A Novel MAPK Phosphatase MKP-7 Acts Preferentially on JNK/SAPK and p38α and β MAPKs*

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Mitogen-activated protein kinases (MAPKs) are inactivated via dephosphorylation of either the threonine or tyrosine residue or both in the P-loop catalyzed by protein phosphatases which include serine/threonine phosphatases, tyrosine phosphatases, and dual specificity phosphatases. Nine members of the dual specificity phosphatases specific for MAPKs, termed MKPs, have been reported. Each member has its own substrate specificity, tissue distribution, and subcellular localization. In this study, we have cloned and characterized a novel MKP, designated MKP-7. MKP-7 is most similar to hVH5, a member of previously known MKPs, in the primary structure. MKP-7 is predominantly localized in the cytoplasm when expressed in cultured cells, whereas hVH5 is both in the nucleus and the cytoplasm. MKP-7 binds to and inactivates p38 MAPK and JNK/SAPK, but not ERK. Furthermore, we have found that MKPs have the substrate specificity toward the isoforms of the p38 family (α, β, γ, and δ). MKP-7 binds to and inactivates p38α and -β, but not γ or δ. MKP-5 and CL100/MKP-1 also bind to p38α and -β, but not γ or δ. Finally, we propose a tentative classification of MKPs based on the sequence characteristics of their MAPK-docking site.

The mitogen-activated protein (MAPK) cascades are well defined signaling modules, which have been revealed to play important roles in a variety of cellular responses, such as cell proliferation, cell differentiation, apoptosis etc. (1–9). To regulate the cellular responses, the timing of the activation and inactivation of MAPKs should be strictly controlled. There are three major subgroups in the MAPK family, ERK, p38 MAPK, and JNK/SAPK. ERK is activated mainly by mitogenic stimuli, whereas p38 MAPK and JNK/SAPK are activated mainly by stress stimuli or inflammatory cytokines. Activated MAPKs phosphorylate several substrates, such as MAPKAPKs (MAPK-activated protein kinases) and transcription factors (1–9). The inactivation of the activated MAPKs is important for normal cellular responses. It has been reported that the forced and sustained activation of ERK leads to oncogenic transformation of the cells (4, 10, 11). MAPKs are activated by MAPK kinases (MAPKKs) through the phosphorylation of threonine and tyrosine residues in the P-loop of the molecules. The phosphorylation of both residues is necessary and sufficient for the activation. Thus, inactivation of MAPKs can be achieved by the dephosphorylation of either the threonine residue or the tyrosine residue, or both. PP2C-type phosphatases are reported to dephosphorylate the threonine residue of p38 MAPK and inactivate it (12, 13). Several tyrosine phosphatases are known to dephosphorylate the tyrosine residue of ERK and p38 and inactivate them (14, 15). Furthermore, there is a group of phosphatases that specifically dephosphorylate both residues of MAPKs (16, 17), which belong to the family of dual specificity phosphatases. Nine members of this group of phosphatase acting specifically on MAPKs, termed MKPs, have been reported, including CL100 (MKP-1) (18–21), PAC1 (22, 23), MKP-2 (hVH2, TYP-1) (24–26), hVH3 (B23) (27, 28), hVH5 (M3/6) (29, 30), MKP-3 (Pyst1, rVH6) (31–33), Pyst2 (34), MKP-4 (Pyst3) (34, 35), and MKP-5 (36, 37). They share sequence homology and are highly specific for MAPKs, but differ in the substrate specificity, tissue distribution, subcellular localization, and inducibility by extracellular stimuli (16, 17).

Here we have identified and characterized a novel MKP, designated MKP-7. MKP-7 shows high homology with all known MKPs and shares all the features of dual specificity phosphatases. MKP-7 is most similar to hVH5 in the primary sequence. Both molecules have a C-terminal stretch, which is not present in other MKPs. MKP-7 possesses a nuclear export signal (NES) sequence in the C-terminal stretch region, which regulates the subcellular localization of the molecule. MKP-7 binds to and inactivates p38 MAPK and JNK/SAPK, but not ERK. Furthermore, we show for the first time that MKPs including MKP-7 have the substrate specificity toward the isoforms of the p38 MAPK family. MKP-7 binds to and inactivates p38α and -β, but not γ or δ. CL100/MKP-1 and MKP-5 also bind to p38α and -β, but not γ or δ. Finally, we propose a tentative classification of MKPs based on their sequences of the MAPK-docking site.

