A Scaffold Protein in the c-Jun NH₂-terminal Kinase Signaling Pathways Suppresses the Extracellular Signal-regulated Kinase Signaling Pathways*  

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We previously reported that c-Jun NH₂-terminal kinase (JNK)/stress-activated protein kinase-associated protein 1 (JSAP1) functions as a putative scaffold factor in the JNK mitogen-activated protein kinase (MAPK) cascades. In that study we also found MEK1 and Raf-1, which are involved in the extracellular signal-regulated kinase (ERK) MAPK cascades, bind to JSAP1. Here we have defined the regions of JSAP1 responsible for the interactions with MEK1 and Raf-1. Both of the binding regions were mapped to the COOH-terminal region (residues 1054–1305) of JSAP1. We next examined the effect of overexpressing JSAP1 on the activation of ERK by phorbol 12-myristate 13-acetate in transfected COS-7 cells and found that JSAP1 inhibits ERK's activation and that the COOH-terminal region of JSAP1 was required for the inhibition. Finally, we investigated the molecular mechanism of JSAP1's inhibitory function and showed that JSAP1 prevents MEK1 phosphorylation and activation by Raf-1, resulting in the suppression of the activation of ERK. Taken together, these results suggest that JSAP1 is involved both in the JNK cascades, as a scaffolding factor, and the ERK cascades, as a suppressor.

The MAP kinase (MAPK) signaling pathway is an intracellular cascade consisting of MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK). Upon stimulation, MAPKKK activates MAPKK by phosphorylation on serine (and/or threonine) residues, which in turn activates MAPK by phosphorylation on threonine and tyrosine residues (1, 2). A variety of MAPK cascades have been identified in organisms from yeast to mammals (3–5). In mammals, five groups of distinguishable MAPK cascades have been identified so far (6). They include the c-Jun NH₂-terminal kinase (JNK) (also known as stress-activated protein kinase (SAPK), p38, and extracellular signal-regulated kinase (ERK) cascades. The JNK and p38 groups of MAPKs are strongly activated in response to proinflammatory cytokines and environmental stresses (7–10). The ERK group of MAPKs is mostly responsive to mitogenic and differentiation stimuli (2, 8). For example, the small G protein Ras is activated by many growth factors, and in turn activates the Raf-MEK-ERK cascades (11).

The mammalian MAPK cascades play key roles in a wide array of cellular processes, including proliferation, differentiation, and apoptosis (7–11). Thus, mechanisms should exist to ensure specific and efficient activation of the MAPK cascades in response to extracellular stimuli. The recent identification of putative scaffold proteins in the MAPK cascades could account for one of the mechanisms (6, 12–15). We proposed previously that JNK/SAPK-associated protein 1 (JSAP1) (also known as JNK-interacting protein 3 (15)) functions as a scaffold factor in the JNK MAPK cascades (14). In the same study, we reported that JSAP1 binds Raf-1 MAPKKK and MEK1 MAPKK, which are involved in the ERK MAPK cascades. Here, we have mapped the MEK1- and Raf-1-binding regions in JSAP1 and examined the effect of JSAP1 on the activation of the ERK MAPK cascades. Furthermore, we have investigated the molecular mechanism underlying JSAP1's effects on the ERK MAPK cascades.

