MKP-3, a Novel Cytosolic Protein-tyrosine Phosphatase That Exemplifies a New Class of Mitogen-activated Protein Kinase Phosphatase*

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MKP-1 (also known as CL100, 3CH134, Erp, and hVH-1) exemplifies a class of dual-specificity phosphatase able to reverse the activation of mitogen-activated protein (MAP) kinase family members by dephosphorylating critical tyrosine and threonine residues. We now report the cloning of MKP-3, a novel protein phosphatase that also suppresses MAP kinase activation state. The deduced amino acid sequence of MKP-3 is 36% identical to MKP-1 and contains the characteristic extended active-site sequence motif VXVHCXGXSRSTXXYAYLM (where X is any amino acid) as well as two N-terminal CH2 domains displaying homology to the cell cycle regulator Cdc25 phosphatase. When expressed in COS-7 cells, MKP-3 blocks both the phosphorylation and enzymatic activation of ERK2 by mitogens. Northern analysis reveals a single mRNA species of 2.7 kilobases with an expression pattern distinct from other dual-specificity phosphatases. MKP-3 is expressed in lung, heart, brain, and kidney, but not significantly in skeletal muscle or tests. In situ hybridization studies of MKP-3 in brain reveal enrichment within the CA1, CA3, and CA4 layers of the hippocampus. Metrazole-stimulated seizure activity triggers rapid (<1 h) but transient up-regulation of MKP-3 mRNA in the cortex, piriform cortex, and some amygdala nuclei. Metrazole stimulated similar regional up-regulation of MKP-3, although this was additionally induced within the thalamus. MKP-3 mRNA also undergoes powerful induction in PC12 cells after 3 h of nerve growth factor treatment. This response appears specific insofar as epidermal growth factor and dibutyryl cyclic AMP fail to induce significant MKP-3 expression. Subcellular localization of epitope-tagged MKP-3 in sympathetic neurons reveals expression in the cytosol with exclusion from the nucleus. Together, these observations indicate that MKP-3 is a novel dual-specificity phosphatase that displays a distinct tissue distribution, subcellular localization, and regulated expression, suggesting a unique function in controlling MAP kinase family members. Identification of a second partial cDNA clone (MKP-X) encoding the C-terminal 280 amino acids of an additional phosphatase that is 76% identical to MKP-3 suggests the existence of a distinct structurally homologous subfamily of MAP kinase phosphatases.

A wide range of cell-surface stimuli, including growth and differentiation factors and cytokines as well as ultraviolet radiation and osmotic shock, trigger rapid and powerful activation of mitogen-activated protein (MAP) kinase family members (1–5). Currently, three major subclasses of MAP kinase can be identified, and these comprise the ERK, SAPK/JNK, and p38/HOG1 families (2, 3, 6). Full activation of MAP kinase requires phosphorylation on critical tyrosine and threonine residues, and several upstream dual-specificity kinases catalyzing this modification have now been identified (1–3, 6). Once activated, MAP kinases phosphorylate and regulate several cellular proteins, including additional protein kinases, cytoskeletal elements, stathmin, phospholipase A2, and transcription factors, notably Myc, Elk-1, J un, and ATF-2 (1, 7–11). This range of substrates indicates a pivotal role for MAP kinases in cellular signal transduction, suggesting that mechanisms regulating the extent and duration of their activation will play a key role in controlling cell function. This is illustrated by mutational activation of the MAP kinase kinase MEK, which leads to constitutive activation of ERK2 accompanied by cellular transformation in fibroblasts or neuronal differentiation in PC12 cells (12). By contrast, inhibition of ERK2 phosphorylation by interfering mutants of MEK suppresses growth factor-stimulated proliferation, reverts oncogene-dependent transformation, and blocks PC12 differentiation by NGF (12). Chemical inhibition of MEK similarly inhibits ERK phosphorylation as well as PC12 cell differentiation (13). These experiments emphasize the critical importance of MAP kinase phosphorylation and activation state in regulating cellular responsiveness and function.

