MKP-7, a Novel Mitogen-activated Protein Kinase Phosphatase, Functions as a Shuttle Protein*

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Mitogen-activated protein kinase (MAPK) phosphatases (MKPs) negatively regulate MAPK activity. In the present study, we have identified a novel MKP, designated MKP-7, and mapped it to human chromosome 12p12. MKP-7 possesses a long C-terminal stretch containing both a nuclear export signal and a nuclear localization signal, in addition to the rhodanese-like domain and the dual specificity phosphatase catalytic domain, both of which are conserved among MKP family members. When expressed in mammalian cells MKP-7 protein was localized exclusively in the cytoplasm, but this localization became exclusively nuclear following leptomycin B treatment or introduction of a mutation in the nuclear export signal. These findings indicate that MKP-7 is the first identified leptomycin B-sensitive shuttle MKP. Forced expression of MKP-7 suppressed activation of MAPKs in COS-7 cells in the order of selectivity, JNK > p38 > ERK. Furthermore, a mutant form MKP-7 functioned as a dominant negative particularly against the dephosphorylation of JNK, suggesting that MKP-7 works as a JNK-specific phosphatase in vivo. Co-immunoprecipitation experiments and histological analysis suggested that MKP-7 determines the localization of MAPKs in the cytoplasm.

The activation of the mitogen-activated protein kinase (MAPK) cascade plays a key role in transducing various extracellular signals to the nucleus to induce responses such as gene expression, cell proliferation, differentiation, cell cycle arrest, and apoptosis (1, 2). MAPKs consist of three major subfamilies, extracellular signal kinases (MAPK/ERK), stress-activated kinase/c-Jun N-terminal kinases, and homologues of the budding yeast HOG1 protein (p38). For full activation of these MAPKs, phosphorylation of both threonine and tyrosine residues found in TXY motifs is required by dual specificity kinases, known as MAPK kinases. Thus dephosphorylation of the TXY motif is critical for negative regulation of MAPK activity (3).

Recently, a family of dual specificity protein phosphatases, DSPs, which dephosphorylate both threonine and tyrosine residues of TXY motifs, has been identified and termed MAPK phosphatases (MKPs). So far 10 MKPs have been identified, including MKP-1 (4, 5), MKP-2 (6), HOG1/2 (7, 8), MKP-3 (9), MKP-X (9), MKP-4 (10), MKP-5 (11–13), HOG1/3/4 (14, 15), PAC-1 (16), and MKP-6 (17). This gene family conserves a rhodanese-like domain, which is also known as a CDC25-like domain, as well as an active site motif required for DSP activity (18). MKPs differ in terms of substrate specificity, tissue distribution, subcellular localization, and target gene regulation, all of which allow fine regulation of MKP by extracellular stimuli (19).

MKPs are potential negative regulators of MAPK cascades and as such are assumed to be involved in carcinogenesis by regulating cell proliferation and apoptosis. As a result, all human MKP genes have been mapped. Among them, MKP-2 and MKP-3 are mapped to a gene locus encoding tumor suppressors for prostate and pancreatic cancer, respectively (20, 21). MKP-X and MKP-5 are mapped to 3p21 (21) and to 1q41 (13), respectively, where frequent deletions are reported in a number of different tumors. Activation/phosphorylation of MAPKs leads to their nuclear translocation and phosphorylation of certain DNA-binding proteins that contribute to transcriptional regulation. The mechanism of nuclear-cytoplasmic transport of MAPKs is, however, not clear. Recent reports indicate that several different proteins contain an intrinsic nuclear export signal (NES) motif mediating their subcellular localization and nuclear-cytoplasmic shuttling through association with the export receptor, CRM1/exportin 1 (22–24). Among them, MEK1, one of MAPK kinases (MAPKKs) has been well characterized (25, 26). Its NES motif was shown to function as an anchor protein of ERK in the cytoplasm when cells are unstimulated, thereby suppressing cell transformation. Leptomycin B (LMB), a specific inhibitor of nuclear export that blocks binding between the NES and CRM1 (27–29), caused nuclear accumulation of MEK1. Substitutions of crucial leucines in the NES motif with alanines caused nuclear accumulation of MEK1 and ERK (30).

