JSAP1/JIP3 Cooperates with Focal Adhesion Kinase to Regulate c-Jun N-terminal Kinase and Cell Migration

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Cell adhesion to the extracellular matrix regulates many cellular functions, including cell differentiation, proliferation, apoptosis, and migration (1). Integrin stimulation by extracellular matrix proteins such as FN2 leads to activation of MAPKs, including JNK and extracellular signal-regulated kinase (ERK) in a variety of cell types. Among the types of MAPK signaling transmitted by integrins, JNK activation is believed to correlate particularly with increased cell migration and invasion (2, 3). FN stimulation of cells is known to activate JNK through an FAK/MEK4, 5, and MAPK kinase kinase1 (MEKK1)-deficient mice are impaired in cell migration (3, 11–13). Recent studies have demonstrated that JNK regulates cell migration by phosphorylating paxillin and regulating microtubule assembly by phosphorylating microtubule-associated protein 2 (14–16). However, the mechanism by which JNK is recruited and activated in association with focal adhesions is largely unknown.

JIP family members (JIP1, -2, and -3) were originally identified as JNK-binding proteins and are thought to serve as scaffold factors for MAPK signaling cascades (17–19). JSAP1 binds not only to MAPK pathway constituents, including all JNK isoforms, MAPK kinases (MKK) 1, 4, and 7, MEKK1, mixed-lineage protein kinase 3, and c-Raf-1, but also to the motor protein kinesin (17–19). We found previously that JSAP1 interacts with FAK (20). This association was enhanced by c-Src, and it promoted cell spreading on FN. FAK is known to be essential for cell migration and for transmitting integrin-stimulated signals to MAPK (1, 6). FAK activated by cell adhesion to extracellular matrix undergoes autophosphorylation at Tyr-397 and thereby associates with Src family kinases, leading to enhancement of its tyrosine phosphorylation and kinase activity. Binding of FAK to c-Src also induces the formation of a multimolecular signaling complex in which FAK functions as a scaffold (21). Notably, JNK activation and FAK autophosphorylation at Tyr-397 are both attenuated in JSAP1 knock-out mice (22), suggesting that JSAP1 might be involved in FAK-mediated JNK activation. Although many studies have suggested critical roles for JNK signaling in tumor cell migration and invasion, the role of JNK scaffold proteins in tumor malignancy is not well examined. Here, we provide evidence that the JSAP1-FAK complex functions as an effective scaffold for the JNK pathway and particularly for stimulating cell migration, and that JSAP1 mRNA is elevated in brain tumors. These results suggest that JSAP1 may also participate in the acquisition of malignancy in brain tumors.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The human astrocytoma cell line U87MG (American Type Culture Collection) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. Transient transfections were performed by a calcium phosphate method or by using Trans IT (PanVera). Reagents used were FN (Asahi Techno Glass), SP600125 (Alexis Biochemicals), poly-I-lysine (Sigma), and rhodamine-phalloidin...
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(Commercial Probes). Immunological reagents used were anti-myc, antiphosphotyrosine, anti-paxillin, anti-FAK, and anti-p130Cas antibodies (BD Biosciences; anti-FAK[pY^397] antibody (BIOSOURCE); anti-myc antibody (Cell Signaling); anti-active JNK antibody (Promega); anti-FAK, anti-His, anti-glutathione S-transferase (GST), and anti-JNK1 antibodies (Santa Cruz Biotechnology); anti-a-tubulin, anti-VSV, anti-FLAG M2 antibodies (Sigma); an anti-Src antibody (Upstate); horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies (Amersham Biosciences); and AlexaFluor 488, 546, and 563 anti-mouse and anti-rabbit IgG antibodies (Molecular Probes); and an anti-JSAP1 rabbit antisera (from Dr. M. Ito, Kitasato University, Japan).