MAP kinase phosphorylation is a reversible process, indicating that protein phosphatases play a crucial role in controlling cellular activities. Among the large number of protein-tyrosine phosphatases currently identified (14–16), an emerging class of dual-specificity phosphatase may regulate directly and specifically MAP kinase family members. The prototypic member of this class is the vaccinia virus VH1 phosphatase (17, 18), although mammalian homologs of this gene have now been identified. The dual-specificity phosphatase family is exemplified by MKP-1 (also known as CL100, 3CH134, Erp, and hVH-1),

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† The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; SAPK/JNK, stress-activated protein kinase; nNOS, N-terminal kinase; MEK, MAP kinase/ERK kinase; MKP, MAP kinase phosphatase; NGF, nerve growth factor; EGF, epidermal growth factor; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); MBP, myelin basic protein.

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which dephosphorylates MAP kinases at both the Tyr and Thr residues necessary for enzymatic activity (19–24). Activity toward phosphorylated Tyr and Thr is abolished when a single active-site cysteine is mutated, suggesting a common catalytic residue necessary for enzymatic activity (19–24). Activity to which dephosphorylates MAP kinases at both the Tyr and Thr residues is increased upon infection of eight serotypes. The template for amplification was single-stranded random-primed cDNA prepared from rat brain poly(A) RNA using SuperScript reverse transcriptase (GIBCO BRL). PCR was initiated by hot start using Ampliwasy (Perkin Elmer) in a reaction buffer consisting of 50 mM KCl, 3 mM MgCl₂, 0.2 mM dNTPs, 10 mM Tris-HCl, pH 8.3, using a 2.7 μM concentration of each primer pair and 1 unit of Taq polymerase (Perkin-Elmer) in a final volume of 50 μl. PCR was performed in a thermocycler (Perkin Elmer) for 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 50°C, and 45 s at 72°C, followed by extension at 72°C for 5 min. The bands of interest were ~300 bp for the combination IF-4 with IR-1 or IR-12 and ~210 bp for the other combinations. PCR products were resolved using a 2% agarose gel and subcloned into EcoRI digested pBluescript SK(−) (Stratagene). A total of 70 clones were sequenced from both T7 and T3 primers using an Applied Biosystems Model 370 automated sequencer. Of these, 24 clones were rat MKP-1, two corresponded to PAC-1, one was B23, and three represented a novel gene encoding the extended active-site sequence motif VXVHCXXGXSRXXTXXXAYLM (where X is any amino acid) common to all known dual-specificity phosphatases. This clone was named MKP-X.

**Screening of cDNA Libraries and Isolation of Full-length MKP-3**

A random 150,000×10^6 pJCTP-radiolabeled MKP-X probe was prepared using a 210-bp EcoRI PCR product, and this was used to screen a commercial random- and oligo(dT)-primed rat lung cDNA library (CLONTECH). The library was plated onto 10 NZY plates (GIBCO BRL, Basel, Switzerland) and allowed to grow at 37°C to a density of ~150,000 recombinants/plate (41). The plaques were transfected in duplicate into nitrocellulose filters, which were then alkali-treated, neutralized, and baked. Filters were prehybridized at 42°C for 1–2 h in hybridization buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 20% formamide, 50 μg/ml denatured salmon sperm DNA, and 50 μg/ml yeast RNA). Boiled MKP-X probe (1–2 × 10⁶ cpm/ml) was then added to the hybridization buffer and incubated overnight at 42°C. Filters were then washed in 2 × SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) containing 0.1% SDS for 2 h at 50°C with three changes of the solution, air-dried, and autoradiographed. A total of 24 clones were plaque-purified and subcloned as EcoRI fragments in pBluescript SK(−). Clone 3, containing the largest insert, was sequenced completely and found to contain an open reading frame of 735 bp. This sequence lacked the start codon of MKP-X. A longer probe was then prepared from this clone and used to screen an oligo(dT)-primed rat superior cervical ganglion cDNA library (provided by Dr. G. Buell, Gurdon Institute, Cambridge, United Kingdom). Conditions were such that filters were washed three times at 60°C in 2 × SSC containing 0.1% SDS, followed by one wash in 0.4 × SSC with 0.1% SDS. Three positive clones were isolated. One of these (clone 310) encoded the C-terminal 280 amino acids of MKP-X, although the open reading frame was again missing the start codon. Two additional clones contained inserts encoding a gene product whose predicted amino acid sequence is similar to, but distinct from, MKP-X. One of these was a partial clone (clone 23), although the other (clone 36) contained a 2.5-kb insert encoding a novel dual-specificity phosphatase, which we have called MKP-3. This was sequenced twice, revealing a full-length open reading frame extending 1146 bp. Comparison of MKP-3 with the GenBankTM data bank demonstrated the closest homology to dual-specificity phosphatases.