EXPERIMENTAL PROCEDURES

5′- and 3′-Rapid Amplification of cDNA Ends—A Super Script Human fetal brain cDNA library (Life Technologies, Inc.) was used as a template. The vector sequence (5′-caacaacacgattagacct-3′) and a gene specific primer (5′-gtggttcgtaggtacacc-3′) were used for the first PCR of 5′-rapid amplification of cDNA ends, and the T7 primer and a gene specific primer (5′-ggtctgcttcagttctgctg-3′) were used for the first PCR of 3′-rapid amplification of cDNA ends. The SP6 primer and gene-specific primers (5′-gcaacagctgctgcagttctg-3′ or 5′-ctgctc-
caagcttatgaagcgaaggtgc-3') were used for nested PCR. Klentaq polymerase mix (CLONTECH) was used in PCR reactions and PCR products were subcloned into TOPO TA cloning vector (Invitrogen). The full-length MKP-7 was obtained by the PCR method using Super Script human fetal brain library as a template. The primers used were 5′-agatctatggcccatgagatgattggaactc-3′ and 5′-agatcttcaggagacctcaatgatttccatgctg-3′.

Plasmid Constructions—cDNAs encoding CL100/MKP-1 (human), MKP-3 (rat), and hVH5 (human) were kindly provided by S. Keyse, S. Arkinstall, and J. E. Dixon, respectively. They were subcloned into SRα-Myc (3X) vector. The full-length of MKP-7 was cloned into SRα-Myc (3X) vector. The deletion mutants of MKP-7 were produced by the PCR method. The primers used were 5′-agatctatggcccatgagatgattggaactc-3′ and 5′-agatcttcaggagacctcaatgatttccatgctg-3′ for MKP-7Dc, and 5′-agatctcaccagactggagcatcagggcc-3′ and 5′-agatcttcaggagacctcaatgatttccatgctg-3′ for MKP-7Dn. The catalytically inactive mutant of MKP-7Dc, in which Cys-244 was replaced by Ser, was constructed by a PCR-based mutagenesis. The primers used were 5′-gtgttctagtgcactcttagctgggatctccc-3′ and 5′-gggagatcccagctaaagagttcactagaacac-3′. MKP-7DcINsRα-Myc vector was used as a template.

Phosphatase Assay—A catalytic activity of Myc-MKP-7 toward p-nitrophenyl phosphate (pNPP) (Sigma) was measured at 37 °C. Fig. 1. cDNA cloning of MKP-7. A, nucleotide and deduced amino acid sequences of human MKP-7 cDNA. Nucleotides and amino acids are numbered on the left and right sides, respectively. The asterisk indicates a stop codon. The solid box corresponds to the active site sequence, and the dotted box indicates the MAPK-docking site. B, sequence comparison of MKP-7 with hVH5. Identical amino acids and homologous amino acids are indicated by the solid and shaded boxes, respectively. The amino acids boxed with solid lines indicate HC1 and HC2. The asterisks indicate the hydrophobic amino acids which are supposed to be important for the NES function. The amino acids boxed with a dotted line indicate PEST-like sequences. C, schematic representation of MKP-7. A Cdc25-like domain (residues 1–139), a phosphatase catalytic domain (residues 158–300), HC1 (residues 373–406), HC2 (residues 596–665), and a PEST-like region (residues 442–463) are indicated. A serine-rich region is shown as Ser rich (residues 552–588).
MKP-7 protein was prepared from the cells transfected with SRα-Myc-MKP-7 by immunoprecipitation using anti-Myc antibody (9E10). The precipitate was washed once with lysis buffer, twice with TBS, and once with Tris-HCl, pH 7.4. Reactions were performed for the indicated hours in 200 μl of 50 mM imidazole, pH 7.5, containing 10 mM dithiothreitol, 20 mM pNPP, and the indicated amounts of Myc-MKP-7 protein. Reaction was stopped by the addition of 0.1 N NaOH, and pNPP hydrolyzed was measured by absorbance at 405 nm with microplate reader (Life Technologies, Inc.).

