Phosphorylation-dependent Scaffolding Role of JSAP1/JIP3 in the ASK1-JNK Signaling Pathway

A NEW MODE OF REGULATION OF THE MAP KINASE CASCADE*†‡

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JSAP1 (also termed JIP3) is a scaffold protein that interacts with specific components of the JNK signaling pathway. Apoptosis signal-regulating kinase (ASK) 1 is a MAP kinase kinase kinase (MAPKKK) that activates the JNK and p38 mitogen-activated protein (MAP) kinase cascades in response to environmental stresses such as reactive oxygen species. Here we show that JSAP1 bound ASK1 and enhanced ASK1- and H2O2-induced JNK activity. ASK1 phosphorylated JSAP1 in vitro and in vivo, and the phosphorylation facilitated interactions of JSAP1 with SEK1/MKK4, MKK7 and JNK3. Furthermore, ASK1-dependent phosphorylation was required for JSAP1 to recruit and thereby activate JNK in response to H2O2. We thus conclude that JSAP1 functions not only as a simple scaffold, but it dynamically participates in signal transduction by forming a phosphorylation-dependent signaling complex in the ASK1-JNK signaling module.

In response to various extracellular stimuli, mitogen-activated protein (MAP)1 kinase kinase kinase (MAPKKK) phosphorylates and activates MAP kinase kinase (MAPKK), and activated MAPKK immediately activates MAP kinase (MAPK). The MAPK signal transduction cascade (MAPKKK-MAPKK-MAPK) is evolutionarily conserved from yeast to humans and plays pivotal roles in many cellular processes including cell growth, differentiation, and apoptosis. In mammalian cells, at least three MAPKs have been identified: the ERKs, the JNKs and the p38s. JSAP1 and p38 are preferentially activated by proinflammatory cytokines and environmental stresses (1, 2).

Apoptosis signal-regulating kinase (ASK) 1 is a member of the MAPKKK group that activates SEK1 (also termed MKK4)/MKK7-JNK and MKK3/MKK6-p38 signaling cascades (1, 2). Overexpression of ASK1 induces apoptosis in various cells through mitochondria-dependent caspase activation (3–5). We have shown by deleting ASK1 in mice that tumor necrosis factor- and oxidative stress-induced sustained activation of JNK/p38 and apoptosis are lost in ASK1−/− cells (6). In addition, ASK1 was recently shown to be required for endoplasmic reticulum stress-induced apoptosis (7). These observations indicate that ASK1 plays an essential role in stress- and cytokine-induced apoptosis. On the other hand, ASK1 may also regulate cell differentiation; low and high expression of exogenous ASK1 in certain cells induced differentiation and apoptosis, respectively (8, 9). These results suggest that ASK1 has a broad range of biological activities depending on cell types, cellular context, or the extent of ASK1 activation.

The maintenance of signaling specificity, efficiency, and integrity in the MAPK cascades appear to be accomplished by specific molecular interactions among kinases, substrates, and scaffold proteins (10). Studies in yeast have established that the components of mating MAPK pathway interact with the scaffold protein Ste5p that is essential for the mating signal (11). Recent studies of the JNK signal transduction pathway have identified many types of potential scaffold proteins, which include CrkII, Filamin, β-arrestin, JNK-interacting proteins (JIPs), and JNK/stress-activated protein kinase-associated protein (JSAP1) (10). CrkII has been demonstrated to bind SEK1 and JNK through SH3 domain and represents a scaffold protein in the Rac1-mediated JNK signaling pathway (12). Filamin also binds to SEK1 and TRAF2 and appears to be a putative scaffold protein in the tumor necrosis factor signaling pathway (13, 14). In contrast, a MAPK phosphatase termed Skp1 was recently shown to interact with ASK1 and MKK7 but not with SEK1 and to enhance ASK1-MKK7 axis (15). The arrestin family proteins bind to G-protein-coupled receptors and function as signaling molecules of ligand-activated G-protein-coupled receptors (16). β-Arrestin 2 has recently been shown to interact with JNK3, ASK1, and indirectly with SEK1 and thereby facilitate JNK3 activation in response to angiotensin II (17). The JIP group proteins such as JIP1 (also known as IB1) and JIP2 have been demonstrated to function as scaffold proteins in JNK signaling cascades (10). JIP1 and JIP2 bind to JNK, MKK7, and a MAPKKK termed mixed-lineage protein kinases (MLKs) (18–22). A genetic evidence for the critical role of JIP1 in JNK signals has recently been provided by deleting JIP1 in mice in which excitotoxic stress-induced JNK activation and apoptosis were suppressed (23). Another scaffold pro-

