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MKP-8, a novel MAPK phosphatase that inhibits p38 kinase ☆,☆☆

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Abstract

Intracellular signaling pathways and their relationship to malignant progression have become a major focus of cancer biology. The dual-specificity phosphatase (DSP) family is a more recently identified family of intracellular signaling modulators. We have identified a novel protein phosphatase with a well-conserved DSP catalytic domain containing the DSP catalytic motif, xHCxxGxSRS, and mitogen-activated protein kinase phosphatase (MKP) motif, AYLM. Because of these unique characteristics, the protein was named mitogen-activated protein kinase phosphatase-8 (MKP-8). This protein is approximately 20 kDa in size and mainly localizes to the nuclear compartment of the cell. MKP-8 is expressed in embryonal cancers (retinoblastoma, neuroepithelioma, and neuroblastoma) and has limited expression in normal tissues. MKP-8 displays significant phosphatase activity that is inhibited by a cysteine to serine substitution in the catalytic domain. When co-expressed with activated MAPKs, MKP-8 is able to inhibit p38 kinase phosphorylation and downstream activity.

Keywords: Dual-specificity phosphatase; Mitogen-activated protein kinase phosphatase; p38 kinase

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☆☆ *Abbreviations:* AP-1, activator protein 1; ATF-2, activating transcription factor 2; CDC25, cell-division cycle 25; DSP, dual-specificity phosphatase; DUSP, dual-specificity protein phosphatase; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione-*S*-transferase; HVH, homologue of vaccinia virus H1 phosphatase gene; IL-1β, interleukin-1β; JSP, JNK stimulatory phosphatase; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein; MEKK, MAPK/ERK kinase kinase; MKK, MAPK kinase; MKP, MAPK phosphatase; PAC, phosphatase of activated cells; PYST, phosphorylates tyrosine serine threonine; SAPK, stress activated protein kinase; SKRP, SAPK pathway-regulating phosphatase; TMDP, testis and skeletal muscle-specific dual-specificity phosphatase; UV, ultraviolet; VHR, vaccinia virus H1 phosphatase gene-related phosphatase; YVH, homologue of yeast vaccinia virus H1 phosphatase gene.

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The three major subclasses of the MAPK family are extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase. Each MAPK is the final activated component of a three-kinase module that results in dual phosphorylation of the MAPK at the threonine-X-tyrosine motif within the activation domain [1]. The MAPK then translocates to the nucleus in order to activate transcription factors such as members of the activator protein-1 (AP-1) family [2]. ERK and JNK have been more frequently studied in cancer due to their mutual relationship to Ras-induced cellular transformation [3,4]. However, p38 has been shown to have a more prominent role in promoting tumor apoptosis in response to genotoxic stress [5–7]. It is now recognized that these complex phosphorylation-dependent signaling pathways depend on a balance between kinase and phosphatase activity. A newly discovered family of phosphatases, called dual-specificity

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phosphatases (DSP), has emerged with the unique ability to dephosphorylate peptides at both the tyrosine and serine/threonine sites. These phosphatases are thought to play a key role in regulating the MAPK pathways.

Initially, the cell-division cycle 25 (CDC25) family of DSPs received the most attention due to their involvement in cell cycle regulation. These phosphatases affect cell cycle progression by activating the cyclin-dependent kinases allowing quiescent cells to progress past the cell cycle checkpoints, such as G_1/S, and divide [8]. The DSP family consists of about 20 other members each containing the distinctive DSP catalytic domain with "xHCxxGxSRS" motif. This catalytic domain is consistently able to down-regulate components of the MAPK pathways. The more extensively studied MAPK kinase phosphatase (MKP) -1 or dual-specificity protein phosphatase (DUSP) 1 and vaccinia virus H1 phosphatase gene-related phosphatase (VHR, DUSP3) are both able to down-regulate ERK and JNK activity; moreover, MKP-1 is able to down-regulate p38 activity [9–13]. Due to these functional characteristics, many of the DSPs have been shown to function as oncoproteins or tumor suppressors in cancer through alterations in the MAPK pathway [14–18].

As part of an ongoing effort to identify novel molecular targets involved in the development and progression of cancer, we now describe the cloning and characterization of a novel DSP, MAPK phosphatase-8 (MKP-8).[1] By searching the human genome cDNA database for novel proteins that may serve as potential therapeutic targets for cancer, we identified the MKP-8 coding region, which contains the highly conserved DSP catalytic domain. The experiments described below show that MKP-8 has limited expression in normal tissues with expression prevalence in some common pediatric malignancies. Also, MKP-8 has an active phosphatase domain that can directly inhibit p38 kinase phosphorylation and downstream activity.

Materials and methods

Cell culture. COS-7 and human embryonic kidney 293T (293T) were obtained from American Type Culture Collection (ATCC). Both of these cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum and 100 U/mL streptomycin/penicillin. All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

Identification of MKP-8. The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) cDNA database was searched for a "hypothetical protein" particular to cancer cell lines and tissues. The identified cDNA/protein sequence was analyzed with BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/) to identify conserved, functional domains and with HomoloGene (http://www.ncbi.nlm.nih.gov/HomoloGene/) to identify potential interspecies homologies. In addition, BLASTp, Scanps 2.3 (http://www.ebi.ac.uk/scanps/), and ClustalW 1.82 online (http://www.ebi.ac.uk/clustalw/) were used to screen and align all known homologous proteins sharing similar conserved domains.

