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A Novel Dual Specificity Phosphatase SKRP1 Interacts with the MAPK Kinase MKK7 and Inactivates the JNK MAPK Pathway

IMPLICATION FOR THE PRECISE REGULATION OF THE PARTICULAR MAPK PATHWAY*

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Mitogen-activated protein kinases (MAPKs) are activated in response to various extracellular stimuli, and their activities are regulated by upstream activating kinases and protein phosphatases such as MAPK phosphatases (MKPs). We report the identification and characterization of a novel MKP termed SKRP1 (SAPK pathway-regulating phosphatase 1). It contains an extended active site sequence motif conserved in all MKPs but lacks a Cdc25 homology domain. Immunoblotting analysis revealed that SKRP1 is constitutively expressed, and its transcripts of 4.0 and 1.0 kb were detected in almost tissues examined. SKRP1 was highly specific for c-Jun N-terminal kinase (JNK) in vitro and effectively suppressed the JNK activation in response to tumor necrosis factor α or thapsigargin. Endogenous SKRP1 was present predominantly in the cytoplasm and co-localized with JNK. However, SKRP1 does not bind directly to its target JNK, but co-precipitation of SKRP1 with the MAPK kinase MKK7, a JNK activator, was found in vitro and in vivo. Furthermore, we found that SKRP1 did not interfere with the co-precipitation of MKK7 with JNK. Together, our findings indicate that SKRP1 interacts with its physiological substrate JNK through MKK7, thereby leading to the precise regulation of JNK activity in vivo.

Mitogen-activated protein (MAP) kinase pathways are evolutionarily conserved in eukaryotic cells (1–5). The pathways are essential for physiological processes, such as embryonic development and immune response (3, 4, 6, 7), and regulate cell survival, apoptosis, proliferation, differentiation, and migration (3, 6–8).

In mammals, three major classes of MAP kinases (MAPKs) have been identified, which differ in their substrate specificity and regulation (1–6, 8). These subgroups compose the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) (7), and the p38/RK/CSBP kinases. ERKs are activated by a range of stimuli including growth factors, cell adhesion, tumor-promoting phorbol esters, and oncogenes (1, 2), whereas JNK and p38 are preferentially activated by proinflammatory cytokines, and a variety of environmental stresses such as UV and osmotic stress (1, 3, 6, 8). For this reason, the latter are classified as stress-activated protein kinases (SAPKs).

Activation of the MAPKs is achieved by dual phosphorylation on threonine and tyrosine residues within a Thr-Xaa-Tyr motif located in the kinase subdomain VIII. This phosphorylation is mediated by a dual specificity protein kinase, MAPK kinase (MAPKK), and MAPKKK in turn activated by phosphorylation mediated by a serine/threonine protein kinase, MAPKK kinase (1–3, 5, 8). In addition to these activating kinases, several types of protein phosphatases have been also shown to control MAPK pathways by dephosphorylating the MAPKs or their upstream kinases. These protein phosphatases include tyrosine-specific phosphatases (9, 10), serine/threonine-specific phosphatases (11, 12), and dual specificity phosphatases (DSPs) (13–30). Therefore, the activities of MAPKs can be regulated by upstream activating kinases and protein phosphatases (31–33). DSPs are one emerging subclass of the tyrosine-specific phosphatase family and dephosphorylate both phosphothreonine and an adjacent phosphotyrosine residue. Most of them inactivate MAPKs by dephosphorylating the critical phosphothreonine and phosphophotyrosine residues within a Thr-Xaa-Tyr motif and therefore are termed MAPK phosphatases (MKPs) (31–34).

Here we have characterized a novel dual specificity MKP that is highly specific for JNK in vitro and negatively regulates the activation of the JNK pathway. We also found that the enzyme interacts physically with the MAPKK MKK7, a JNK activator, but not with JNK. Thus, these findings suggest that the enzyme interacts indirectly with its physiological target JNK through MKK7, consequently leading to the specific regulation of JNK signaling pathway. Therefore, we have termed this novel enzyme SKRP1, as SAPK pathway-regulating phosphatase 1.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise noted, routine chemical materials were obtained from either Sigma or Wako (Osaka, Japan). Molecular standards and restriction enzymes were purchased from New England Biolabs (Beverly, MA).