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tein in the JNK cascades, JSAP1 (also known as JIP3), has been identified by the yeast two-hybrid screening using JNK3 as a bait (24, 25). JSAP1/JIP3 is structurally unrelated to JIP1 or JIP2 and has at least four spliced isoforms (25, 26). Although biochemical studies of JSAP1 have yielded several conflicting results, JSAP1 appears to interact with JNKs, SEK1, MKK7, full-length MEKK1, MLK2, and MLK3 in transfected cells (24, 25). Recently, it was found that Caenorhabditis elegans homolog of JSAP1, called UNC-16, interacts genetically and physically with JNK-1 and JKK-1 that have the highest sequence similarity to mammalian JNK3 and MKK7, respectively (27). JSAP1 thus serves as a functional scaffold that may be required for appropriate signaling of JNK. It was also shown that JSAP1 is phosphorylated in vivo (25); however, the role of phosphorylation of JSAP1 has not been elucidated. Moreover, little is known in general about the functional significance of posttranslational modification of scaffold proteins.

In the present study, we have investigated the role of JSAP1 in the ASK1-JNK signaling pathway. Endogenous JSAP1 was found to bind ASK1 in PC12 cells, and exogenous JSAP1 en-
hanced ASK1- and H$_2$O$_2$-induced JNK3 activation. Interestingly, ASK1 phosphorylated JSAP1 in vitro and in vivo, and phosphorylated JSAP1 recruited SEK1, MKK7, and JNK3 into the JSAP1-ASK1 signaling complex. Moreover, phosphorylation of JSAP1 by ASK1 was found to be required for the enhancement of JNK activation by JSAP1. We demonstrate for the first time that stimulation-dependent phosphorylation of JSAP1 facilitates formation of a functional JNK signaling module. This may represent a novel mode of regulation of the MAPK signal transduction cascades.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Expression Plasmids, and Transfections**—Rat pheochromocytoma cell line PC12 and human embryonic kidney cell line HEK293 cells were maintained as described (8, 28). ASK1$^{+/+}$ and ASK1$^{-/-}$ MEFs were obtained from E12.5 embryos (29). pcDNA3-HA-ASK1, pcDNA3-HA-ASK1KM, pcDNA3-Flag-ASK1, and pcDNA3-HA-JNK3–1 have been described (4, 28, 30). HA-ERK1, Flag-ERK1, HA-SEK1, HA-MKK7, and HA-JNK3â‘£ were constructed in pcDNA3. Mutations were introduced to replace amino acids serine 287, threonine 291, and serine 293 with glutamic acid in pcDNA3-HA-MKK7EEE and with alanine in pcDNA3-HA-MKK7AAA. Mutations were also introduced to replace threonine 221 with alanine and tyrosine 223 with phenylalanine in pcDNA3-HA-JNK3APF and the ATP-binding lysine 93 and 94 with arginine in pcDNA3-HA-JNK3KR. Five copies of the Myc tag were inserted at the N terminus of JSAP1 (Myc-JSAP1) in pcDNA3. Transfection was performed with FuGENE6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

**Antibodies and Immunoblotting Analysis**—Affinity-purified rabbit polyclonal antibody raised against active ASK1 (phospho-ASK1) was recently described (31). Antisera to ASK1 (28) and JSAP1 (32) have been described. Immunoblotting analysis has been performed as previously described (6). Blots were probed with antibodies to phospho-ERK, phospho-JNK, phospho-ASK1, and JSAP1.