By screening an EST library, we identified a human cDNA clone encoding a novel member of the MKP family, MKP-7.
Interestingly MKP-7 contains predicted functional motifs such as a nuclear export signal (NES) and nuclear localization signals (NLSs), suggesting that it functions as a shuttle protein and a MAPK phosphatase. In this report, the substrate specificity, subcellular localization, and regulation of MKP-7 are presented and discussed.

**EXPERIMENTAL PROCEDURES**

**Identification of a Novel MKP cDNA**—By using the amino acid sequence of human MKP-4, we screened an expressed sequence tag data base, dEST, and identified a novel mRNA. A human cDNA (GenBank accession number AI274662, IMAGE clone ID 19896459) and mouse clone (GenBank accession number AA879894, IMAGE clone ID 1230637) had high sequence homology to human MKP-4. The human and mouse clones were obtained from Research Genetics, Inc. (Huntsville, AL), and their nucleotide sequences were determined using the dideoxycte chain termination method on a 373A DNA sequencer (Applied Biosystems, Foster City, CA), with a Dynamic dye terminator cycle sequencing kit (Amersham Pharmacia Biotech). We performed 5′- and 3′-RACE using cDNA derived from Jurkat cells as template and primers based on the human clone. Nucleotide sequencing analysis showed that the deduced amino acid sequences of the PCR product are identical to those of clone AI 274662 with the presence of some polymorphism in 5′-UTR region (Fig. 1). The obtained PCR fragment had an ORF of 1995 base pairs. All RACE methods were performed with a SMART™ RACE cDNA amplification kit (CLONTECH, Palo Alto, CA) according to the manufacturer's protocol.

**Northern Blot Analysis**—Total RNAs from various tissues of 6-week-old male mice were isolated by acid guanidinium thiocyanate extraction (31). The RNAs were fractionated on a 1.5% formaldehyde-agarose gel and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were hybridized with a 32P-labeled 1.8 kb insert of mouse MKP-7 DNA, which contains the full-length ORF. Hybridization was performed at 42°C in 50% formamide, 0.65 M NaCl, 5 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, 0.1 M PIPES, pH 6.8, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA. The membranes were washed twice with 2× SSC containing 0.1% SDS at room temperature for 5 min, followed by sequential washes with 0.5× SSC containing 0.1% SDS and 0.2× SSC containing 0.1% SDS at 50°C, each for 15 min. The filters were exposed to an x-ray film using an intensifying screen at −80°C.

**MKP-7 Expression Plasmids**—To construct pEGFP-MKP-7, the coding region of human MKP-7 cDNA was amplified by PCR to introduce a BglII site on the 5′ end and a SaI site on the 3′ end using Platinum Pfx DNA polymerase (Life Technologies, Inc.) and ligated to BglII and SaI-digested pEGFP-C2 vector (CLONTECH) in frame with the EGFP-coding sequence. To construct pFLAG-MKP-7, the same region was amplified by PCR to introduce a NorI site on the 5′ end and a SauI site on the 3′ end using Platinum Pfx DNA polymerase and ligated to NorI- and SauI-digested pFLAG-CMV2 vector (Sigma) in frame with the FLAG epitope sequence. Several constructs encoding MKP-7 mutant proteins, including DA (D213A), CS (C244S), and DS (D213A, C244S), were generated by PCR and ligated into pEGFP-MKP-7. To screen for novel MKP-7 clones, we used a dual specificity phosphatase (DSP) catalytic site motif, VAYAYXSRSXRTXXXAYXM, which is essential for phosphatase activity, was found in this clone (32). A MAPK-docking motif composed of a kinase-interacting motif (33) at residues 51–65 and δ-like domain (34–36) at residues 161–169 were also present. Since this ORF contains these two essential sequences, we designated the clone a new member of MKP gene family (Fig.