EXPERIMENTAL PROCEDURES

Plasmids—The full-length human Ha-Ras cDNA was obtained from Health Science Research Resources Bank, Osaka, Japan. The open reading frame (ORF) of Ha-Ras was amplified by polymerase chain reaction (PCR). The product was subcloned into BamHI/Xhol-digested pcDNA3-Myc to generate pcDNA3-Myc-Ha-Ras. For the expression of a constitutively active Myc-Ha-Ras, a glycine residue corresponding to codon 12 of Ha-Ras was replaced by a valine residue using overlapping PCR (14). The resulting plasmid was termed pcDNA3-Myc-Ha-Ras(V12). The COOH-terminal region (residues 316–648) of Raf-1 was amplified by PCR with pcDNA3-Flag-Raf-1 (14) as the template. The product was subcloned into EcoRI/Xhol-digested pcDNA3-Myc (14) to generate pcDNA3-Myc-Ha-Ras(C)-C. The COOH-terminal region (residues 1054–1305) of JSAP1 was amplified by PCR with pcDNA3-His-S-JSAP1 (14) as the template. The product was subcloned into NcoI/BamHI-digested pET32b (Novagen, Madison, WI) to generate an expression vector for thioredoxin (Trx)-histidine (His)-S-JSAP1 (residues 1054–1305). The resulting plasmid was termed pET32-JSAP1-C. The region encoding residues 115–504 of JSAP1 was amplified by PCR with pcDNA3-His-S-JSAP1 (14) as the template. The product was subcloned into NcoI/BamHI-digested pET32b (Novagen, Madison, WI) to generate pET32-JSAP1-C. The ORF of human MEK1 was amplified by PCR with pcDNA3-Flag-MEK1 (14) as the template. The product was subcloned into HindIII/NotI-digested pET32b to generate pET32-MEK1. To prepare a kinase-negative Trx-His-S-MEK1, a lysine residue corresponding to codon 97 of MEK1 was replaced by an arginine residue using overlapping PCR. The resulting plasmid was termed pET32-MEK1(R97). All the amplified sequences were verified by DNA sequencing. Other expression vectors used in this study were described previously (14).
Cell Culture and Transfection—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui, Tokyo) supplemented with 10% fetal calf serum in a 5% CO₂ atmosphere at 37 °C. COS-7 cells (1 × 10⁶) in a 35-mm dish were transfected with plasmid DNA using FuGene6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. P19 embryonal carcinoma (EC) cells were maintained in minimum essential medium-α (Life Technologies, Inc.) supplemented with 10% fetal calf serum in a 5% CO₂ atmosphere at 37 °C.

Antibodies—Anti-Flag M5 monoclonal antibody, anti-His polyclonal antibody, anti-c-Myc 9E10 monoclonal antibody, and anti-Raf-1 P5 monoclonal antibody were purchased from Sigma, Santa Cruz Biotechnology Inc. (Santa Cruz, CA), Roche Molecular Biochemicals, and Transduction Laboratories (Lexington, KY), respectively. Phospho-specific anti-MEK1/2 polyclonal antibody was obtained from New England Biolabs Inc (Beverly, MA). Trx-His-S-JSAP1 (residues 115–504) protein was expressed in Escherichia coli and purified with S-protein-agarose (Novagen) according to the manufacturer’s instruction. The protein was further purified by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and extracting the ~85 kDa fusion protein from the gel. The purified protein was injected into Khl JW rabbits to raise anti-JSAP1 antiserum.

Protein Binding and Kinase Assays—Protein binding assays in COS-7 cells in vitro and immune-complex kinase assays were performed as described previously (14, 16). P19 EC cells were lysed at 4 °C in buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 0.5% Nonidet P-40, 0.3 mM dithiothreitol) and precipitated with an anti-Raf-1 monoclonal antibody or anti-JSAP1 antiserum immobilized on Sepharose-coupled protein G (Life Technologies, Inc.). The precipitates were then analyzed by immunoblotting with an anti-JSAP1 antiserum or anti-Raf-1 monoclonal antibody, respectively. Myelin basic protein (MBP) purchased from Sigma was used as substrate to measure the kinase activity of ERK. In some experiments, COS-7 cells were treated with phorbol 12-myristate 13-acetate (PMA) (Sigma) for 10 min to activate ERK.