**Northern Analysis—** Northern analysis was performed using ~2 μg of rat tissue poly(A)⁺ RNA separated on a denaturing formaldehyde–agarose (1.2%) gel, transferred to nylon membranes, and fixed by ultraviolet irradiation (CLONTECH). The blots were prehybridized for 2 × 10⁶ cpm/ml of rat liver poly(A) RNA separated on a denaturing formaldehyde–agarose (1.2%) gel, transferred to nylon membranes, and fixed by ultraviolet irradiation (CLONTECH). The blots were prehybridized for 1 h in hybridization buffer containing 5 × SSPE, 50 μg/ml heat-denatured rat liver poly(A) RNA, 0.5% SDS, and 1% sodium dodecyl sulfate, followed by two washes in 0.5× SSC containing 0.1% SDS at 50°C. The filters were then hybridized with a 32P-labeled probe (5000 Ci/mmol) and washed three times at 60°C in 0.1× SSC, containing 0.1% SDS, followed by one wash in 0.4× SSC with 0.1% SDS. Three positive clones were isolated. One of these (clone 310) encoded the C-terminal 280 amino acids of MKP-X, although the open reading frame was again missing the start codon. Two additional clones contained inserts encoding a gene product whose predicted amino acid sequence is similar to, but distinct from, MKP-X. One of these was a partial clone (clone 23), although the other (clone 36) contained a 2.5-kb insert encoding a novel dual-specificity phosphatase, which we have called MKP-3. This was sequenced twice, revealing a full-length open reading frame extending 1146 bp. Comparison of MKP-3 with the GenBankTM data bank demonstrated the closest homology to dual-specificity phosphatases.

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A single band of ~2.7 kb was detected after overnight exposure at ~70 °C using X-Omat film (Eastman Kodak Co.).

PC12 Cell Culture and Northern Blot Analysis—PC12 cells were grown in 9-cm collagen-coated culture dishes using RPMI 1640 medium supplemented with 10% (v/v) horse serum, 5% (v/v) fetal calf serum, and antibiotics under 7.5% CO2. Cells were washed in RPMI 1640 medium 18 h before transfection, and cultures were continued utilizing 10% (v/v) NaCl, 1% (v/v) streptomycin, 1% (v/v) glutamine, 1% (v/v) penicillin, and 0.1% (v/v) horse serum. NGF, EGF, or dibutyryl AMP was then added at the concentrations indicated, and this was repeated every 2 days without further medium exchange. Total RNA was extracted at the indicated times using the RNeasy total RNA extraction kit (Qiagen, Basel) according to the manufacturer’s protocol. For Northern analysis, 5 µg of total RNA were electrophoresed in a 1% formaldehyde-agarose (1%) gel, transferred to nylon membranes (Hybond N, Amersham International), and probed using an antisense riboprobe obtained using clone 36 following removal of an XhoI fragment and an SphI/T7 transcription kit (Boehringer Mannheim). Hybridization was performed overnight at 70 °C using 50% formamide/Denhardt’s solution in 5× SSC buffer (41). The hybridized membrane was washed twice for 30 min in 2× SSC containing 0.1% SDS at 75 °C and twice for 30 min in 0.1× SSC containing 0.1% SDS at the same temperature. The washed membrane was exposed to Kodak XAR-5 films at ~70 °C.

In Situ Hybridization—MKP-3 and CL100 mRNAs were synthesized (Applied Biosystems Model 394 synthesizer), purified by gel electrophoresis, and diluted to a concentration of 0.25–0.3 mg/ml in double-autoclaved sterile water. Probes were end-labeled using [32P]-dATP (42). In situ hybridization was performed using fixed brain sections (12-µm thickness) that were prewarmed to room temperature for 15 min. [32P]-Labeled oligonucleotide probes (3× 106 cpm/ml) were diluted in 100 µl of hybridization buffer (50% [v/v] formamide, 4× SSC, 5× Denhardt’s solution, 25 mM NaPO4, 1 mM NaH2PO4, 10% [w/v] dextran sulfate, 10 µg/ml denatured salmon sperm DNA, 5 µg of polyadenylic acid) containing 10 mM dithiothreitol. Sections were covered with 50–24-mm coverslips, and hybridization was performed in a humidified chamber for 16–24 h at 42 °C. After hybridization, coverslips were removed under 1× SSC at room temperature, and slides were washed first for 30 min in 2× SSC at 52 °C and then for 1 min in 1× SSC and for 1 min in 0.1× SSC at room temperature. Slides were then dehydrated by a sequential immersion in 70% (v/v) and 100% (v/v) ethanol for 3 min, after which they were air-dried and exposed to Amersham Hyperfilm at room temperature for 1–6 days.