Cell Cultures and Transfection—NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% calf serum. COS7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. These cells were maintained in 5% CO2 at 37 °C. Cells were split on 35- or 60-mm dishes at 2 × 10^5 or 5 × 10^5 cell number per dish.
After 19 h, cells were transfected using Lipo- 
fectAMINE Plus reagent (Life Technologies, Inc.) according to the man-
ufacturer’s protocol.

Cell Staining—NIH3T3 cells and COS7 cells were transfected with 
Myc-tagged MKP-7 or Myc-tagged hVH5. After 24 h, cells were fixed 
with 3.7% formaldehyde in PBS for 10 min at room temperature. After 
three times wash with PBS, cells were permeabilized in 0.5% Triton 
X-100 in PBS and washed with PBS three times. And then, cells were 
incubated with anti-Myc antibody (9E10) (Santa Cruz) in 3% bovine 
serum albumin in PBS for 16 h at 4 °C. Cells were washed three times 
with PBS, and incubated with anti-mouse IgG secondary antibody in 
3% bovine serum albumin in PBS for 1 h at 37 °C. After three times 
wash with PBS, and with Milli-Q water, coverslips were mounted 
with mowiol. Fluorescence was viewed with a Zeiss fluorescent 
microscope.

Co-immunoprecipitation—Cells were lysed in Nonidet P-40 buffer 
(50 mM Hepes, pH 7.4, 10% glycerol, 2 mM EGTA, 2 mM MgC2, 1% 
Nonidet P-40, 1 mM phenylmethysulfonyl fluoride, and 20 μg/ml apro-
tinin). Tagged proteins were immunoprecipitated from cell lysates 
(about 1 × 10^6 cells in each sample) by incubation with 2 μg of appropri-
te antibody and protein A-Sepharose beads (15 μl) (Amersham 
Pharmacia Biotech) for 2 h at 4 °C. Each precipitate was washed twice 
with TBS (20 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 1 mM phenylmethyl-
sulfonyl fluoride, 2 mM dithiothreitol, 1 mM sodium vanadate, and 20 
μg/ml aprotinin), and then washed with Tris buffer (20 mM Tris-HCl, 
pH 7.5). The washed beads were mixed with substrates in a kinase 
reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 2 mM 
EDTA, 10 mM NaF, 1% Triton X-100, 2 mM dithiothreitol, 1 mM phen-
ylthysulfonyl fluoride, 1 mM sodium vanadate, and 20 μg/ml aprotinin). 
The precipitates were then washed twice with lysis buffer. The proteins 
were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting.

Kinase Assays—Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 
7.5, 12 mM 2-glycerophosphate, 150 mM NaCl, 1.5 mM MgCl2, 2 mM 
EGTA, 10 mM NaF, 1% Triton X-100, 2 mM dithiothreitol, 1 mM phen-

RESULTS

Isolation of MKP-7 cDNA—We previously identified a mem-
ber of MKP, MKP-5, and revealed its MAPK-docking site (36, 
50). Using the primary sequence of the MAPK-docking site of 
MKP-5 (CADKISRRRLQQGKITV), we searched the EST 
cDNA bank for cDNAs encoding a protein containing a similar 
sequence. We identified one clone, that has a sequence 
CSKLMKRRLQQD. By performing the 5’- and 3’-rapid ampli-
fication of cDNA ends/PCR method in the human fetal brain 
cDNA library, we obtained a 2345-base pair cDNA fragment

![Fig. 3. Subcellular localization of 
Myc-MKP-7.](http://www.jbc.org/)