**RESULTS**

**Isolation of a Human and Mouse MKP-7**—To search for novel MKPs, we screened a human dEST using the amino acid sequence of human MKP-4 as a probe. This clone (GenBank accession number AI274662, IMAGE clone ID 19896459) also showed high sequence homology to human MKP-4. The nucleotide sequence of this clone was determined, and then 5′-RACE was performed to identify the first methionine codon and sequences of the 5′-UTR region. The full-length clone was obtained by reverse transcriptase-PCR using mRNA from Jurkat cells. The nucleotide sequences were verified by comparing them to independent clones (Fig. 1). The open reading frame (ORF) of this cDNA was predicted to encode a 665 amino acid sequence. A dual specificity phosphatase (DSP) catalytic site motif, VAYAYXSRSXRTXXXAYXM, which is essential for phosphatase activity, was found in this clone (32). A MAPK-docking motif composed of a kinase-interacting motif (33) at residues 51–65 and δ-like domain (34–36) at residues 161–169 was also present. Since this ORF contains these two essential sequences, we designated the clone a new member of MKP gene family (Fig.

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- The phosphorylation status of activated MAPKs was monitored by an anti-phospho-ERK antibody (New England Biolabs, Beverly, MA), an anti-ACTIVE JNK antibody (Promega, Madison, WI), or an anti-phospho-p38 antibody (New England Biolabs), followed by a horse-radish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Chemicon International, Temecula, CA). The expression level of HA-tagged MAPKs and FLAG-tagged MKP-7 variants was monitored by anti-HA (12CA5) monoclonal antibody (Roche Molecular Biochemicals) and anti-FLAG M2 monoclonal antibody (Sigma), respectively, followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Signals were detected by enhanced chemiluminescence using the ECL reagent (Amersham Pharmacia Biotech).

**Co-immunoprecipitation**—Transfected COS-7 cells were lysed on a plate (300 µl/60-mm plate) in co-IP buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin). The IP was performed at 4°C with 5 nM LMB (provided by Dr. M. Yoshida) for the indicated periods.

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1). To date, 6 species among 12 DSPs have been designated MKP-1 to MKP-6 based on their structural similarity and substrate specificity toward MAPKs. Following this nomenclature, we named our novel DSP MKP-7 (Fig. 1).

MKP-7 exhibits several other predicted functional motifs. Two bipartite NLS motifs (37) were located at amino acid residues 296–313 (NLS1) and 610–627 (NLS2). One leucine-rich NES motif was located at amino acid residues 376–385.

The presence of a NES as well as an NLS suggested that MKP-7 acts as a shuttle protein. PEST sequences, which are thought to be involved in rapid degradation through ubiquitin-mediated proteolysis (38), were found at residues 332–353 and 441–462 residues. The presence of these motifs suggested that a C-terminal stretch of MKP-7 is important for localization and stability of the protein.

To obtain the mouse homologue, we screened mouse dbESTs and found one clone (GenBankTM accession number AA879894, IMAGE clone ID 1230637) that had high sequence homology to human MKP-7 but lacked the 5'/H11032-half of the ORF. We obtained a full-length cDNA clone by RACE using mouse cerebellum RNA as a template. The nucleotide sequence was verified using three independent reverse transcriptase-PCR fragments covering the entire ORF of mouse MKP-7.

The alignment of domain structures of MKP-7 with those of other MKPs is shown in Fig. 2B. MKP-7 has similar domain structures and the highest sequence similarity to hVH5. The similarities between human MKP-7 and hVH5 are 76.1, 59.6, and 30.5% in the DSP domain, rhodanese-like domain, and C-terminal stretch, respectively.

Chromosomal Location of the MKP-7 Gene—The location of the MKP-7 gene on human chromosomes was determined by the identification of MKP-7 cDNA between two sequence-tagged site markers, G24001 and G41293, in Homo sapiens 12p BAC RP11-253I19 (GenBankTM accession number AC007619.22). This gene was localized to human chromosome 12p12 as shown in Fig. 3A. By comparing the nucleotide sequences of the MKP-7 genomic clone with the cDNA, the MKP-7 gene was shown to be composed of at least seven exons (Fig. 3B). The catalytic core and C-terminal stretch were each encoded on a single exon, whereas the rhodanese-like domain was contained on three exons. Since the 5'-UTR
sequence did not match any sequences in the NCBI data base or others, we did not identify the promoter region of MKP-7.