MKP-3 Expression Plasmids—For cellular expression, MKP-3 was subcloned into pMT-SM (supplied by Dr. A. Ashworth, Institute of Cancer Research, London), which is derived from pMT2 (43) and contains three upstream stop codons in all three reading frames and a multiple cloning site. pMT-SM/MKP-3 was obtained by subcloning an XhoI-EcoRI fragment of ~2.5 kb carrying the complete coding region together with the 3′-end of clone 36 into SacI-EcoRI present in the multiple cloning site of pMT-SM. MKP-3 bearing the C-terminal Myc epitope was constructed using the same procedure and a synthetic oligonucleotide encoding the Myc epitope, a stop codon, and a BglII site. This was phosphorylated in vitro using T4 polynucleotide kinase and ligated to an XhoI-BsaII fragment carrying the complete coding sequence of MKP-3. This fragment was ligated within the SacI-EcoRI site of linearized plasmid pMT-SM to obtain pMT/MKP-3/Pac-1.

COS Cell Culture and Transfection—COS-7 cells were grown under 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 2 mM glutamine. Cells were grown to 60% confluency in 6-well plates and transfected with 2 µg of plasmid DNA using 20 µg of Lipofectamine (GIBCO BRL) according to the manufacturer’s instructions. Transfections were performed using combinations of the following plasmids: pMT-SM (control) and pMT-SM carrying MKP-3, pMT-SM/Myc, or TYP-1/Myc with or without pEX3 expressing ERK2/Myc. Following 6 h of exposure to Lipofectamine, cells were washed and grown for 24 h before starvation by incubation in serum-free medium for a further 18 h. Cells were then stimulated for 10 min with EGF (10 nm), phorbol 12-myristate 13-acetate (100 nm), or serum (15%) added directly to the medium; washed once with 2 ml of cold phosphate-buffered saline; and immediately frozen over a dry ice/methanol mixture together with 300 µl of buffer T (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1.5% [v/v] Triton X-100, 1 mM leupeptin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM sodium pyrophosphate, 5 mM sodium vanadate, and 10 mM calyculin). Cells were scraped into Eppendorf tubes and homogenized using a probe sonicator at full power for 5 s.

Western Blotting—For immunodetection of endogenous ERK2 as well as heterologously expressed ERK2/Myc, Myc-3/Mkp-3, and TYP-1/Myc, or their controls (20 µg of total RNA were electrophoresed using SDS-polyacrylamide gel electrophoresis using 10% acrylamide with 0.165% bisacrylamide as a separating gel). To observe bands accompanying ERK2 activation, samples were migrated until the 30-kDa protein standard marker was near the gel base. Electrophoresis onto nitrocellulose membranes was at 100 V for 1 h using 50 mM Tris containing 380 mM glycine, 1.0% SDS, and 20% (v/v) methanol. Membrane blocking and washing as well as antibody incubation and detection by enhanced chemiluminescence were performed as described (44). Endogenous ERK2 was detected using antibody 122 (supplied by Professor C. Marshall, Institute of Cancer Research). All heterologously expressed Myc-tagged proteins were detected using monoclonal antibody 9E10 (Dr. Glaser AG).

Cloning and Characterization of the MAP Kinase Phosphatase MKP-3—Identification and Cloning of MKP-3—To identify novel dual-specificity phosphatases expressed in brain, we employed reverse transcription-PCR amplification using single-stranded random-primed cDNA prepared from rat brain poly(A)+ RNA. Alignment of MKP-1, PAC-1, and B23 showed regions of high homology, and these were used to design degenerate primers. 5′-Primers were synthesized based on the sequences (W/H/F/N/Q/E/A/I and CPHNF, while 3′-primers corresponded to NF/S/G/FM (see “Experimental Procedures”). Sequence analysis of the PCR products revealed that out of 70 clones, three represented a novel sequence, which was termed MKP-X. A 210-bp EcoRI insert encoding MKP-X was then radiolabeled to screen a rat lung cDNA library. One of the positive clones from this library was identified by hybridization with a 5′-end-labeled DNA probe.