Expression Plasmids—pcDNA-myc-JSAP1, pcDNA-FLAG-JSAP1, pcDNA-His-S-JSAP1, pcDNA-FLAG-JNK1, pRK-green fluorescent protein (GFP), pRK-FLAG-FAK, pRK-VSV-FAK, and pSG-c-Src were constructed as described previously (20, 23, 24). A cDNA encoding JIP1 (GenBankTM accession number NM_005456) was obtained by reverse transcription-PCR with human placenta cDNA and was cloned into the pEAK-FLAG expression vector (pEAK-FLAG-JIP1). The expression plasmids for p130Cas (pSSR-p130Cas) and its deletion mutants (deleted SH3 domain, ΔSH3; substrate domain, ΔSD; Src binding domain, ΔSB) (25) were kind gifts from Dr. H. Hirai (Tokyo University, Japan), and the FLAG-epitope was inserted at their N terminus by site-directed mutagenesis. The pHA246pur puromycin-resistance plasmid was kindly provided by Hein te Riele (Netherlands Cancer Institute, Amsterdam, The Netherlands).

Immunoprecipitation Analyses—At 36 h after transfection, cells were washed twice with ice-cold phosphate-buffered saline and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 2 mM NaF, and 1% Nonidet P-40. In experiments to analyze the association between JSAP1 and FAK, the lysis buffer contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 2 mM NaF, 1% Triton X-100, and 10% glycerol. Lysates were used for precipitation with the indicated antibodies or S-protein-agarose (Nova-SAP, Library, Tokyo, Japan). The classification of human brain tumors used in this study was based on the revised WHO criteria for tumors of the central nervous system (27).

Measurement of FAK Activity—FAK activity was measured using the SAPK/JNK assay kit from Cell Signaling. Briefly, the cell lysates were incubated with GST-c-Jun (amino acid residues 1–89), and precipitates were incubated in the presence of ATP for 30 min at 30 °C. Phosphorylation of c-Jun on Ser-63 by JNK bound to GST-c-Jun was detected by immunoblotting using anti-phospho-c-Jun (Ser 63) antibody.

Embryonic Stem Cell Culture—The murine ES cell line E14K (gift of Dr. Hiroshi Nishina, Tokyo Medical and Dental University) was maintained as described previously (26) with the minor modification that KnockOut Serum Replacement (Invitrogen) was used instead of fetal calf serum. The generation of Jsp1(−/−) ES cell lines will be described elsewhere.

Clinical Samples and Histology—Under an institutional review board-approved protocol, fresh human brain tumor tissues were obtained from 26 patients with astrocytic tumors who underwent therapeutic removal of brain tumors. Normal brain tissues were obtained from three patients undergoing temporal lobectomy for epilepsy. Histological diagnosis was made by standard light-microscopic evaluation. The classification of human brain tumors used in this study was based on the revised WHO criteria for tumors of the central nervous system (27). The 26 astrocytic tumors consisted of 7 low-grade astrocytomas, 8 anaplastic astrocytomas, and 11 glioblastomas. All of the tumor tissues were obtained at primary resection, and none of the patients had been subjected to chemotherapy or radiation therapy before resection.

Quantitative reverse transcription-PCR—Real-time quantitative PCR was performed using a LightCycler (Roche Diagnostics) with SYBR green fluorescence signal detection after each cycle of amplification as described previously (28). Briefly, total RNA was isolated from human brain or brain tumor tissues. PCR was performed using the following primers: JIP1, sense (5′-TCTCTGCTATTACGCACTC-3′) and antisense (5′-CACCACCGACGGTGAT-3′) (amplon size, 238 bp); JIP2, sense (5′-CCTCAGGAGCATCATC-3′), and antisense (5′-GGGACCCAGGGAGGAGTT-3′) (amplon size, 188 bp); JSAP1, sense (5′-TGAAGACACCGAAAGAC-3′) and antisense (5′-GGAATGCTGAGCTGAAAC-3′) (amplon size, 245 bp); and histone H3.3, sense (5′-CCCTCTGATGGATGCT-3′), and antisense (5′-GCTGTTGCTAGCTGAAAC-3′) (amplon size, 215 bp) (GenBankTM accession numbers AF074091, AF136382, and NM_002107 for JIP1, JIP2, and histone H3.3, respectively). The PCR data were analyzed with LightCycler analysis software as previously described (28). Quantification was based on the number of cycles necessary to produce a detectable amount of product above background. The difference in the cycle number (d) was normalized to the housekeeping gene histone H3.3 and then used to calculate the fold-difference in copy number according to the formula f = 2^d, where f = -fold difference in specific gene expression and d = cycle number difference between compared sources of mRNA (corrected for differences in histone H3.3).