Theoretically, DSPs have tumor suppressor activity. The Recurrent Chromosome Aberrations in Cancer Data base searcher (www.cgap.nci.nih.gov/Chromosomes/Recurrent Aberrations) showed that chromosome 12p12 is a region where deletions often occur in acute lymphoblastic leukemia, acute and chronic myeloid leukemia, and myelodysplastic syndrome, suggesting that tumor suppressor genes for leukemia lie within this region.

Tissue Distribution of MKP-7 mRNA—The expression pattern of MKP-7 mRNA in mouse tissues was examined by Northern blot analysis using mouse MKP-7 cDNA containing the entire ORF as a probe. As shown in Fig. 4, two mRNA species of 4.1 kb as a major transcript and 2.1 kb as a minor transcript were detected. The 4.1-kb transcript was abundantly expressed in the brain, kidney, intestine, and testis but expressed at low levels in the thymus, spleen, and bone marrow. The 2.1-kb transcript was detected only in the testis.

MKP-7 Suppresses JNK1 Activation in COS-7 Cells in Vivo—To determine the substrate specificity of MKP-7 as a MAPK phosphatase, we analyzed the effect of MKP-7 on activation of HA-ERK2, HA-JNK1, or HA-p38 in vivo (Fig. 5). An inhibitory effect of MKP-7 on activation of HA-JNK1 was substantial (Fig. 5B). Co-transfection of 0.06 μg of MKP-7 expression plasmid reduced the level of HA-JNK1 activation to an unstimulated level (Fig. 5B, compare lanes 3–6), and further inactivation was obtained in a dose-dependent manner, to trace levels (Fig. 5B, lanes 6–9). HA-p38a was also inactivated by MKP-7, although the effect was not as substantial (Fig. 5C).
Co-transfection with 0.6 g of MKP-7 expression plasmid was required to reach the basal level of HA-p38 activation (Fig. 5, compare lanes 3–8). In contrast, HA-ERK2 was not inactivated by MKP-7 (Fig. 5A, lanes 5–12).

In order to analyze specificity further, we used two types of catalytically inactive proteins with mutations in conserved residues, MKP-7-CS (C244S) and MKP-7-DA (D213A). Both mutant proteins significantly enhanced phosphorylation of HA-JNK1 but had little effect on activation of HA-ERK2 and HA-p38 (Fig. 5B, lanes 10 and 11), indicating that both mutant proteins function as dominant negatives toward JNK. Under the same conditions, MKP-5 inactivated JNK1 and p38 more strongly than ERK2, as reported (Fig. 5, A–C, lane 12, and Refs. 11 and 12). Therefore, MKP-7 blocked activation of MAP kinases in the order of selectivity, JNK1 > p38α > ERK2.

In Vivo Interaction between MKP-7 and MAPKs—Since MKP-7 inactivated JNK1 and p38α in vivo, we asked whether MKP-7 binds MAPKs in vivo. We tested an in vivo interaction between MKP-7 and MAPKs by co-immunoprecipitation experiments to determine whether MKP-7 has a binding preference among MAPKs and, if a direct interaction occurs, whether MAPKs must be phosphorylated for that interaction.

FLAG-MKP-7 and HA-MAPKs (either HA-ERK2, HA-JNK1, or HA-p38α) were co-expressed in COS-7 cells (Fig. 6A). As expected, HA-JNK1 was co-immunoprecipitated with FLAG-MKP7 (Fig. 6A, lanes 10–13); however, stimulation did not affect this interaction (Fig. 6A, compare lanes 12 and 13). The interaction was observed even under culture conditions lacking starvation or stimuli (Fig. 6A, lane 10). When we expressed FLAG-MKP-7DA, an inactive mutant, interaction of FLAG-MKP-7DA and HA-JNK1 was similar to that of FLAG-MKP-7.

FIG. 3. Chromosomal localization and structure of the human MKP-7 gene. A, mapping of the MKP-7 gene on human chromosome 12 by schematic representation. The location of the MKP-7 gene and its proximal sequence-tagged site markers are indicated. This region had already appeared in GenBank under accession number AC007619.22. B, genomic structure of the MKP-7 gene. MKP-7 is encoded on at least seven exons. The exon including the initiation codon is designated exon I.