RESULTS AND DISCUSSION

Identification and Cloning of MKP-3—To identify novel dual-specificity phosphatases expressed in brain, we employed reverse transcription-PCR amplification using single-stranded random-primed cDNA prepared from rat brain poly(A)+ RNA. Alignment of MKP-1, PAC-1, and B23 showed regions of high homology, and these were used to design degenerate primers. 5′-Primers were synthesized based on the sequences (W/H/F/N/Q/E/A/I and CPHNF, while 3′-primers corresponded to NF/S/G/FM (see “Experimental Procedures”). Sequence analysis of the PCR products revealed that out of 70 clones, three represented a novel sequence, which was termed MKP-X. A 210-bp EcoRI insert encoding MKP-X was then radiolabeled to screen a rat lung cDNA library. One of the positive clones from this
A protein of 381 amino acids with a predicted molecular mass of 42.3 kDa (Fig. 1). The partial clone MKP-X does not contain a predicted translation initiation site, but encodes the C-terminal 280 amino acids of a protein displaying 76% amino acid identity to MKP-3 (Fig. 2). Alignment of the deduced amino acid sequences of MKP-3 and MKP-X with the GenBank™/EMBL Data Bank revealed the greatest homology to the dual-specificity phosphatases MKP-1, PAC-1, B23, hVH-2, and VHR. The overall predicted amino acid sequence identity between this gene family and MKP-3 is 32–37%, although this value masks greater homology within C-terminal regions (Fig. 3A). Within this region, MKP-X displays 83% primary amino sequence identity to MKP-3 (Fig. 3A). As with all other dual-specificity phosphatases, the C-terminal domains of both MKP-3 and MKP-X contain the extended active-site sequence motif VXXVHCXXGXSRTXXAYLM (where X is any amino acid) (Fig. 3B). This motif contains Cys-293 and Ser-300 (numbering according to MKP-3), which, together with Asp-262, are cognate to Cys-124, Ser-131, and Asp-92 of VHR and are likely to participate in the catalytic mechanism underlying dual-specificity phosphatase activity (47, 48). Indeed, Cys-124 of VHR and presumably also Cys-293 of MKP-3 are likely to serve as the active-site nucleophile that forms a covalent thiol-phosphate intermediate during catalysis (48). Despite lower homology within N-terminal regions, MKP-3 does contain stretches, termed CH2 domains, conserved with two segments flanking the active site within the Cdc25 phosphatase (Fig. 3C). MKP-X also possesses one CH2 domain within the most N-terminal sequence hitherto identified (Fig. 3C). These CH2 domains have also been identified within the N-terminal regions of other dual-specificity phosphatases (49, 50), although their functional significance is currently unknown.

MKP-3 Expression and Block of Mitogen-stimulated ERK2 Activity—To test whether MKP-3 displays functional properties expected of a dual-specificity phosphatase, we measured mitogen-stimulated ERK2 phosphorylation state and enzymatic activity in transfected COS cells. In agreement with its predicted molecular size, MKP-3/Myc was immunodetectable as a major band migrating at 42 kDa (Fig. 4A). An additional minor protein band was also detected at ~44 kDa. TYP-1/Myc was also readily detected by Western analysis (Fig. 4A) and migrates at 43 kDa as predicted by its deduced amino acid sequence. Cells transfected with control plasmid (pMT-SM) or untagged MKP-3 were negative using the anti-Myc monoclonal antibody 9E10 (Fig. 4A).