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To identify novel DSPs, we employed degenerate PCR-based method and EST database searching using consensus DSP phosphatase domain as a template, and we isolated several clones encoding a novel sequence. By concatenating overlapping EST clones, we readily obtained putative full-length cDNA sequences, including SKRP1. The full-length SKRP1 sequence was...

Fig. 1. SKRP1 is a novel member of DSP family. A, nucleotide and deduced amino acid sequences of mouse SKRP1 cDNA. The conceptually translated amino acid sequences are presented in single-letter code below the nucleotide sequences. The stop codon is shown by an asterisk. The numbers on the left or the right denote the positions of nucleotides or amino acids, respectively. The active site sequence motif conserved in all DSPs is indicated by a shaded box. The catalytic cysteine is double-circled. Note that the conserved Ser in the active site sequence motif is replaced by Ala (a circle at the amino acid position 156). The peptide sequence spanning residues 80–97 of the mouse SKRP1 protein was used to generate the rabbit polyclonal antiserum against SKRP1 and is shown by a double underline in A–C. B, alignment of amino acid sequences of mouse and human SKRP1. The numbers denote the amino acid positions from the first Met. The active site sequence motif conserved in all DSPs is indicated by a shaded box. The catalytic cysteine is indicated by an asterisk. C, comparison of the phosphatase catalytic domains of mouse SKRP1 and other members of DSP family (13–30) using the ClustalX software. To optimize homology, gaps were inserted as denoted by dots. The black boxes or the gray boxes indicate identities or similarities, respectively. The catalytic cysteine is indicated by an asterisk. D, schematic representation of mouse SKRP1 amino acid identity with other members of DSP family. CH2 domains and phosphatase catalytic domains are indicated by black and shaded boxes, respectively. The percentages indicate the amino acid identity with mouse SKRP1 within the phosphatase catalytic domain. E, a radial dendrogram representing the relation between DSP family (13–30).
amplified from the mouse embryonic cDNA library with a sense primer 5’-GTGCTTCTTCTGGGAAG-3’ and an antisense primer 5’-TCCG-GATCTTTAAGTTATATATTGAG-3’, corresponding to the both ends of cDNA. The coding sequence of SKRP1 was amplified with a sense primer 5’-GGATCCATTGCACTGCTCCTCCACAAACAACTC-3’ and an antisense primer 5’-GCGGAGGTGGACGAGATCATCATCATCAAG-3’, having a NsiI site to bear an in-frame fusion to epitope-tagged expression vectors. To isolate the human homologue of SKRP1, we screened human EST data base using mouse full-length SKRP1 cDNA as a probe, and we obtained overlapping clones that match the 5’- and 3’-ends of the SKRP1 sequence.

Based on the sequences from these clones, we designed a sense primer 5’-TCTGCAACAAAGCTTATATAATAGG-3’ and an antisense primer 5’-AGCATTTAATCAGCATTAT-3’ and cloned putative full-length cDNA from 293 cells by reverse transcriptase-PCR.

Protein Expression—GST and GST fusion proteins GST-SKRP1, GST-SKRP1 C149S, GST-SKRP1 A156S, GST-SKRP1 C1145S, GST-SKP1 JSP-1 C88S, GST-MKP6, GST-MKP6 C111S, GST-JSP-1 (1-79), or GST-MKK7 were expressed in Escherichia coli DH5α using the pQE vector (American Biosciences) to generate His-tagged affinity chromatography skrp1 C149S mutants were generated by site-directed mutagenesis using a Quick Change Site-Directed Mutagenesis Kit (Stratagene) as described previously. His-tagged proteins His-SKRP1, His-JNK2, and His-MKK7 were expressed in E. coli BL21 (DE3) using pET-32b vector (Novagen) and purified using a nickel-nitrotriacetic acid-agarose chromatography (Qiagen) according to the manufacturer’s instructions.