**Co-immunoprecipitation Analysis**—For endogenous co-immunoprecipitation analysis, $1 \times 10^7$ of PC12 cells were lysed in the lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 1% Triton X-100, 1% deoxycholate, 12 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1.5% aprotinin. Cell lysates were immunoprecipitated (IP) with anti-Myc antibody. Beads were treated (PPase) or untreated with phosphatase for 30 min at 30 °C, washed with a washing buffer, and subjected to SDS-PAGE. Interactions were analyzed by immunoblotting with anti-HA antibody (top panels in A, B, and C).

**Fig. 2.** ASK1-dependent interactions of JSAP1 with SEK1, MKK7, and JNK3. WT but not kinase-inactive (KM) ASK1 enhances the interactions of JSAP1 with SEK1 (A), MKK7 (B), and JNK3 (C). HEK293 cells were transiently co-transfected with indicated plasmids. Lysates were immunoprecipitated (IP) with anti-Myc antibody. Beads were treated (PPase) or untreated with phosphatase for 30 min at 30 °C, washed with a washing buffer, and subjected to SDS-PAGE. Interactions were analyzed by immunoblotting with anti-HA antibody (top panels in A, B, and C). The presence of Myc-JSAP1, HA-ASK1WT, HA-ASK1KM, HA-SEK1, HA-MKK7, and HA-JNK3 in the same lysates was verified by immunoblotting analysis. ASK1-induced HA-JNK3 activation was detected by immunoblotting with anti-phospho-JNK antibody (C, WB:JNK). WT and EEE MKK7s, but not kinase-inactive (AAA) MKK7 (D), or HA-tagged WT, unphosphorylatable (APF), or kinase-inactive (KR) JNK3 (E) were transiently co-transfected with Myc-JSAP1 and HA-ASK1 in HEK293 cells. Cell lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA antibody. The presence of Myc-JSAP1, HA-ASK1, HA-MKK7, and HA-JNK3 in the same lysates was verified by immunoblotting. These experiments were performed more than three times with similar results (Fig. 2).
Fig. 3. ASK1-dependent phosphorylation of JSAP1 enhances its scaffolding activity. A, ASK1 phosphorylates JSAP1 in vivo. Myc-JSAP1 was cotransfected in HEK293 cells with ASK1 WT or ASK1 KM in the indicated combinations. After 24 h, cells were metabolically labeled with [32P]orthophosphate for 3 h, and cells were lysed with lysis buffer. Cell lysates were immunoprecipitated with anti-Myc antibody, and phosphorylation of JSAP1 was analyzed (top panel). The amount of JSAP1 was confirmed by immunoblotting with anti-JSAP1 antibody. The presence of HA-ASK1WT and HA-ASK1KM in the same lysates was verified by immunoblotting. B, recombinant ASK1 directly phosphorylates JSAP1 in vitro. GST-ASK1NWT or GST-ASK1NKR proteins (0.1 μg) were incubated with or without GST-JSAP1 (0.1 μg) and GST-MKK6KN (0.1 μg) in the presence of γ-[32P]ATP for 20 min at 30°C. The presence of GST fusion proteins in the same samples was verified by immunoblotting with an anti-GST antibody. This experiment was performed more than three times with similar results. C and D, ASK1 and JNK3 bind and phosphorylate different regions of JSAP1 in vitro. GST pull-down (C) was performed using recombinant GST-JSAP1 (1–744) and GST-JSAP1 (744–1305) from HEK293 cell lysates transfected with HA-ASK1 and HA-JNK3 plasmids. Co-purified material was analyzed by immunoblotting with anti-HA antibody. Immunoprecipitated ASK1 and JNK3 from transfected HEK293 cells were subjected to in vitro phosphorylation analysis (D, top panel) using recombinant GST-JSAP1 (1–744), GST-JSAP1 (744–1305), and GST-MKK6KN as substrates. Phosphorylation by ASK1 was quantified in comparison with ASK1KM, and relative values were indicated as n-fold. Essentially the same results were obtained from three independent experiments. E, requirement of ASK1 for H2O2-induced phosphorylation of JSAP1 in vivo. ASK1+/+ and ASK1−/− MEFs were infected with Ad-Myc-JSAP1 at an m.o.i. of 1.0. After 16 h, cells were metabolically labeled with [32P]orthophosphate for 3 h, and cells were lysed after treatment with or without indicated concentration of H2O2 plus 25 mM aminotriazole (catalase inhibitor) for 60 min. Lysates were immunoprecipitated with anti-Myc antibody, and phosphorylation of JSAP1 was analyzed (top panel). The amount of immunoprecipitated JSAP1 was confirmed by immunoblotting with anti-JSAP1 antibody. The intensity of JSAP1 phosphorylation was quantified and represented by a graph. Data are means (± S.E.) of three independent experiments. F, requirement of ASK1 for JNK-dependent activation of JNK. ASK1+/+ and ASK1−/− MEFs were infected with Ad-β-galactosidase (β-gal) or Ad-Myc-JSAP1 at an m.o.i. of 0.1. After 16 h, cells were treated with 0.2 mM H2O2 plus 25 mM aminotriazole for the indicated time periods. Lysates were subjected to immunoblotting with anti-phospho-JNK, anti-phospho-p38, and anti-phospho ERK antibodies. Membrane was reprobed with antibodies to total JNK, p38, and ERK. G, requirement of ASK1 for H2O2-induced JNK recruitment to JSAP1. ASK1+/+ and ASK1−/− MEFs were infected with Ad-Myc-JSAP1 at an m.o.i. of 1.0. After 16 h, cells were treated or untreated with 0.2 mM of H2O2 for 60 min. Cells were lysed and immunoprecipitated with control (C) or anti-JNK antibody (anti-JNK), and the immunoprecipitates were analyzed by immunoblotting with indicated antibodies. Essentially the same results were obtained in three independent experiments.
buffer containing 10 mM glutathione, 50 mM Tris-HCl, pH 8.0, and 25 mM MgCl₂. The beads fused with 0.1 μg of GST-JSAPl or GST-MKK6KN were incubated with or without 0.1 μg of GST-AASK1N or GST-AASK1N-KR in the presence of 0.3 μCi of [γ-32P]ATP for 20 min at 30 °C. Kinase activities were stopped by adding SDS sample buffer. Phosphorylation of GST-JSAPl and GST-MKK6KN was analyzed by image analyzer. The amount of GST fusion proteins was determined by immunoblotting with antibody to GST (Santa Cruz).