A Novel MAPK Phosphatase MKP-7

Co-immunoprecipitation—Cells were lysed in Nonidet P-40 buffer 
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with TBS (20 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 1 mL phenylmethyl-
sulfonyl fluoride, 2 mL dithiothreitol, 1 mL sodium vanadate, and 20 
μg/ml aprotinin), and then washed with Tris buffer (20 mL Tris-HCl, 
pH 7.5). The washed beads were mixed with substrates in a kinase 
reaction buffer (20 mL Tris-HCl, pH 7.5, 10 mL MgCl2, 100 mL ATP 
(2 μCi of [γ-32P]ATP)), and incubated for 15 min at 37 °C. The reaction 
was stopped by addition of Laemmli’s sample buffer (38). Substrate 
phosphorylation was detected by autoradiography and BAS 2500 (Fuji 
Film) after SDS-polyacrylamide gel electrophoresis.
a protein of 665 amino acids with a calculated molecular mass of 73 kDa (Fig. 1B). It resembles all the known MKPs. We then designated it MKP-7. In its N-terminal portion, there is a “Cdc25-like domain,” in which the two regions showing amino acid similarity to the Cdc25 phosphatase and the MAPK-docking site (CSKLMKRLQQD) exist (Fig. 1A and B). In the middle portion, there appears to be a phosphatase catalytic domain, in which the active site sequence VLVHCLAGISRSA-TIAIAYIM (residues 240–261) exists (Fig. 1A and B) (16, 17). The two amino acids, Cys-244 and Ser-231 in this motif, together with Asp-203, are likely to participate in the catalytic mechanism of dual specificity phosphatase activity (39, 40). MKP-7 is most similar to hVH5 (29) (Fig. 1B). Both molecules have a long C-terminal stretch which is not present in other MKPs. In the C-terminal stretch region (residues 300–665), two portions show high similarity to hVH5 in the primary sequence. We named them HC1 and HC2 (highly conserved regions 1 and 2) (HC1, residues 373–406; HC2, residues 596–665) (Fig. 1B and C). Near HC1, there is a PEST-like sequence in both MKP-7 and hVH5 (indicated as dotted box in Fig. 1B).
MKP-7 gene localizes to human chromosome 12 between the BAC clones, RPCI11-180M15 (accession number AC008115) and RP11-161A14 (accession number AC022276) (Fig. 2, left). The organization of the exon and intron of the gene was also determined (Fig. 2, right). It is similar to that of the hVH5 gene (data not shown).

Subcellular Localization of MKP-7—Different members of MKPs show different patterns of subcellular localization (16, 17). We expressed Myc-tagged MKP-7 or Myc-tagged hVH5 in COS7 cells, C2C12 cells, and NIH3T3 cells. Indirect immunofluorescence with anti-Myc antibody showed that Myc-MKP-7 predominantly localized in the cytoplasm in these cells (Fig. 3A, upper), whereas Myc-hVH5 localized in both the cytoplasm and the nucleus (Fig. 3A, lower), the pattern of which being different depending on the cell type, as previously described (29, 30). While Myc-hVH5 localized predominantly in the nucleus in COS7 cells, when expressed in C2C12 cells it showed pan-cellular distribution in some cells but nuclear localization in other cells. When expressed in NIH3T3 cells, Myc-hVH5 showed almost the same subcellular localization pattern as in C2C12 cells, although the NIH3T3 cells expressing Myc-hVH5 became small (Fig. 3A, lower). Essentially the same results were obtained using GFP-tagged MKP-7 and hVH5 (data not shown). In the C-terminal portion of MKP-7, there are two regions (HC1 and HC2) that show high homology with hVH5 in the primary sequence, as described above (Figs. 1A and 3D). In HC1, there exists a putative NES sequence. Then, the cytoplasmic localization of MKP-7 in cultured cells might be caused by this putative NES sequence. Then, the cytoplasmic localization of MKP-7 in cultured cells might be caused by this putative NES sequence (Figs. 1B and 3B). To test this idea, we constructed and expressed deletion mutants of MKP-7 and examined their subcellular localizations and the effect of leptomycin B (LMB) (a specific inhibitor of NES-mediated active nuclear export) on them in COS7 cells. While full-length MKP-7 and MKP-7AN (residues 300–665), in which the N-terminal portion including the phosphatase domain is deleted (see Fig. 3D), localized predominantly in the cytoplasm, MKP-7D (residues 1–317), in which the C-terminal stretch is deleted (see Fig. 3D), localized both in the cytoplasm and the nucleus (Fig. 3C). Furthermore, full-length MKP-7 and MKP-7AN became localized to the nucleus after treatment with LMB (Fig. 3C). These results indicate the existence of a functional NES in the C-terminal portion of MKP-7. To narrow down a region responsible for nuclear export of MKP-7, we made several additional deletion mutants of MKP-7 and examined their subcellular localization. The obtained results summarized in Fig. 3D indicate that HC1 is required for the cytoplasmic localization of MKP-7. Essentially the same results were obtained by using C2C12 or NIH3T3 cells (data not shown). Therefore, these results, taken together, suggest that the putative NES sequence in HC1 is functional and regulates the cytoplasmic localization of MKP-7 in cultured cells.