Phosphotase Assay—GST-SKRP1, GST-SKRP1 C149S, GST-SKRP1 A156S, GST-SKP1 JSP-1 C88S, GST-MKP6, and GST-MKP6 C111S were incubated at the indicated concentrations for 1 h at 37°C in a reaction buffer containing 50 mM imidazole (pH 7.5), 20 mM p-nitrophenol (pNPP), and 5 mM dithiothreitol. The reaction was stopped by the addition of 0.1 N NaOH, and the pNPP hydrolysis was measured by absorbance at 405 nm.

Expression Plasmids—pSR/H9251-HA1-ERK2 (Xenopus MAPK, MPK1) (36), pSR/H9262-HA1-JNK2, pSR/H9251-Roche cDNA from 293 cells by reverse transcriptase-PCR.

Site-directed Mutagenesis—SKRP1 C149S mutants were generated in a standard two-step megaprimer PCR with modified oligonucleotides as primers and full-length SKRP1 complementary DNA as a template. The PCR products were used in a nested PCR that span the mutation site. Finally, PCR products were cloned into appropriate vectors, and mutations were verified by DNA sequencing.

Cell Culture and Transfection—COS-7 and 293 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. NIH3T3 or BALB/3T3 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium or minimum essential medium, respectively, supplemented with 10% fetal bovine serum and 100 units/ml penicillin, 0.1% glucose, and 100 units/ml streptomycin. COS-7 cells were transfected using LipofectAMINE (Invitrogen), according to the manufacturer’s protocol. The total amount of DNA in each reaction was kept constant by supplementation with empty vector DNAs.

In Vitro Dephosphorylation of MAPs—COS-7 cells were transfected with HA-MAPks or GST-MAPks. After culture for 24 h, the cells were left untreated or treated with 250 ng/ml 12-O-tetradecanoylphorbol-13-acetate after serum deprivation for 8 h (for ERK2) or 10 μg/ml anisomycin (for JNK2 and p38α) and then incubated for 20 min. Tagged MAPks were precipitated from the cell lysates by incubation with anti-HA antibody (clone 12CA5, Roche Diagnostics) or mouse monoclonal anti-His antibody (Qiagen) coupled to keyhole limpet hemocyanin.

Kinase Assays—Cells were lysed on ice in the following buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 12 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM EGTA, 3 mM dithiothreitol, and a protease inhibitor mixture (Complete, Roche Diagnostics). Lysates were sonicated for 15 s (for JNK2 and p38α) or 15 min (for ERK2) on ice before being centrifuged at 16,000 g for 15 min. The supernatants were clarified immediately or stored at −80°C. Immunoprecipitations or glutathione-Sepharose precipitations from cell lysates were carried out using anti-HA antibody (12CA5, Roche Diagnostics) together with protein G-Sepharose beads (Amersham Biosciences) or using glutathione-Sepharose 4B (Amersham Biosciences), respectively. After incubation for 3 h at 4°C, the complexes were washed three times with lysis buffer and then twice with kinase reaction buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 12 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1 mM dithiothreitol. The kinase assays were initiated by the addition of the substrate protein (1.0 mg) and 1 μCi of [γ-32P]ATP in a final volume of 25 μL. The reactions were terminated after 20 min at 30°C by addition of Laemmli sample buffer. Samples were boiled for 5 min, and the phosphorylate of the substrate proteins was examined by SDS-PAGE with a Fujif BAS2000 image analyzer.