**In Vivo Phosphorylation of JSAPl—HEK293 cells were transfected with pcDNA3-Myc-JSAPl and HA-ASK1. Twenty-four h after transfection, cells were washed and incubated with phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum containing 1 μCi/ml [32P]orthophosphate for 3 h at 37 °C. Cells were lysed with the lysis buffer, and cell lysates were immunoprecipitated with anti-Myc antibody (9E10). Proteins were resolved by SDS-PAGE under reducing conditions followed by electrophoretic transfer onto polyvinylidene difluoride membrane. Phosphorylation of JSAPl was analyzed by an image analyzer. The amount of Myc-JSAPl was determined by immunoblotting with anti-Myc antibody and quantified as described above. For MEF cells, recombinant adenoviruses encoding β-galactosidase (Ad-β-gal) and Myc-JSAPl (Ad-Myc-JSAPl) were constructed as described (4). Sixteen h after infection with Ad-Myc-JSAPl, MEFs were washed and incubated with phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum containing 1 μCi/ml [32P] orthophosphate for 3 h at 37 °C. Phosphorylation of JSAPl was determined as described above.

**Pull Down Analysis—Bacterially expressed GST-JSAPl(1–744) and GST-JSAPl(744–1305) proteins bound to glutathione-Sepharose beads were mixed with HEK293 cell lysates overexpressing ASK1 or JNK3, rotated 2 h at 4 °C, spun, and washed with washing buffer. The beads were analyzed by immunoblotting.