RESULTS

Mapping of MEK1- and Raf-1-binding Regions in JSAP1—To define the region of JSAP1 responsible for its interaction with MEK1, Flag-tagged full-length MEK1 was transiently coexpressed with His-S-tagged full-length or deletion mutants of JSAP1 in COS-7 cells. The His-S-JSAP1 proteins, His-S-JSAP1-FL (residues 1–1305), His-S-JSAP1-1C (residues 1–1053), or His-S-JSAP1-C (residues 1054–1305), were recovered from the cell extracts by affinity binding to S-protein-agarose, and the precipitates were examined for the presence of Flag-MEK1 by immunoblotting with an anti-Flag antibody (Fig. 1A). The full-length JSAP1 (JSAP1-FL) and JSAP1-C, which contains only the COOH-terminal region of JSAP1, but not JSAP1-1C, in which the COOH-terminal region of JSAP1 is deleted, interacted with MEK1, indicating that the MEK1-binding region of JSAP1 is located between amino acid residues 1054–1305 in JSAP1.

We also mapped the Raf-1-binding region in JSAP1 using similar JSAP1 constructs. Because we reported previously that the COOH-terminal half of Raf-1 (residues 316–648, Raf-C) is required for binding to JSAP1 (14), Raf-C was used for this experiment. Flag-Raf-C was copurified with His-S-JSAP1-FL (residues 1–1305) or His-S-JSAP1-C (residues 1054–1305), but not with His-S-JSAP1-1C (residues 1–1053) from transiently cotransfected COS-7 cells (Fig. 1B). These results indicate that the COOH-terminal region (residues 1054–1305) of JSAP1 is also responsible for the interaction with Raf-1.

Binding of MEK1 and Raf-1 to JSAP1 in Vitro—We next carried out in vitro binding assays between MEK1 and JSAP1 (Fig. 2, upper panel) and Raf-1 and JSAP1 (Fig. 2, lower panel). A Trx-His-S-JSAP1-C (residues 1054–1305) fusion protein was expressed in bacteria, purified using S-protein-agarose, and assayed for the ability to bind MEK1 and Raf-1. The agaroase-bound fusion protein was mixed with in vitro translated ³⁵S-labeled MEK1 or Raf-1, recovered, and analyzed by SDS-PAGE and autoradiography. The results indicate that JSAP1 binds Raf-1, but not MEK1 in vitro, suggesting that JSAP1 directly binds Raf-1 but binds MEK1 indirectly. However, we cannot rule out the possibility that the binding of MEK1 to JSAP1 is direct, because JSAP1 may adopt different conformations in vivo and in vitro.

Association of Endogenous JSAP1 and Raf-1 in Vivo—We

![Fig. 1. Mapping of the binding regions for MEK1 (A) and Raf-1 (B) in JSAP1 and schematic representation of the mapping data (C).](http://www.jbc.org/)
Effect of JSAP1 on Activation of ERK by PMA—The observations that JSAP1 binds Raf-1 and MEK1, which are MAPKK and MAPKK, respectively, in the ERK MAPK cascades, suggest the involvement of JSAP1 in the ERK cascades. To examine this possibility, we first analyzed the effect of overexpressing JSAP1 on the activation of ERK by PMA. Flag-ERK2 was transiently coexpressed with or without His-S-JSAP1-FL, His-S-JSAP1-ΔC, or His-S-JSAP1-C in COS-7 cells. After treating the cells with PMA (100 ng/ml) for 10 min, the tagged ERK2 was immunoprecipitated with an anti-Flag antibody, and the kinase activity was measured using MBP as the substrate (Fig. 4A). The activation of ERK2 was completely inhibited by the overexpression of JSAP1-FL and JSAP1-ΔC, both of which contain the MEK1- and Raf-1-binding region. In contrast, JSAP1-C, which lacks this region, showed only a marginal effect on the activation of ERK2. These results suggest that JSAP1 suppresses the activation of ERK by PMA through its interaction with Raf-1 and/or MEK1.