Immunofluorescence Staining—NIH3T3 or BALB/3T3 cells were seeded in 8-well culture slides (BIOCAT, BD PharMingen) overnight and then fixed in 4% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature, followed by permeabilization in phosphate-buffered saline containing 0.2% Triton X-100. The cells were incubated with primary antibodies in blocking buffer (phosphate-buffered saline containing 0.2% Triton X-100 and 3% bovine serum albumin) overnight at 4°C. After washing three times, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies (Zymed Laboratories Inc. laboratories) for SKRP1 staining and Cy3-conjugated goat anti-mouse antibodies (Zymed Laboratories Inc. laboratories) for JNK2 staining, respectively, to detect bound primary antibodies. Coverslips were mounted on slides with Permafluor (Lipshaw Immunon). Fluorescent images were Kalman-averaged and analyzed by using a MicroRadiance laser-scanning confocal microscope (Bio-Rad).

In Vitro Binding Assays—GST or GST fusion proteins (1.0 μg) immobilized on 15 μl of glutathione-Sepharose beads were incubated with the indicated His-tagged proteins or cell lysates in lysis buffer for 3 h at 4°C. After washing the beads extensively with the same buffer, glutathione-Sepharose precipitates were immunoblotted for the presence of His-tagged proteins or HA-tagged MKK7 derivatives using mouse monoclonal anti-His antibody (Qiagen) or rabbit polyclonal anti-HA antibody (MBL), respectively, followed by Coomassie staining. Some of the proteins used in binding reactions was also loaded as a positive control (Input).

Metabolic Labeling—Metabolic labeling of cells was performed in methionine-free medium supplemented with [35S]EasyTag (Perkin-Elmer) at concentrations to 0.2 μCi/μL for 5 h. Cell lysates were prepared in lysis buffer and precipitated with 1.0 μg of GST or GST-SKRP1 fusion proteins. Glutathione-Sepharose precipitates were subjected to 10% SDS-PAGE, followed by autoradiography.

Co-presentation Analysis—Cell lysates from COS-7 cells transfected with the appropriate combinations of expression plasmids were precipitated using glutathione-Sepharose 4B as described previously (38–44). The glutathione-Sepharose precipitates were then washed five times with lysis buffer, resolved on SDS-PAGE, and examined by immunoblotting using appropriate antibodies.
FIG. 2. Phosphatase activity and substrate specificity for MAPKs of SKRP1. A, intrinsic phosphatase activity of SKRP1 protein. Phosphatase activities of GST-SKRP1, GST-JSP-1, and GST-MKP-6 either in the absence (○) or presence (●) of 1 mM sodium vanadate and of their...
RESULTS

Identification of SKRP1 as a Member of MKP Family—DSPs display a high degree of sequence similarity within an active site sequence motif. Therefore, to identify novel DSPs, we used both degenerate PCR-based methods and EST data bases for cDNA fragments containing this conserved motif. Several genes were isolated from a mouse embryo and human embryonic kidney (293) cells, including JSP-1 (30) and MKP-6 (26). One clone encodes a novel protein, which we have termed SKRP1. The sequence of the mouse SKRP1 cDNA (GenBankTM accession number AB051896) revealed an open reading frame of 220 amino acids with a predicted molecular weight of 25,000 (Fig. 1A). The human (GenBankTM accession number AB063186) SKRP1 cDNA has 83% sequence identity with full-length mouse SKRP1 cDNA (data not shown) and encodes protein of 217 amino acids that have 81% sequence identity with the mouse SKRP1 protein (Fig. 1B). The SKRP1 protein contains the extended active site sequence motif conserved in all DSPs, within which the cysteine residue is essential for catalytic activity (Fig. 1C) and displayed 33–40% identity to the phosphatase catalytic domains of other members of the DSP family (Fig. 1D). However, the predicted amino acid sequence of SKRP1 does not contain two N-terminal Cdc25 homology domains (CH2 domains), characteristic of almost other members of DSP family (Fig. 1D). The radial dengrogram showed that SKRP1, JSP-1 (30), MKP-5 (24, 25), and hVHR (14) constitute a subfamily of DSPs (Fig. 1E).