FIG. 4. Expression of MKP-7 mRNA in mouse tissues. Total RNA (20 μg per lane) from various mouse tissues as indicated was separated on a 1% formaldehyde-agarose gel, transferred to a nitrocellulose membrane, and hybridized with a 32P-labeled mouse MKP-7 cDNA probe. The rRNA pattern is shown in the lower panel as a loading control.
and HA-JNK1 (data not shown), suggesting that MKP-7 binds the dephosphorylated as well as the phosphorylated protein. It should be noted that MKP-7 binds not only JNK1 but also ERK2 and p38/H9251. Under standard conditions, we did not observe any binding preference of MKP-7 toward a specific MAPK. Under these conditions, the MAPK binding specificity of MKP-5 (Fig. 6B) and MKP-2 (data not shown) was confirmed as already reported (11, 39), which excludes a possibility that the binding of MKP-7 and MAPKs is due to be artificial by overexpression. Taken together, we conclude that MKP-7 interacts with ERK2 and p38α as well as JNK1 with similar preference in vivo and that such interaction does not depend on the phosphorylation state of MAPKs.

An Inactive Mutant of MKP-7 Acts as a Dominant Negative against the Dephosphorylation of MAPKs—Since MKP-7 interacts with ERK2, p38/H9251, and JNK1 under all conditions exam-
MKP-7 Is Localized in the Cytoplasm — In order to understand the function of MKP-7, we investigated the subcellular localization of MKP-7. The localization of EGFP-MKP-7 in HeLa cells is shown in Fig. 8A. The control EGFP protein was distributed evenly in transfected cells, whereas EGFP-MKP-7 was specifically localized in the cytoplasm (Fig. 8A, a and c). In a separate experiment, we used FLAG-tagged MKP-7 to ensure that localization of MKP-7 to the cytoplasm was not an artificial result of the green fluorescent protein domain being fused to the phosphatase (Fig. 8A, e). We examined the subcellular distribution of FLAG-MKP-7 in several cell lines, including COS-7, NIH3T3, and 293 cells. FLAG-MKP-7 was localized exclusively in the cytoplasm of all these cell lines (data not shown).

MKP-7 Is an LMB-sensitive Shuttle Protein — To determine whether the predicted NES is functional, we examined the effect of LMB on distribution of FLAG-MKP-7 when the cells were starved (Fig. 8B). Without LMB treatment, FLAG-MKP-7 was localized exclusively in the cytoplasm, but it accumulated in the nucleus in a manner proportional to incubation time with LMB. By 120 min of LMB treatment, FLAG-MKP-7 had exclusively accumulated in the nucleus. Similar results were obtained in cells without starvation (data not shown). These data suggested that MKP-7 shuttles between the nucleus and the cytoplasm, and that nuclear export of MKP-7 is LMB-sensitive.

Analysis of the Sequences Required for Shuttling — To determine the importance of C-terminal stretch for nuclear transport and export, we analyzed localization of the following three deletion mutant proteins: FLAG-MKP-7-delC1, FLAG-MKP-7-delC2, and FLAG-MKP-7-delC3 (Fig. 9A). In sharp contrast to the wild type protein, FLAG-MKP-7-delC1 lost its specific localization and was evenly distributed in the cell. It also lacked sensitivity to LMB (Fig. 9), strongly suggesting that the cytoplasmic localization and LMB sensitivity of MKP-7 is determined by the C-terminal stretch. FLAG-MKP-7-delC2 localized mainly in the nucleus and its localization was not affected by LMB, whereas FLAG-MKP-7-delC3 was localized in the cytoplasm and this localization was sensitive to LMB. These data show that the region (residues 291–370) containing NLS1 functions for nuclear import, and the region containing the NES functions for LMB-sensitive nuclear export. NLS2 appears not to be critical since the localization of FLAG-MKP-7-delC3 is the...
same as that of the wild type protein.

To verify the importance of the NES motif, FLAG-MKP-7-LA, which has crucial three leucines substituted with alanines, was expressed in HeLa cells. This mutant protein was completely accumulated in the nucleus. These results were observed in other cell lines such as COS-7, NIH3T3, and 293 cells (data not shown).