MKP-7 Binds To and Inactivates p38α and JNK2 but Not ERK2—It has been shown that docking interactions play an important role in regulating the efficiency and specificity of the enzymatic reactions in the MAPK cascades (15, 41, 43–51). We examined the docking ability of MKP-7 to the three major members of MAPKs (ERK2, p38α, and JNK2) using a co-immunoprecipitation assay. p38α and JNK2 co-immunoprecipitated well with MKP-7, but ERK2 did not (Fig. 4A). As the MAPK-docking site and the catalytic domain are both located in the N-terminal portion (see Fig. 1), the N-terminal half of the molecule was supposed to be sufficient to bind to MAPKs. We then examined the docking ability of both the N-terminal half and the C-terminal half of MKP-7 (MKP-7AC and MKP-7AN; see Fig. 3D). MKP-7AC, like full-length MKP-7, co-immunoprecipitated well with p38α and JNK2, but not with ERK2 (Fig. 4B). The docking ability of MKP-7AN was also confirmed (Fig. 4B). These results indicate that the N-terminal portion is sufficient for docking to these MAPKs. As the catalytic domain is located in the C-terminal half, we examined the docking site(s) by constructing and expressing deletion mutants of MKP-7 and then examining their subcellular localizations and the effect of leptomycin B (LMB) on them in COS7 cells. While full-length MKP-7 and MKP-7AN localized predominantly in the cytoplasm, MKP-7D (residues 1–317), in which the C-terminal stretch is deleted (see Fig. 3D), localized both in the cytoplasm and the nucleus (Fig. 3C). Furthermore, full-length MKP-7 and MKP-7AN became localized to the nucleus after treatment with LMB (Fig. 3C). These results indicate the existence of a functional NES in the C-terminal portion of MKP-7. To narrow down a region responsible for nuclear export of MKP-7, we made several additional deletion mutants of MKP-7 and examined their subcellular localization. The obtained results summarized in Fig. 3D indicate that HC1 is required for the cytoplasmic localization of MKP-7. Essentially the same results were obtained by using C2C12 or NIH3T3 cells (data not shown). Therefore, these results, taken together, suggest that the putative NES sequence in HC1 is functional and regulates the cytoplasmic localization of MKP-7 in cultured cells.

Near HC2, a serine-rich sequence exists (residues 552–588) (Fig. 1C). The C-terminal stretches of MKP-7 and hVH5 including both HC1 and HC2 show no sequence similarity to other proteins.

Chromosomal Localization and Genomic Organization of the MKP-7 Gene—During the course of this study, a BAC clone RP11-253I19 (accession number AC007619), in which the cDNA of MKP-7 is included, was reported. Then, we determined the chromosomal location of the gene for MKP-7. The cDNA of MKP-7 is included, was reported. Then, we determined the chromosomal location of the gene for MKP-7. The organization of the exon and intron of the gene was also determined (Fig. 2, right). It is similar to that of the hVH5 gene (data not shown).
ERK2 (12 or 24 h). Furthermore, MKP-7 DC inactivated p38 for each time point. C was divided equally and used MKP-7 60-mm dishes) transfected with Myc-MKP-7 from COS7 cells (10 plates of 60-mm dishes) transfected with Myc-MKP-7 C. Myc-MKP-7 C was prepared by immunoprecipitation from COS7 cells expressing it. The phosphatase activity of MKP-7DC was measured as pNPP hydrolysis in the presence of GST-p38α or GST-ERK2 (12 or 24 μg) for the indicated hours (h). The immunoprecipitate from the lysates of NIH3T3 cells (10 plates of 60-mm dishes) transfected with Myc-MKP-7DC was divided equally and used for each time point.