To determine whether SKRP1 possesses intrinsic phosphatase activity, GST-SKRP1 fusion proteins were assayed for enzymatic activity toward p-nitrophenyl phosphate (pNPP), compared with JSP-1 (30) and MKP-6 (26), two members of DSP family. These GST fusion proteins hydrolyzed pNPP in a dose-dependent manner, and sodium vanadate, a potent inhibitor of tyrosine-specific phosphatase, effectively inhibited this catalytic activity (Fig. 2A). A point mutation in the catalytic cysteine of SKRP1 (C149S) abolished the phosphatase activity as seen in JSP-1 and MKP-6 (Fig. 2A), indicating that SKRP1 C149S is a catalytically inactive mutant of SKRP1. Similar phosphatase activities were obtained with His-tagged SKRP1 proteins (data not shown). Furthermore, these studies showed that SKRP1 was 10-fold less active toward pNPP compared with JSP-1 and MKP-6 (Fig. 2A). Three amino acids, Asp, Cys, and Ser, in the active site sequence motif (Fig. 1A, shaded box) have been shown to contribute to the catalytic mechanism of DSPs (45–49). Therefore, to examine whether the natural replacement S156A of SKRP1 protein is responsible for its low phosphatase activity, we compared the catalytic activity of SKRP1 with that of SKRP1 A156S using in vitro phosphatase assay. However, these studies showed that both proteins exhibited the same enzymatic activities in vitro (Fig. 2B).

Since each DSP has its own substrate specificity for MAP kinases (MAPKs) (31–33), we tested the activity of SKRP1 toward different MAPKs in vitro. Dually phosphorylated HA-MAPKs were immunoprecipitated from the transfected cells that had been treated with 12-O-tetradecanoylphorbol-13-ace-
Fig. 4. Inactivation of JNK pathway by SKRP1. A, effect of SKRP1 on the activation of JNK pathways by distinct stimuli. GST-JNK2 was transiently transfected into COS-7 cells together with increasing amounts of Myc-SKRP1, as indicated. After culture for 24 h, the cells were either...
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Co-localization of SKRP1 and JNK—To examine the subcellular localization of exogenously expressed SKRP1, NIH3T3 cells were transiently transfected with Myc-SKRP1, followed by indirect immunofluorescence staining using anti-Myc antibody. The result showed that exogenously expressed SKRP1 was localized to the cytoplasm (Fig. 5A, right panel). We next investigated the localization of endogenous SKRP1 in NIH3T3 cells using preimmune serum or antiserum to SKRP1 described in Fig. 3A, and we found that endogenous SKRP1 was also detected predominantly in the cytoplasm (Fig. 5A, left and middle panels). Furthermore, together with the result as shown in Fig. 3A, these findings revealed that SKRP1 was constitutively expressed.

As shown in Fig. 4, SKRP1 suppressed the activation of the JNK pathway. Therefore, to examine whether endogenous JNK can be a physiological target for endogenous SKRP1, NIH3T3 and BALB/3T3 cells were immunostained for their subcellular localizations using anti-SKRP1 and anti-JNK2 antibodies. These studies showed that endogenous SKRP1 co-localized with the endogenous JNK2 in both cells (Fig. 5B). To test whether SKRP1 interacts directly with JNK2, we performed in vitro binding assays using recombinant proteins, and we found that SKRP1 did not bind directly to JNK2 (Fig. 5C). Thus, SKRP1 co-localizes with but does not interact directly with JNK2. These findings raised the possibility that SKRP1 interacts indirectly with JNK2 through unidentified molecules in vivo.

Interaction of SKRP1 with the MAPKK MKK7—To identify molecules related to the JNK pathway that associate with SKRP1, metabolically labeled extracts were precipitated with GST or GST-SKRP1 fusion proteins, followed by SDS-PAGE analysis. This study showed that three major proteins of 46, 48, and 54 kDa were specifically precipitated by GST-SKRP1 and GST-SKRP1 C149S fusion proteins but not by GST protein alone (Fig. 6A).