Effect of JSAP1 on Activation of ERK by Constitutively Active Raf-1—Because Raf-1 binds directly to JSAP1, and the COOH-terminal half of Raf-1, which contains the kinase domain, is involved in the binding, we investigated whether overexpression of JSAP1 inhibits the activation of ERK2 by truncated Raf-1 (Raf-C; residues 316–648), a constitutively active Raf-1.
MEK1 phosphorylation and activation by Raf-1. COS-7 cells were transiently transfected with 0.4 μg of pcDNA3-His-S-MEK1 alone or with 0.1 μg of pcDNA3-Myc-Raf-C in the absence or presence of 1 μg of pcDNA3-His-S-JSAP1-FL, pcDNA3-His-S-JSAP1-3C, or pcDNA3-His-S-JSAP1-C as indicated. Total DNA was kept at 1.5 μg per transfection with pcDNA3-His-S empty vector. After 34 h, cell lysates were precipitated with S-protein-agarose (S-PA) and analyzed by immunoblotting with a phospho-specific anti-MEK (P-MEK) antibody. The expression of Myc-Raf-C, His-S-MEK1, His-S-JSAP1-FL, His-S-JSAP1-3C, and His-S-JSAP1-C was confirmed by immunoblotting one-tenth of the cell lysates used in the experiment.

Inhibition of Kinase Activity of Ha-Ras-activated Raf-1 by JSAP1—We proceeded to confirm the above conclusion. Flag-tagged full-length Raf-1 was transiently coexpressed with or without Myc-tagged Ras(V12), a constitutively active Ha-Ras (18), in COS-7 cells. The tagged Raf-1 was immunoprecipitated from the cell extracts with an anti-Flag antibody and examined for its ability to phosphorylate a Trx-His-S fusion protein of MEK1(R97), a kinase-negative MEK1, in the presence or absence of Trx-His-S-JSAP1-C (residues 1054–1305) (Fig. 6). The Raf-1 kinase activity was inhibited by the JSAP1-C fusion protein in a dose-dependent manner.

DISCUSSION

In the present study, we found that JSAP1 prevents the activation of ERK by PMA or a constitutively active Raf-1 and that it does so by interfering with the phosphorylation and activation of MEK1 by Raf-1. Our and R. J. Davis’s groups previously reported that JSAP1 works as a putative scaffold protein in the JNK cascades (14, 15). Therefore, JSAP1 might be involved in determining to what extent the ERK and JNK cascades can be activated. A higher level of JSAP1 would lower the activation of the ERK cascades, and in contrast, would facilitate the activation of the JNK cascades.

Recently, Yeung et al. (19) reported that the Raf kinase inhibitor protein (RKIP) interferes with MEK1 phosphorylation and activation by Raf-1. Moreover, they showed that RKIP dissociates a Raf-1-MEK1 complex and behaves as a competitive inhibitor of MEK1 phosphorylation (19). JSAP1 also exerted its inhibitory effects at the Raf-1-MEK1 interface, and both the MEK1- and Raf-1-binding regions were mapped to the COOH-terminal region (residues 1054–1305) of JSAP1. JSAP1, therefore, may inhibit Raf-1 in a similar way to RKIP. It is also possible that JSAP1 simultaneously binds MEK1 and Raf-1. If this were the case, JSAP1 might keep Raf-1 separate from MEK1 by forming a complex that prevented MEK1 phosphorylation and activation by Raf-1. Further study will be required to clarify this issue.

The rat pheochromocytoma cell line PC12 serves as a useful model of neuronal differentiation. Sustained, but not transient, activation of ERK leads to differentiation of PC12 cells (11). Interestingly, the expression level of JSAP1 increased during differentiation induced by nerve growth factor2 (15). At present it is not clear whether or how JSAP1 is involved in the process of differentiation. After the commitment of PC12 cells to differentiation, the cells would not require prolonged and high levels of ERK kinase activity. JSAP1’s inhibition of the ERK cascades might be required for the post-commitment processes. In addition, JSAP1 expression might be up-regulated by ERK, which in turn would lead to the suppression of the activation of ERK.

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