**RESULTS AND DISCUSSION**

**Interaction of JSAPl with ASK1**—To investigate the potential roles of JSAPl in the ASK1 signaling pathway, we first examined whether JSAPl physically interacts with ASK1 in transfected cells (Fig. 1, A and B). Myc-tagged JSAPl was communoprecipitated with differentially tagged ASK1 (FLAG-ASK1 and HA-ASK1) in HEK293 cells (Fig. 1, A and B, lane 2), but not with p38 (Fig. 1A, lane 3 and Fig. 1B, lane 4) or ERK (Fig. 1A, lane 4 and Fig. 1B, lane 6). These results strongly suggested that JSAPl and ASK1 can form a complex in vivo. Although many components in the JNK signaling module such as JNK, SEK1, MKK7, full-length MEKK1, MLK2, and MLK3 have been shown to associate with JSAPl by overexpression (24, 25), their endogenous interactions have not been reported. To examine whether JSAPl interacts with ASK1 in untransfected cells, lysates from PC12 cells were immunoprecipitated with nonimmune, anti-ASK1 or anti-JSAPl antisera, and the immunoprecipitates were analyzed by immunoblotting with anti-JSAPl and anti-ASK1 (Fig. 1C). Endogenous JSAPl was communoprecipitated with endogenous ASK1 (Fig. 1C, lane 2; endogenous ASK1 represented by a doublet as previously reported (28)) and *vice versa* (Fig. 1C, lane 3). Quantification of communoprecipitated proteins indicated that ~7–10% of endogenous JSAPl and ASK1 exists as a complex, suggesting that certain portion but not all of JSAPl and ASK1 proteins forms a signaling complex under physiological conditions.

**JSAPl Is a Scaffold Protein in the ASK1-JNK3 Signaling Cascade**—JSAPl has been known to bind JNK1, JNK2, and JNK3 but not p38 or ERK (Refs. 24, 25, and Fig. 1, A and B). JSAPl was also shown to enhance MEKK1- and MLK3-induced activation of JNKs (24, 25). JSAPl and JNK3 are both expressed exclusively in the brain (33), and JSAPl has higher affinity to JNK3 than to other JNK isoforms (24, 25). We thus examined whether JSAPl affects ASK1-induced JNK3 and p38 activations. HA-tagged JNK3 (HA-JNK3) or p38α (HA-p38) were cotransfected with or without FLAG-tagged ASK1 (FLAG-ASK1) and Myc-tagged JSAPl (Myc-JSAPl) in HEK293 cells, and cell lysates were immunoprecipitated with anti-HA antibody. The immune complexes were subjected to *in vitro* kinase assays using GST-εJun and GST-ATF2 as specific substrates for JNK3 and p38, respectively. JSAPl had little effect on the basal activity of JNK3 (Fig. 1D, lane 3), ASK1-induced JNK3 activation (Fig. 1D, lanes 5 and 6) but not p38 activation (Fig. 1E, lanes 5 and 6) was markedly enhanced by JSAPl in a dose-dependent manner, suggesting that JSAPl may serve as a scaffold protein in the ASK1-JNK3 signaling cascade. ASK1 is activated by H₂O₂ and specifically required for H₂O₂-induced sustained activation of JNK (6). If JSAPl potentiates ASK1-JNK module as a scaffold, we would expect the expression of JSAPl to increase the activation of JNK caused by an ASK1-specific stimulus, H₂O₂. Adenovirus-mediated expression of JSAPl (Ad-Myc-JSAPl), but not control β-galactosidase (Ad-β-gal), enhanced activation of endogenous JNKs (Fig. 1F, third panel), especially JNK3 (Fig. 1F, top panel) in PC12 cells. In contrast, JSAPl did not potentiate p38 or ERK activation by H₂O₂ (Fig. 1F). Importantly, exogenous JSAPl had no effect on the activity of endogenous ASK1 itself as determined by anti-phospho-ASK1 immunoblotting analysis (Fig. 1F, fifth panel from top). These results suggest that JSAPl functions as a molecular scaffold that specifically facilitates H₂O₂-induced and thus ASK1-induced JNK3 activation.