We therefore searched for such candidates related to JNK, with the molecular mass of 46–54 kDa. One candidate is the JNK activator MKK7 (40, 51–59), which is a MAPKK recognized as 47–48-kDa protein by immunoblotting in various mammalian cells containing NIH3T3 cells (52, 53). We then examined the ability of SKRP1 to interact with MKK7. When His-MKK7 was incubated with GST, GST-SKRP1, or GST-SKRP1 C149S in vitro, His-MKK7 did not interact with GST.
protein alone but did interact with GST-SKRP1 and GST-SKRP1 C149S (Fig. 6B).

Next, to determine whether SKRP1 specifically interacts with MKK7 in vivo, GST-SKRP1 was co-expressed in COS-7 cells together with either HA-MKK4, another MAPKK for JNK, or HA-MKK7, followed by co-precipitation analysis using glutathione-Sepharose as described previously (38–44). This study showed that MKK7 was specifically detected in SKRP1 precipitates whereas MKK4 was not (Fig. 6C). These findings suggest that SKRP1 interacts selectively with the MAPKK MKK7 in vivo.

Binding of MKK7 to SKRP1 Independently of JNK2—Because MKK7 binds to JNK (40, 54), the SKRP1-MKK7 direct interaction (Fig. 6B) raised the possibility that SKRP1 interacts indirectly with JNK through MKK7. We therefore tested the effect of SKRP1 on the physical interaction between MKK7 and JNK2. When GST-MKK7 was used as affinity reagents, JNK2 and SKRP1 mixed together bound to GST-MKK7 with more efficiency than that of each individual protein alone (Fig. 7A). Consistent results were obtained from co-transfection experiments, in which cells were transfected with MKK7, JNK2, and increasing amounts of SKRP1. Expression of SKRP1 did not interfere with the co-precipitation of MKK7 with JNK2 (Fig. 7B). Thus, JNK2 and SKRP1 did not compete for binding to MKK7. To confirm this conclusion further, we mapped the domains in MKK7, which are necessary for SKRP1 binding. Analysis of various MKK7 derivatives demonstrated that MKK7 residues 128–214 were required for interaction with SKRP1 (Fig. 7C). Previous study (40) showed that three isoforms (α, β, and γ) of MKK7 with different N termini are created by alternative splicing, and its substrate JNK binds to the N-terminal extension that is present in MKK7β but not MKK7α isoforms. This region is also present in the residues 1–89 of MKK7γ isoform used in our study and therefore can account for the interaction with JNK (Fig. 7, A and B). Furthermore, these findings indicate that SKRP1 and JNK2 can interact independently with MKK7 to form ternary complexes.

**DISCUSSION**

To date many MKPs have been identified, and their precise regulation is considered to confer the specificity of MAPK signaling pathways. They share the extended active site sequence motif (DX_3+1VHCXAGXSRX, where X is any amino acid), and previous studies (45–49) showed that three amino acids, Asp, Cys, and Ser, in this motif contribute to the catalytic mechanism of them. The predicted amino acid sequence of mouse and human SKRP1 revealed that one of their critical residues, Ser, is naturally replaced by Ala at amino acid positions 156 and 157, respectively (Fig. 1B). Therefore, to test whether this replacement affects the SKRP1 catalytic activity, we performed in vitro phosphatase assay of GST-SKRP1 and GST-SKRP1 A156S using pNPP as a substrate. These studies showed that both proteins exhibited the similar enzymatic activities in vitro (Fig. 2B). In vitro studies as described previously (45) showed that a substitution of Ala for Ser conserved in the active site sequence motif had essentially no effect on substrate binding, intermediate formation, but dramatically affected the rate of intermediate hydrolysis. Therefore, these findings suggest that the rate-limiting step in the catalytic mechanism is completely converted from intermediate formation in the native enzyme to intermediate hydrolysis in the SA mutant. However, the physiological significance of Ala-156 in SKRP1 protein remains to be known. We next compared the in vitro enzymatic activity of recombinant SKRP1 with two other DSP members, JSP-1 and MKP-6, which have been isolated in the cloning process. These studies showed that SKRP1 is 10-fold less active toward pNPP compared with JSP-1 and MKP-6 (Fig. 2A). In addition, unlike almost other MKPs, SKRP1 is constitutively expressed in cells (Figs. 3A and 5A), and its inducibility in response to various stresses was not detected (data not shown). Together, these findings suggest that as yet unknown mechanism may exist to regulate the phosphatase catalytic activity of SKRP1 in vivo.