4B). But, MKP-7ΔN did not co-immunoprecipitate with p38α (Fig. 4C) or JNK2 (data not shown) even when the expression level of MKP-7ΔN was increased (Fig. 4C). These results have clearly shown that MKP-7ΔC specifically binds to p38α and JNK2, not to ERK2, through the N-terminal portion of the molecule. Previously, we have identified the domain on MAPKs, termed the CD domain, which is commonly utilized for binding to MAPKs, MAPKAPKs, and MKPs (50, 51). To examine whether the CD domain is also utilized in the docking interactions with MKP-7, we used the CD domain-disrupted mutant of p38α (p38CDm), in which Asp-313, Asp-315, and Asp-316 in the CD domain were replaced by asparagines (50, 51). As shown in Fig. 4D, while wild-type p38α co-precipitated well with MKP-7ΔC, p38CDm did not. Therefore, the CD domain is indispensable for the docking interaction between MKP-7 and p38 MAPK. Next, we examined the catalytic activity of MKP-7 toward MAPKs using a co-expression assay. Co-expression of full-length MKP-7 efficiently suppressed the activation of p38α or JNK2, but did not affect the activation of ERK2 (Fig. 4E). As the expression level of full-length MKP-7 in cultured cells was too low to examine its activity in higher expression levels (compare the lower panels of Fig. 4, E and F), we used MKP-7ΔC, in which both the MAPK-docking domain and the phosphatase catalytic domain are present (see Fig. 3D). MKP-7ΔC also efficiently inactivated p38α or JNK2, but not ERK2 (Fig. 4F). Furthermore, MKP-7ΔC inactivated p38α or JNK2 in a dose-dependent manner, but not ERK2 (Fig. 5A). These results demonstrate that MKP-7 binds to and efficiently inactivates p38α and JNK2, but not ERK2. Thus, we can conclude that there is good correlation between the docking ability of MKP-7 to MAPKs and its enzymatic activity toward them. It is known that when a Cys residue in the catalytic active site (VXXVH-CXXGSRXSXXAYLM) is replaced by Ser, dual specificity phosphatases are converted to catalytically inactive forms. We tested whether MKP-7 was made catalytically inactive by such a mutation. As shown in Fig. 5B, the mutant form of MKP-7 (C244S) could not inactivate JNK2.

Catalytic Activity of MKP-7 in Vitro—To determine whether MKP-7 has a phosphatase activity, we examined the enzymatic activity of MKP-7 against pNPP, a well known artificial substrate of phosphatases. Myc-tagged MKP-7ΔC was prepared by immunoprecipitation from the cells transfected with SRα-Myc-MKP-7ΔC. Myc-MKP-7ΔC hydrolyzed pNPP in a dose-dependent manner, and sodium vanadate, a potent inhibitor of tyrosine phosphatase, strongly inhibited the activity of MKP-7 toward pNPP (Fig. 6A). We then examined whether p38 is directly inactivated by MKP-7 in vitro. Activated p38α was prepared by incubating GST-p38α with His-MKK6 in the presence of ATP. The activated p38α was then incubated with immunoprecipitated Myc-MKP-7ΔC and the kinase activity of p38α was measured by using myeline basic protein as a substrate. As shown in Fig. 6B, p38α was inactivated by Myc-MKP-7ΔC. These results clearly demonstrate that MKP-7 has an intrinsic phosphatase activity and directly dephosphorylates p38α. It has previously been reported that the binding of ERK to MKP-3/Pyst1/vH6, Pyst2, or MKP-4/Pyst3 enhances the activity of these phosphatases (52). We then assayed the activity of Myc-MKP-7ΔC in the presence of GST-p38α (12 or 24 μg). Incubation of Myc-MKP-7ΔC with GST-p38α stimulated the phosphatase activity of MKP-7ΔC toward pNPP significantly (Fig. 6C). However, incubation of Myc-MKP-7ΔC with
GST-ERK2, which did not bind to MKP-7, did not stimulate the phosphatase activity markedly (Fig. 6C). MKP-7 Shows the Substrate Specificity Toward the Isoforms of the p38 MAPK Family—There are four isoforms in the p38 MAPK family, namely, α, β, γ, and δ (53). We examined the docking ability of MKP-7 and MKP-5, a member of MKPs specific for p38 MAPK and JNK/SAPK (36), toward these four isoforms of the p38 family. Both MKP-7ΔC and MKP-5 co-immunoprecipitated well with p38α and -β, but not p38γ or -δ. The experiments were performed as in Fig. 4E, using appropriate p38 isoforms. C) MKP-5 inactivates the p38α and β, but not p38γ or -δ. The experiments were performed as in Fig. 4E, by using MKP-5.