**Wild-type but Not Kinase-inactive ASK1 Potentiates the Interactions of JSAPl with SEK1, MKK7, and JNK**—Although the mechanism was unknown, activation of the components of the JNK signaling pathway has been reported to associate with increased formation of JSAPl complexes with SEK1 and JNK (24, 25). To examine whether enhancement of ASK1-induced JNK3 activation by JSAPl may involve increased formation of JSAPl complexes with the downstream components, we investigated the effect of ASK1 on JSAPl binding to SEK1, MKK7, and JNK3. HA-SEK1, HA-MKK7, or HA-JNK3 was transiently cotransfected with Myc-JSAPl in HEK293 cells with or without wild-type (WT) and kinase-inactive (KM) forms of ASK1. In the absence of transfected ASK1, JSAPl was confirmed to associate with SEK1, MKK7, and JNK3 (Fig. 2, A, B, and C, lane 2) as previously reported (24, 25), but not with the components of the p38 signaling pathway (MKK3, MKK6, and p38; data not shown). Interestingly, these interactions were strongly increased by ASK1WT (Fig. 2, A, B, and C, lane 3), but not by the catalytically inactive mutant of ASK1 (ASK1KM; Fig. 2, A, B, and C, lane 4). In different sets of experiment, ASK1-dependent interactions were shown to be suppressed by the treatment of immunoprecipitates with protein phosphatase (Fig. 2, A, B, and C, lanes 5–7), suggesting that phosphorylation of the components within the signaling complex is required for enhanced interactions of JSAPl with SEK1, MKK7, and JNK3.

**Activation of MKK7 or JNK3 Does Not Increase the Affinity with JSAPl**—There were at least two possible explanations how ASK1 enhanced the interactions of JSAPl with the downstream components. First, the downstream kinases activated by ASK1 might have higher affinity to JSAPl; or second, JSAPl might be phosphorylated by the components of ASK1-JNK signaling module, and phosphorylated JSAPl might have higher affinity to the downstream components. To test the first
hypothesis, we compared the affinity of active and inactive mutants of MKK7 to JSAP1. MAPKKs require phosphorylation at conserved Ser/Thr residues in the activation loop of the kinase domain for activation. Substitution of these residues with charged or uncharged amino acids has been reported to generate constitutively active or inactive forms of MAPKK, respectively. Thus, we examined the bindings of JSAP1 with wild-type, constitutively active (MKK7EE) and unphosphorylatable (therefore unactivatable; MKK7AAA) forms of MKK7 by communoprecipitation analysis (Fig. 2D) (34). HA-tagged MKK7 mutants were transiently cotransfected into HEK293 cells with or without HA-ASK1 plus Myc-JSAP1, and cell lysates were immunoprecipitated with anti-Myc antibody. JSAP1 was found to associate to a similar extent with MKK7 mutants (Fig. 2D). These results suggest that the activation status of MKK7 does not affect its binding to JSAP1. We next examined whether phosphorylation and activation of JNK3 are required to interact with JSAP1. Unphosphorylatable (therefore unactivatable; JNK3APF) and kinase-inactive (JNK3KR) mutants and wild-type JNK3 were equally bound to JSAP1 in the presence of ASK1 (Fig. 2E, top panel), suggesting that neither activation nor phosphorylation state of JNK3 seemingly associates with its affinity to JSAP1. It was reported that prior activation of JNK3 in COS-7 cells by co-expression with MEKK1 strongly reduced the binding of JNK3 to recombinant JSAP1 in vitro (24). These observations appear to contradict our present study in that activation status of JNK3 does not affect its binding to JSAP1 in vivo. However, this may be partly explained by the different affinity of JSAP1 to JNK3 between in vitro and in vivo (see below).