![Fig. 1. Nucleotide and encoded amino acid sequences of MKP-3 cDNA.](http://www.jbc.org/)
Mitogen-stimulated ERK2 phosphorylation results in a shift in its electrophoretic mobility on SDS-polyacrylamide gels (12, 51). Consistent with this, when ERK2 endogenous to COS cells was studied by Western analysis, EGF induced a clear retardation in gel migration (Fig. 4B). More important, this effect on the ERK2 band shift was abolished in COS cells expressing MKP-3 (Fig. 4B). The EGF-stimulated shift in ERK2 electrophoretic mobility was also inhibited, although not completely abolished, in COS cells expressing TYP-1/Myc (Fig. 4B). To test whether suppressed EGF-stimulated ERK2 phosphorylation correlates with inhibition of enzymatic activity, immune complex assays were performed using myelin basic protein (MBP) and ERK2 immunoprecipitated from transfected COS cells (see "Experimental Procedures"). While ERK2 from cells transfected with control plasmid displayed powerful EGF-stimulated MBP phosphorylation, this was inhibited considerably in cells transfected with either MKP-3/Myc or untagged MKP-3 (Fig. 5, A and B). Consistent with partial suppression of ERK2 phosphorylation state by TYP-1/Myc (Fig. 4B), this dual-specificity phosphatase inhibited ERK2 activity by 60% (Fig. 5, A and B). Differences in MBP phosphorylation reflect altered ERK2 activation state as similar levels of ERK2 were immunoprecipitated in these comparative experiments between transfected COS cells (Fig. 5C). In additional experiments (data not shown), we have shown that MKP-3/Myc expression also results in near complete inhibition of ERK2 activation by 15% serum and the phorbol ester phorbol 12-myristate 13-acetate at 100 nM. Identical results were also obtained when we performed immune complex assays on ERK2/Myc cotransfected in COS-7 cells together with untagged MKP-3 (data not shown). Together, these experiments demonstrate that MKP-3 displays functional activity expected for a dual-specificity phosphatase in its ability to abolish mitogen-stimulated activation of ERK2.

Fig. 3. MKP-3 homology to other dual-specificity phosphatases. A, schematic representation of MKP-3 amino acid identity to other members of the dual-specificity phosphatase family. Predicted amino acid sequences (MKP-1, GenBankTM/EMBL accession number X84004 (M. Muda, unpublished data); hVH-2 (30); PAC-1, GenBankTM/EMBL accession number L11329 (26); B23, GenBankTM/EMBL accession number U15932 (28); and VHR, GenBankTM/EMBL accession number L05147 (25)) were compared with MKP-3 using the GAP routine of the Wisconsin Genetics Computer Group sequence analysis software package. Values shown are percentage identities (boxed) for independent comparisons of N- and C-terminal regions based on residue numbers as indicated. N- and C-terminal domains were defined based on a division on either side of the highly conserved PV(E/Q)IL residues. B, conservation of the extended active-site motif shared between dual-specificity phosphatase enzymes. Amino acids used for comparison are indicated on the left of the sequence, and identical residues are boxed. C, alignment of two N-terminal homology domains (CH2-N and CH2-C) conserved between dual-specificity phosphatase family members and human Cdc25 phosphatase (GenBankTM/EMBL accession number P30307) (49, 50). Amino acid numbers used for comparison are indicated, and identical residues are boxed.
MKP-3 mRNA Is Widely Expressed in Tissues—Using a random-primed probe prepared from a 410-bp SmaI fragment from the 5′-end of the MKP-3 clone, a single 2.7-kb mRNA species was observed by Northern analysis (Fig. 6). This is consistent with the size of MKP-3 clone 36. MKP-3 mRNA was detected at high levels in lung and at lower levels in heart, brain, spleen, liver, and kidney. MKP-3 mRNA was undetectable in skeletal muscle and testis. This expression pattern overlaps with other dual-specificity phosphatases in some tissues, while in others, there are clear distinctions. For instance, MKP-1 mRNA is present at moderate levels in skeletal muscle (29, 31), while it is barely detectable in brain and kidney (29, 30, 50). Also, in contrast to MKP-3 expression, MKP-2 is undetectable in liver (31); hVH-3 is present only at low levels in lung (29); hVH-5 is highly abundant in brain and skeletal muscle (32); and PAC-1 is undetectable in all tissues except for spleen and thymus (26).