Mammalian expression constructs and cloning. A full-length cDNA clone containing the MKP-8 gene was obtained from ATCC (IMAGE Consortium ID 3535215). MKP-8 was localized from the cDNA clone using polymerase chain reaction (PCR) amplification with the following primers: 5′-CACCAAGCTTACCATGTGCCCTGGTAACTGG-3′ and 5′-GAGCCAGGGACCTACCCA-3′, and cloned into the pcDNA3.1D-TOPO vector (Invitrogen) (MKP-8wt). From this primary construct, MKP-8 was subcloned into p3xFLAG-CMV-10 (FLAG-MKP-8wt) (Sigma) using *Hin*dIII/*Xba*I. The following primer set, 5′-CACCGGATCCATGTGCCCTGGTAACTG-3′ and 5′-GAGCCA GGGACCTACCCA-3′, was used to subclone MKP-8wt into pGEX-KG (Pharmacia) using *Bam*HI/*Xba*I. This bacterial expression plasmid (pGEX-MKP-8wt) was used to purify glutathione-*S*-transferase (GST)-MKP-8wt (GST-MKP-8wt) from *Escherichia coli*. The SRα3HA-ERK2, SRα3-RafBxB, SRα3HA-JNK1, and SRα3HA-MAPK/ERK kinase kinase (MEKK) 3 plasmids were contributed by Dr. B. Su (M.D. Anderson Cancer Center, Houston, TX). The pcDNA3-FLAG-p38 and pcDNA3-MAPK kinase (MKK) 6b expression plasmids were contributed by Dr. J. Han (The Scripps Research Institute, La Jolla, CA). The following primer set, 5′-CACCAAGCTTATGTCTCAGTCG-3′ and 5′-TTAGTCTCCAAGAATCAGTT-3′, and *Hin*dIII/*Xba*I digestion were used to clone MKK6b into p3xFLAG-CMV-10. The control plasmid, pCMV6 (empty), was used to balance all DNA concentrations for transfection and also used as a control plasmid for all negative controls used within individual experiments.

Synthesis of MKP-8-c151s mutant. To create a catalytically inactive MKP-8, a serine was substituted for the cysteine at position 151 using a PCR-directed mutagenesis technique as described previously [19]. Primary PCR was used to create two DNA fragments containing the mutation using the initial cloning primers and the following two primers: (the underline indicates the nucleotide mutation for the serine substitution): 5′-CCCACAGCACTATGCACCAG-3′ and 5′-CTGGTGCAT AGTGCTGTGGG-3′. The MKP-8-c151s mutant gene was then cloned into the pcDNA3.1D-TOPO vector (MKP-8-c151s), pGEX-KG (pGEX-MKP-8-c151s), and p3xFLAG-CMV-10 (FLAG-MKP-8-c151s) as described before.

Northern blot. The full-length cDNA corresponding to the MKP-8 open reading frame was used to create a [^{32}P]dCTP labeled probe with the Rediprime II random prime labeling kit (Amersham Biosciences) in accordance with the manufacturer's protocol. The MKP-8 probe was hybridized to a multiple tissue blot containing 2 μg polyA$^+$ RNA from 12 different types of normal adult tissues (Clontech) and developed by autoradiography.

First-strand cDNA synthesis for cell lines. Total RNA was extracted from cell lines grown to ⩾80% confluency using Trizol (Invitrogen) according to the manufacturer's protocol. RNA for the following human cell lines was collected: Jurkat, Molt (T-cell leukemia); Raji (B-cell lymphoma); D54, U87, U373, U251, U343, and A172 (glioma); SNB19 (glioblastoma); HT-29, SW480 (colon cancer); BxPC3 (pancreatic cancer); MCF7, MDA-MB231, MDA-MB435, MDA-MB468, SK-BR3 (breast cancer); UCD, 11B (melanoma); NCI-H226, NCI-H157, NCI-H1792, NCI-H460, NCI-H1299, NCI-H322, A549 (non-small cell lung cancer); NCI-H69, NCI-H526, NCI-H1688 (small cell lung cancer); Y79 (retinoblastoma); SK-N-MC (neuroepithelioma); and NB-16, SK-N-SH, SH-SY5Y, IMR-32, LAN-1, and SMS-KCN (neuroblastoma). The total RNA (4 μg), random hexamers, and Superscript II reverse transcriptase (Invitrogen) were used to synthesize first-strand cDNA for the above cell lines according to the manufacturer's protocol.

Reverse transcriptase-polymerase chain reaction. Conventional reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out for 32 cycles with the following reaction mixture: 1× PCR buffer

[1] Nucleotide and protein sequence data for MKP-8 are available in the DDBJ/EMBL/GenBank databases under the Accession No. AY902194.