Phosphorylation of JSAP1 by ASK1 in Vitro and in Vivo—To test the second hypothesis, we examined whether ASK1 phosphorylates JSAP1 (Fig. 3A). Myc-JSAP1 was cotransfected with or without ASK1WT and ASK1KM into HEK293 cells, and cells were metabolically labeled with [32P]orthophosphate. Expression of ASK1WT but not ASK1KM directly phosphorylated JSAP1 in HEK293 cells, suggesting that ASK1 phosphorylates JSAP1 in vitro. Although JSAP1 has been reported to be phosphorylated at Thr-234/266, Thr-244/276, and Thr-255/287 residues (numbers in JSAP1a and JIP3, respectively) by JNK (24, 25), phosphorylation of these sites was shown to be unrelated to the affinity between JSAP1 and JNK (24, 25). To examine whether ASK1 can directly phosphorylate JSAP1, we performed an in vitro phosphorylation analysis using recombinant JSAP1 protein. Bacterially expressed GST fusion protein of JSAP1 (GST-JSAP1) was clearly phosphorylated by GST-ASK1ΔN (a constitutively active form of ASK1), but not by GST-ASK1ΔN-KR (a kinase-inactive mutant) (Fig. 3B, left panels). GST-JSAP1 (1–744), which contains the known phosphorylation sites by JNK (25, 26), was bound to (Fig. 3C) and phosphorylated by JNK3 (Fig. 3D, lane 1) but not ASK1 (Fig. 3D, lane 3), whereas, GST-JSAP1 (744–1305) was clearly bound to (Fig. 3C) and phosphorylated by ASK1 in vitro (Fig. 3D, lane 6). These results indicated that ASK1 directly phosphorylates JSAP1 on sites different from those phosphorylated by JNK.

Requirement of ASK1 for Phosphorylation-dependent Scaffolding Activity of JSAP1—To examine whether ASK1 is required for the phosphorylation of JSAP1, ASK1+/+/ and ASK1−/− MEFS were metabolically labeled with [32P]orthophosphate. Treatment with H2O2 clearly phosphorylated JSAP1 in ASK1+/+ MEFS in a dose-dependent manner but only marginally in ASK1−/− MEFS (Fig. 3E). These results indicate that ASK1 is an essential component for H2O2-induced phosphorylation of JSAP1. We next assessed the role for ASK1-induced phosphorylation of JSAP1 in its scaffolding activity. We have recently shown that ASK1 is specifically required for sustained activation of JNK induced by H2O2 (6). Fig. 3F shows that H2O2-induced activation of JNK was maintained after 60 or 120 min in ASK1+/+ MEFS (Fig. 3F, lanes 3, 4) but not ASK1−/− MEFS (Fig. 3F, lanes 11, 12) as reported (6). When JSAP1 was expressed, sustained activation of endogenous JNK but not p38 (third panel from top) and ERK (fifth panel from top) was enhanced in ASK1+/− MEFS but not at all in ASK1−/− MEFS (Fig. 3F, lanes 7, 8, 15, and 16). Moreover, H2O2-induced interaction between JNK and JSAP1 was enhanced only in ASK1+/+ but not ASK1−/− MEFS (Fig. 3G). These results strongly suggest that upon phosphorylation by ASK1 JSAP1 specifically facilitates JNK activation by recruiting downstream components of the ASK1-JNK cascade in response to oxidative stress.

CONCLUSION

Here we represented a novel regulatory mechanism for JSAP1 scaffold. Phosphorylated JSAP1 by ASK1 appears to gain higher affinity to the downstream components. Our data suggest that activated ASK1 phosphorylates JSAP1 and thereby triggers the conformational change of JSAP1 from "inactive" to "active" configuration, which in turn induces the formation of a signaling complex composed of ASK1, JNK, SEK1/MKK7, and JNK3. JSAP1 thus functions not only as a simple scaffold but it dynamically participates in signal transduction by forming a stimulation-dependent functional complex in the ASK1-JNK module. Compared with a conventional idea in that necessary components are preset on the scaffold in order for efficient and specific signaling, this new mode of regulation of MAPK cascade appears to be advantageous for cells to prevent leaky signals generated under unstimulated conditions. Although we have not determined the phosphorylation site(s) of JSAP1 by ASK1, such a study will lead to our understanding of precise mechanism of phosphorylation-dependent recruitment of JNK's signaling module. In addition, targeted disruption of JSAP1 will enable evaluation of the role of JSAP1 in stress-induced ASK1-JNK signaling pathway. Finally, whether this novel mode of regulation of MAPK cascade is applied to other MAPK pathways will be an interesting issue to be determined.

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