Discussion

We have isolated a cDNA clone encoding a novel MKP, termed MKP-7. MKP-7 possesses all the features of the dual specificity MAPK phosphatase. MKP-7 is most similar to hVH5 in the primary sequence. Both molecules have a C-terminal stretch, which is not present in other MKPs. In this portion, we noticed two regions (HC1 and HC2), whose sequences are highly homologous between these two molecules. There is an NES sequence in the N-terminal portion of HC1. This NES sequence regulates the cytoplasmic localization of MKP-7. Although the sequence of the NES portion of hVH5 is also very similar to that of MKP-7, subcellular localization of hVH5 in cultured cells differs from that of MKP-7. hVH5 is not exclusively cytoplasmic. hVH5 might have a nuclear localization signal or some binding proteins in the nucleus, or the activity of its NES might be weaker. HC2 is featured by clusters of positively charged amino acids. GFP-tagged HC2 fragment of MKP-7 (residues 596–665) did not show any distinct localization but showed pan-cellular localization. We may speculate, however, that the HC2 region is important for some common function, not defined yet, of the two molecules. MKP-7 has a PEST-like sequence in its C-terminal stretch, and hVH5 also has a PEST-like sequence, the sequence being not homologous to that of MKP-6, at the corresponding site. Furthermore, we
noticed a serine-rich region in the C-terminal stretch of MKP-7, which is not present in hVH5. However, the mouse ortholog of hVH5, M3/6, also has a serine- and glycine-rich region at the corresponding site (29). Puckered (a Drosophila phosphatase specific for JNK/SAPK) also has a serine-rich region in the C-terminal portion of the molecule (54). The PEST-like sequence and the serine-rich sequence might be involved in protein-protein interactions.

Incubation with the substrate p38α enhances the enzymatic activity of MKP-7, as was reported for MKP-3 (52). Recently, the activity of CL100/MKP-1 was also reported to be stimulated by p38α (55). Furthermore, we have previously shown that incubation with p38α enhances the catalytic activity of MKP-5, although the enhancement is modest (only 1.6-fold) (36). It is likely, therefore, that the binding of the substrate generally enhances the catalytic activity of MKPs. This mechanism might be important for regulation of the specificity of MKPs.