MKP-3 Subcellular Localization—MKP-3 was cloned from a superior cervical ganglion cDNA library (see “Experimental Procedures”). To test MKP-3 subcellular localization within its native cellular environment, we microinjected pMT-SM carrying MKP-3/Myc into superior cervical ganglion sympathetic neurons. Interestingly, immunoreactive MKP-3/Myc was exclusively cytosolic and excluded from the nucleus (Fig. 7, A and B). This contrasts with TYP-1/Myc, which appears to be restricted to the nuclear compartment under identical conditions (Fig. 7, C and D). Other dual-specificity phosphatases, including MKP-1, hVH-2, hVH-3, and PAC-1, have been reported previously to be localized to the nucleus (26, 29, 30, 35). For the dual-specificity phosphatase B23, a bipartite RRAR-(14)-RRAR motif was suggested to function as a nuclear localization signal (28). Neither this motif nor other potential nuclear targeting sequences (52) can be identified in MKP-3, and this could account for its cytosolic localization. Regardless of the molecular mechanism, our study of MKP-3 subcellular localization in sympathetic neurons is the first demonstration of a cytosolic compartmentalization for a member of this dual-specificity phosphatase family. This distinct subcellular localization indicates a novel and specific role regulating MAP kinases. For instance, MKP-3 may play a selective role in inactivating MAP kinases with cytosolic phosphorylation targets. Alternatively, MKP-3 may inactivate MAP kinase family members within the cytosol and thereby block their translocation into the nucleus. Such nuclear translocation for MAP kinase has been observed in PC12 cells following stimulation with agents triggering differentiation (53).

MKP-3 mRNA Expression Is Induced by NGF in PC12 Cells—In PC12 pheochromocytoma cells, NGF-stimulated activation of MAP kinase is a critical event underlying induction of neuronal morphology (12, 37, 54). As part of an investigation of the functional significance of MKP-3 in neuronal differentiation, we assessed levels of MKP-3 mRNA in PC12 cells following NGF stimulation. While MKP-3 mRNA was not detected in undifferentiated cells, NGF stimulation (50 ng/ml) resulted in a powerful biphasic induction, reaching peak levels 3 and 24 h after NGF treatment (Fig. 8). This delayed time course is not consistent with expression of an immediate early gene, and in this regard, MKP-3 induction appears distinct from other dual-specificity phosphatase genes. Indeed, increased MKP-1, MKP-2, and hVH-5 mRNA expression has been reported to occur within 1 h of NGF treatment in PC12 cells (31, 32). Also, if MKP-3 was an immediate early gene, its rapid induction by a number of stimuli may be expected as observed for MKP-1, MKP-2, and hVH-5 (31, 32). This, however, is clearly not the case as EGF (150 ng/ml) and dibutyryl cAMP (0.5 mM) had little effect on MKP-3 mRNA levels over the same time course (Fig. 8). Interestingly, in PC12 cells, NGF stimulates both sustained
Regulated MKP-3 Brain Expression following Seizure Activity—Several dual-specificity phosphatases are transcribed rapidly in response to growth factors and exposure to cellular stress (22, 23, 26, 28, 29, 30, 32–34). This, together with reports that immediate early genes undergo rapid expression (22, 23, 26, 28, 29, 30, 32–34). This, together with reports that immediate early genes undergo rapid and prolonged induction in NGF-stimulated PC12 cells could indicate cell- and stimulus-specific mechanisms of regulated expression of MKP-3 mRNA.

In summary, we have identified a novel member of the dual-specificity phosphatase family, MKP-3, which is rapidly induced by growth factors and by exposure to cellular stress. MKP-3 expression is detected in the hippocampus, thalamus, and hypothalamus, and its induction is distinct from that of MKP-1 mRNA. These findings provide insight into the regulation of MKP-3 expression and may contribute to our understanding of the role of MKP-3 in neuronal plasticity and neuronal injury.

Fig. 9. Localization of MKP-3 and MKP-1 mRNAs in rat brain by in situ hybridization. Shown are autoradiograms of adult rat coronal brain sections hybridized with MKP-3 (A) or MKP-1 (B) 35S-end-labeled antisense oligodeoxynucleotides. Brain sections were prepared from rats 1 h following either saline (Control) or Metrazole (40 mg/kg intraperitoneal injection) treatment. The localization of the following brain regions is indicated: layers of the hippocampus (CA1 and CA3–4), piriform cortex (Pir), amygdala (A), and thalamus (T).