We also investigated the involvement of the rhodanese-like domain in subcellular localization. FLAG-MKP-7-delR and FLAG-MKP-7-delRLA did not translocate to the nucleus even with LMB treatment. These results also support the idea that MKP-7 is a shuttle protein between the nucleus and the cytoplasm. NLS1, in collaboration with rhodanese-like domain, seems function to allow nuclear import, and the NES in the C-terminal stretch is critical for the nuclear export.

**DISCUSSION**

By using EST cloning, we identified a novel MKP designated MKP-7. Our results indicate that MKP-7 is a JNK phosphatase since MKP-7 binds to JNK and efficiently inhibits the activation/phosphorylation of JNK. Inactive MKP-7 mutants also function as dominant negatives against dephosphorylation of JNK. MKP-7 has both structural and functional characteristics of a shuttle protein. Nuclear-cytoplasmic transport of MKP-7...
MKP-7 is likely to function as a JNK phosphatase. MKP-7 was more effective toward phosphorylated and activated JNK1 than ERK2 and p38α (JNK1 ≫ p38α > ERK2). Also the finding that inactive mutants of MKP-7 worked as strong dominant negatives against dephosphorylation of JNK supports the idea that MKP-7 functions as a JNK phosphatase in vivo. The substrate specificity of MKP-7 toward MAPKs was similar to that of hVH-5/M3/6 (JNK ≈ p38 ≫ ERK) (47) and MKP-5 (JNK ≈ p38 ≫ ERK) (11, 12) but very different from that of PAC-1 (ERK = p38 > JNK) (48–50), MKP-2 (ERK = JNK > p38) (50), MKP-3 (ERK ≫ JNK = p38) (9, 47), and MKP-4 (ERK > p38 = JNK) (10). MKP-7 and hVH-5/M3/6 have high sequence homology, similar domain structures, and similar substrate specificities toward MAPKs. Compared with the wild type protein, FLAG-MKP-7delC2 showed higher activity toward p38α, although its activity toward JNK1 and ERK2 was unchanged (data not shown). These results suggest that 1) the high specificity to JNK1 depends on conserved sequences between MKP-7 and hVH-5/M3/6, which include the rhodanese-like domain and catalytic domain, and 2) the C-terminal stretch of MKP-7 may interfere with its recognition of p38α as a substrate.

It is unclear why, despite its high specificity toward JNK1, MKP-7 can bind ERK2 and p38α as well as JNK1 with similar affinity. This observation suggests that binding is necessary but not sufficient for determination of substrate specificity. To address this issue, experiments either substituting the catalytic domain of MKP-7 with the corresponding domain from other MKPs or mutating the catalytic domain of MKP-7 will be required. It is of note that MKP-7-active mutants increased levels of phosphorylated HA-ERK2 as well as HA-JNK1, when cells are unstimulated (Fig. 7). MKP-7 may play a role as a gatekeeper for ERK as well as JNK by setting a high threshold for stimulation.

To our knowledge, MKP-7 is the first identified shuttle MKP. By substitution experiments and LMB treatment, we showed that the NES in the C-terminal stretch is functional. It is of interest that the NES motif of MKP-7 (LXXXLXXXLX) is identical to that of MEK1 (25, 30). For nuclear import, the NLS1 region in addition to the rhodanese domain was identified as functional. The rhodanese-like domain may be involved in conformational changes of MKP-7, but the details remain to be clarified. An important question is the function and role of MKP-7 as a shuttle molecule. Based on the results shown in Fig. 10 that MKP-7 trapped MAPKs in the cytoplasm, we propose two models. One model is that MKP-7 translocates into the nucleus and interacts with activated MAPKs and then dephosphorylates and transports them back to the cytoplasm. Another is that MKP-7 remains in the cytoplasm to anchor and dephosphorylates and transports them back to the cytoplasm. To distinguish between these two models, we analyzed the activity of FLAG-MKP-7 LA. This mutant was localized in the nucleus (Fig. 9) and showed activity toward MAPKs similar to that of the wild type protein (data not shown), supporting the former model. Future experiments focusing on MKP-7 will address the question how localization and activity of MAPKs are regulated.

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