Some members of MKP family display the specificity for inactivating MAPKs. It has been reported that the N-terminal CH2 domain of each MAPK mediates the interaction with its preferred MAPK (31), which then induces the catalytic activation of MKP (23, 27, 60, 61) and subsequent dephosphorylation of its bound MAPK. Furthermore, MAPK-docking site of MKP has been mapped to the consensus sequence motif containing more than two consecutive positively charged amino acids (62). However, SKRP1 possesses neither the N-terminal CH2 domain...
nor a sequence motif homologous to the consensus MAPK-docking sequence, and concomitantly we could not find any significant interaction between SKRP1 and MAPKs (data not shown).

In the present study, we demonstrated that SKRP1 interacts directly with the MAPKK MKK7 (Fig. 6B), but not directly with JNK2 (Fig. 5C), raising the possibility that SKRP1 interacts indirectly with its physiological substrate JNK2 through MKK7 in vivo. MKK7 is a MAPKK that specifically activates JNK among MAPKs (51–59). To date there have been reported six isoforms of MKK7, H9251, H9252, H9253, and H9254 (40).
which $\beta$ and $\gamma$ isoforms possess N-terminal JNK-interacting domain at amino acid residues 1–73 and 1–89, respectively (40, 63). As shown in Fig. 7, $A$ and $B$, we actually observed that JNK2 interacted with the MKK7$\gamma 2$ isoform that we have employed in this study and that JNK2 binding on MKK7 was not competed by SKRP1 (Fig. 7B). In line with this result, we have also determined SKRP1-binding domain on MKK7 as shown in Fig. 7C, which differs from JNK-binding domain on MKK7. These results indicated that the selective interaction between SKRP1 and MKK7 within the cells contributes to the specificity in JNK dephosphorylation by SKRP1. However, 10 JNK isoforms have been isolated so far (64); therefore, SKRP1 may have differential dephosphorylation efficiency on these JNK isoforms, according to the affinity of MKK7–JNK interaction that varies among the combinations of use of MKK7 and JNK isoforms. Furthermore, SKRP1 strongly inhibited the activation of JNK2 induced by TNFα or thapsigargin, whereas SKRP1 had only a small influence on the JNK2 activation induced by anisomycin or UV radiation (Fig. 4). These findings are, to our knowledge, the first evidence to show that MKK7 interact directly with a MAPKK and preferentially regulates JNK2 activation on MKK7 as shown in Fig. 7. As shown in Fig. 6A, in vitro binding analysis using both metabolically labeled extracts and GST-SKRP1 fusion proteins as affinity reagents revealed that only three major proteins were detectable in GST-SKRP1 precipitates. However, there is a possibility that SKRP1 can associate with proteins other than them, which may contribute to the regulation of its subcellular localization and function. Interestingly, the cytosolic SKRP1 has been found to translocate to the nucleus in response to osmotic stress, a potent activating stress for the p38 MAPK pathway (data not shown), and we also found that SKRP1 suppresses the basal and stress-induced activities of p38 MAPK in vivo (data not shown). Thus, different compartments of SKRP1 might exist in the cell, which would involve different signaling pathways. Therefore, SKRP1 may function as a target site for cross-talk with other signaling pathway.

Finally, although MKP has been implicated in the negative regulation of MAPK pathways, the general importance in terminating MAPK, the molecular mechanisms that may control its phosphatase catalytic activity, and its signaling specificity for inactivating the particular MAPK pathway signaling remain to be determined. Here our results may provide a hint on these issues and illustrate a new tier of the precise regulation of MAPK signaling pathways.

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