal liver (not shown). We failed to detect any MKP-4 mRNA in the following adult cells and tissues: heart, lung, liver, skeletal...
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MKP-4 Subcellular Localization—Sustained activation of MAP kinases has been reported to trigger their translocation to the nucleus (62, 63). This relocalization places MAP kinases in close proximity with several nuclear target proteins, including the transcription factors Elk-1, c-Jun, ATF-2, and CHOP (11–15). Three other human dual specificity phosphatases, PAC-1, PYST2, and hVH-5, display a highly restricted expression pattern, indicating important roles regulating MAP kinases in a limited complement of cell and tissue types at similar or higher levels (39, 40, 45, 57).

Fig. 1. Chromosomal localization of the human MKP-4 gene by analysis of a panel of human/rodent somatic cell hybrids. *+* indicates that the hybrid contains the chromosome shown on the upper row, while ‘*+*’ indicates the absence of the corresponding chromosome. ‘*+*’ indicates a chromosome translation, extra chromosome, or other modification as follows: MCP6BRA, Xqter-Xq13.6p21–6qter; C4u, 8 and a fragment of 22, 762–8a, 10 and Y; Ia9607+, 12, 21, and X; 289, 13 and fragments of 8, 11, and 12; GM10479, 14 and a fragment of 16; HORLI, 15, 11q, part of Xp, and proximal Xq; GM10478, 20, part of 4, part of 8, 22q, and X; PgME25NU, 22 and a fragment of Xp. The column for chromosome X is outlined in bold to highlight the correlation of this chromosome with the presence of the MKP-4 gene. The pattern of retention of the MKP-4 gene is shown in the final column, where the presence of the gene is indicated by ‘*+*’ and the absence of the gene is indicated by ‘*–*’.

Table 1: HUMAN CHROMOSOMES

| HYBRID       | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X | Y | MKP4 |
|--------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| GM07299      | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | +  |    | +  |
| GM10826B     | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| GM10293      | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| HHW416       | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| GM10114      | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| MCP6BRA      | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| CLONE21E     | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| C4A          | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| GM10611      | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| 762-8a       | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| GICL4        | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| Ia9607+      | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| 289          | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| GM10479      | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| HORLI        | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| 2806H7       | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| PCTBA1.8     | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| DL187S       | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| GM10612      | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| GM10478      | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| THYB1.3      | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| PgME25NU     | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| HORL9X       | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |
| 853          | -  |  + | -  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | -  |    | -  |

hVH-3/B23, which are localized entirely within the nucleus (34, 36, 39), while another dual specificity phosphatase family member, hVH-5/M3–6, is cytosolic or nuclear depending on the cellular environment (41). We have studied the subcellular localization of Myc-tagged MKP-4 following expression in rat sympathetic neurons as well as in COS-7 and NIH 3T3 cell lines. In sympathetic neurons both MKP-3 and MKP-4 were immunodetectable within the cell body cytoplasm, while MKP-4 protein was also clearly detectable within neurites (Fig. 9, A and B). Interestingly, in some neurons, MKP-4 protein appeared to concentrate in regions resembling synaptic swellings (Fig. 9B, indicated by arrows). By electron microscopy these regions possess a high content of vesicular membranes characteristic of premature synaptic structures. MKP-4 was also localized within the cytosol of NIH 3T3 (Fig. 9, C and D) and COS-7 cells (Fig. 9, E and F), although a subset of transfected cells (10–20%) also displayed punctate staining within the nucleus (Fig. 9, D and F). Confocal microscopy confirms an intra-nuclear localization for MKP-4 (data not shown). Although we have not yet been able to test whether localization of heterologously expressed protein reflects exactly compartmentation of endogenous MKP-4, this pattern of subcellular localization is clearly different from other dual specificity phosphatases also expressed as epitope-tagged proteins (34, 36, 39, 41, 43, 45).

Together with MKP-4, several dual specificity phosphatases have now been described as nuclear (see above), although none possess a clear bipartite nuclear targeting motif (66). Despite this, two N-terminal charged clusters exemplified by RRARR-5RAR in hVH-3/B23 (39, 40) can be identified also in the primary amino acid sequences of MKP-1/CL100 (29), hVH-2/TYP-1 (36, 38), and PAC-1 (34) and could contribute to their presence in the nucleus. MKP-4 also contains two clusters of basic amino acids, although these are separated by a longer spacer (RRLRR-(30)-RRRR) (Fig. 1). It remains to be established whether this could account for its distinctive subcellular localization.

3 J. Staple, personal communication.
MKP-4 Co-localization with Promyelocytic Leukemia (PML) Protein—Punctate nuclear staining as seen for MKP-4 has also been reported for PAC-1 (34), although not for CL100, hVH-2, or hVH-3 (36, 39), and may be a distinctive property of a subset of dual specificity phosphatases. Punctate nuclear localization is also reminiscent of the PML protein, which concentrates in M-phase nuclei of dual specificity phosphatases. Punctate nuclear staining is also reminiscent of the PML protein, which concentrates in M-phase nuclei of human cells. It is also reminiscent of the PML protein, which concentrates in M-phase nuclei of human cells. It is also reminiscent of the PML protein, which concentrates in M-phase nuclei of human cells.

MKP-4 Chromosomal Localization—To determine the chromosomal localization of MKP-4, DNA from human/rodent somatic cell hybrids were analyzed for the presence of the human MKP-4 gene by PCR. An MKP-4-specific human fragment of 200 bp was distinguishable from rodent-specific fragments. This human fragment was present in hybrids GM07299, MCP6BRA, 1aA9607, GM10478, THBY13.1, and HORL9, all of which retain the long arm of human chromosome X (Fig. 11). PCR analysis of DNAs from a panel of radiation hybrids (52) confirmed this, assigning the gene for MKP-4 to Xq28 (data not shown).

Southern blot analysis of human male and female genomic DNAs using a fragment from the 3′-untranslated region of MKP-4 as a probe identified a single band (Fig. 12). A stronger signal was detected in the lanes containing female DNA, again indicating that the gene for MKP-4 is X-linked (Fig. 12). No other dual specificity phosphatase genes have been found to map to the Xq28 gene (69, 70), and, based on tissue expression in adult, there are no obvious candidate human diseases assigned to this region that may be caused by mutations in MKP-4.

In this paper we report the identification and initial characterization of MKP-4 as a novel dual specificity phosphatase. Although structurally homologous to other family members, including an extended active site consensus as well as two N-terminal CH2 domains, MKP-4 is unique in terms of both function and distribution. Our studies indicate that MKP-4 inactivates MAP kinases with the selectivity ERK > p38 = JNK/SAPK. This enzymatic specificity is similar to its close homologue MKP-3/PYST1 (ERK > JNK/SAPK = p38) (45, 46), although distinct from hVH-5/M3–6 (JNK/SAPK = p38).

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