MKP-7 has a MAPK-docking site in the N-terminal portion (residues 49–60). Based on the sequence characteristics of the docking site of MKPs, we propose that MKPs can be categorized into three groups. One group (the first group) includes CL100/ MKP-1, MKP-2, and PAC-1, and the second group MKP-3, MKP-4, Pyst-2, and B23, and the third group MKP-5, MKP-7, and hVH5 (Fig. 8A). The consensus sequence of the MAPK-docking site of each group is: XXRRA(K/R) for the first group; XXXRRY(R/K)(Q)R/K(G) for the second group; X(K/R)RRLQQYK for the third group (where X is a hydrophobic residue, and Y is any residue except for K or R). MKPs acting on p38 MAPK (the first and third groups) have three consecutive positively charged amino acids in the MAPK-docking site, while the second group MKPs acting mainly on ERK have two (Fig. 9A). It has previously been shown that MAPKAPKs acting on p38 also have more consecutive positively charged amino acids in the MAPK-docking site than those acting on ERK (45, 48, 50, 51). Notably, there are more negatively charged amino acids in the docking region of p38α than in that of ERK2 (50, 51). In fact, we recently reported that the number of negatively charged amino acids in the docking groove of ERK2 and p38α regulates their docking specificity (51). Thus, the number of charged amino acids in the MAPK-docking site of MKPs might be important for the docking specificity and therefore the efficiency of the catalytic activity of MKPs. Not only the MAPK-docking sites but also other parts show high degree of sequence similarity within each group of MKPs. Thus, each group has its own substrate specificity, as outlined in Fig. 9A. However, the published results are not necessarily in complete agreement with this tentative classification of the substrate specificity of MKPs. Chu et al. (56) reported that while PAC-1 could inactivate JNK/SAPK in COS7 cells, it could not act efficiently on JNKSAPK in NIH3T3 or HeLa cells. Such a difference might be caused by the difference in the expression levels of MKPs in each experiment, as also discussed by Chu et al. (56).
MKPs show the substrate specificity toward the isoforms of the p38 MAPK family. MKP-7 and MKP-5 selectively bind to and inactivate p38α and -β, but not γ or δ. CL100/MKP-1 also binds to p38α and -β, but not γ or δ (Fig. 5B). A selectivity among the isoforms of the p38 MAPK family was already reported for the upstream activating kinases and the downstream substrates (57, 58). MKK6 can activate all the isoforms of the p38 family, whereas MKK3 can activate only p38α. As for the substrate specificity, p38α and -β can phosphorylate and activate MAPKAPK2/3, but p38γ or -δ cannot. We also observed that p38α can bind to MAPKAPK-3, but p38γ cannot.2 According to our tentative classification of MKPs, the first and third groups of MKPs act on p38 (Fig. 8A), and our results show that their representatives, CL100/MKP-1 (the first group), MKP-5 and MKP-7 (the third group), cannot act on p38γ or -δ. Then, inactivation of p38γ and -δ in cells might be achieved by other phosphatases, such as PP2C-type phosphatases or PTPs. The different mode of activation and inactivation of p38γ and -δ suggests that they may function in signaling pathways different from those regulated by other MAPKs. p38γ is reported to have a motif sequence in its C-terminal tail that can associate with a PDZ domain-containing protein, α1-syntrophin (42). We also found that p38γ can bind to a PDZ-containing protein, hDLG, and phosphorylate it. Then, p38γ might regulate PDZ domain-containing proteins. It should be noted that p38γ and -δ are absent from Caenorhabditis elegans or Drosophila melanogaster. They might play some vertebrate specific roles. Adendum—During the course of the revision of this manuscript, the nucleotide sequence of human MKP-7 was deposited by another group.

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Fig. 9. The tentative classification of MKPs. A, a proposed classification of MKPs according to the primary sequence of their MAPK-docking sites. The underlined amino acids are the positively charged amino acids which are supposed to be important for the docking interaction. The consensus sequence of each group is: XRXXRA(R/K) for the group containing CL100/MKP-1; XXXRRY(R/K/Q/R/K/G) for the group containing MKP-3; XKR/RRLQQYK for the group containing MKP-7 (a); K/RRLQQYK for the group containing MKP-7 (b); and RRY(R/K)(Q)R/K(G) for the group containing MKP-7 (c). The docking interaction may serve as an important factor for the specificity determination. Accumulating data indicate a good correlation between the docking ability and the enzymatic activity in actions of MKPs toward MAPKs (36, 43, 50). For example, MKP-7 binds to and inactivates p38α and JNK2, but does not bind to or inactivate ERK2. This is a basis for our tentative classification of MKPs according to the sequence features of the MAPK-docking sites.

Our results here demonstrate, for the first time, the substrate specificity of MKPs toward the isoforms of the p38 MAPK family.
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