Using coronal sections of rat brain, MKP-1 mRNA expression was examined for comparison. An MKP-3 transcript was detected in the hippocampus, where the strongest hybridization signals were observed over cells of the CA1 pyramidal cell layer (Fig. 9A). Weaker hybridization was also found in CA3 and CA4 hippocampal regions, while neurons of the CA2 region were devoid of detectable MKP-3 expression (Fig. 9A). This distribution is distinct from that of MKP-1 mRNA, which in parallel sections was observed in the cortex and thalamus (Fig. 9B). These patterns are partly overlapping, but also distinct from other members of the dual-specificity phosphatase family. For instance, MKP-2 mRNA displays the strongest expression in the dentate gyrus, piriform cortex, and suprachiasmatic nucleus (31), while hVH-5 is broadly expressed at high levels in many brain areas, including the hippocampus and hypothalamus (32).

Regulated MKP-3 Brain Expression following Seizure Activity—Several dual-specificity phosphatases are transcribed rapidly in response to growth factors and exposure to cellular stress (22, 23, 26, 28, 29, 30, 32–34). This, together with reports that immediate early genes undergo rapid induction following seizure activity (57, 58), prompted us to investigate MKP-3 and MKP-1 mRNA expression at early times after treatment with the convulsant Metrazole (pentylenetetrazole, PET). Both MKP-3 and MKP-1 showed rapid increases in mRNA levels within 1 h of Metrazole treatment. Induction of MKP-3 and MKP-1 mRNAs was observed in some brain regions, including the frontal, parietal, and piriform cortex, while in the thalamus, only MKP-1 displayed powerful up-regulation (Fig. 9). Following peak levels of MKP-3 and MKP-1 expression at 1 h after Metrazole treatment, resting levels were re-established rapidly and were indistinguishable from control levels by 6 h (data not shown). This time course of induction for MKP-3 and MKP-1 is reminiscent of expression of a number of immediate early genes, including c-fos, c-jun, junB, and zif/268, following Metrazole treatment (57, 58). This apparent discrepancy with delayed, and prolonged induction in NGF-stimulated PC12 cells could indicate cell- and stimulus-specific mechanisms of regulated expression of MKP-3 mRNA.

In summary, we have identified a novel member of the dual-specificity phosphatase family, MKP-3, which is rapidly induced by growth factors and by exposure to cellular stress. MKP-3 expression is detected in the hippocampus, thalamus, and hypothalamus, and its induction is distinct from that of MKP-1 mRNA. These findings provide insight into the regulation of MKP-3 expression and may contribute to our understanding of the role of MKP-3 in neuronal plasticity and neuronal injury.
specificity phosphatase family which we have called MKP-3. Structurally, MKP-3 displays both an extended active-site sequence motif as well as regions of homology to Cdc25 phosphatase (CH2 domains) shared by all known members of this gene family. MKP-3 also exhibits functional properties expected of a dual-specificity phosphatase in that it blocks mitogen-stimulated activation of the MAP kinase ERK2. Identification of a second partial cDNA clone (MOK-X) encoding an additional phosphatase that is 76% identical to MKP-3 indicates the existence of a distinct subfamily of structurally homologous MAP kinase phosphatase genes. This is also consistent with identification of the Xenopus dual specificity phosphatase X17c, which blocks MAP kinase-dependent embryonic mesoderm formation (59) and is 88% identical to MKP-3. MKP-3 is the first dual specificity phosphatase to show an exclusively cytosolic localization, indicating a unique regulatory role perhaps in inactivating MAP kinases targeting cytoplasmic substrates or blocking nuclear translocation. A novel role for MKP-3 is also supported by a distinct tissue distribution and regulated expression in PC12 cells. An important conclusion from this report as well as other recent publications (23, 27–32) is that molecular diversity within the dual specificity phosphatase family now parallels the number of ERK, SAPK/JNK, and p38/HOG1 MAP kinases providing the opportunity for highly specific regulatory interactions. Mechanisms underlying such specificity remain undefined, although they could include enzymatic substrate selectivity, time course of stimulus-dependent activation and induction, cell type-specific coexpression, or subcellular compartmentalization.

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Note Added in Proof—The predicted amino acid sequence is identical with rVH6 (61).

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MKP-3, a Novel Cytosolic Protein-tyrosine Phosphatase That Exemplifies a New Class of Mitogen-activated Protein Kinase Phosphatase
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