RESULTS

JSAP1 Forms a Scaffold for JNK with FAK—JSAP1 has been identified as a scaffold factor for JNK and we previously reported that JSAP1 also associates with FAK (17, 20). To examine whether formation of the JSAP1 and FAK complex affects JNK signaling, e.g. as a scaffold factor,
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**FIGURE 1.** JSAP1 forms a scaffold for JNK with FAK. A, deletion mutants of JSAP1 (ΔJBD and Δ2) are shown schematically. A His tag and an S tag were inserted at the N terminus of JSAP1 (1305 amino acids). B, 293T cells were co-transfected with 0.5 μg of His-S-ΔJBD, FLAG-JNK1, FAK, c-Src plasmids as indicated. At 36 h after transfection, the cells were lysed, and His-S-ΔJBD and co-precipitated materials isolated using S-protein agarose (Pull down: S-protein agarose) were analyzed by immunoblotting with anti-phosphotyrosine (Blot: pTyr), anti-FLAG (Blot: FLAG), anti-active JNK (Blot: pJNK), and anti-His (Blot: His) antibodies. The whole cell lysates (WCL) were immunoblotted with anti-FLAG (Blot: FLAG) and anti-c-Src (Blot: Src) antibodies. C, 293T cells were transfected as indicated. At 36 h after transfection, the cells were lysed, and immunoprecipitates with anti-FAK antibody (IP: FAK) were immunoblotted with HRP-conjugated anti-FLAG (Blot: FLAG), anti-His (Blot: His), and anti-VSV (Blot: VSV) antibodies. Alternatively, the immunoprecipitates with anti-FAK antibody (IP: FAK) were immunoblotted with anti-VSV (Blot: VSV) or anti-FLAG (Blot: FLAG) antibodies. The WCL were immunoblotted with anti-c-Src (Blot: Src) antibody. D, 293T cells were transfected as indicated. At 48 h after transfection, the cells were trypsinized, suspended for 30 min (Sus) in complete medium, and then replated onto FN for 2 h (FN). The cells were lysed, and proteins co-precipitating with S-tagged JSAP1 in pull-down assays with S-protein-agarose (Pull down: S-protein agarose) were analyzed by immunoblotting with anti-phosphotyrosine (Blot: pTyr), and anti-His (Blot: His) antibodies. The WCL were immunoblotted with anti-FLAG (Blot: FLAG) antibody. E, homogenate of brain from 12-week-old wild-type mouse was immunoprecipitated with control rabbit IgG or anti-FAK rabbit antibody. The precipitates were analyzed by immunoblotting with anti-JSAP1 (Blot: JSAP1), anti-JNK1 (Blot: JNK1), and anti-FAK (Blot: FAK), and anti-rabbit IgG antibodies. 

JSAP1-WT or a mutant lacking the JNK-binding domain of JSAP1 (ΔJBD) was co-expressed with FAK, c-Src, and JNK1 in 293T cells, and their interaction was analyzed by immunoprecipitation. Co-expression of FAK/c-Src significantly stimulated complex formation between JSAP1 and JNK1, and the JNK1 in the complex was phosphorylated (Fig. 1B). Although both JSAP1-WT and JSAP1-ΔJBD were tyrosine-phosphorylated and co-precipitated with FAK, only JSAP1-WT but not JSAP1-ΔJBD was co-precipitated with JNK1. To confirm that both FAK and JNK1 reside on the same JSAP1 scaffold, immunoprecipitation with an antibody against FAK was performed. JNK1 was co-precipitated with FAK only in cells co-expressing JSAP1-WT but not JSAP1-ΔJBD, whereas both JSAP1-WT and JSAP1-ΔJBD were co-precipitated with FAK (Fig. 1C). FAK was co-precipitated with JNK1 only in the cells co-expressing JSAP1-WT but not JSAP1-ΔJBD. The tyrosine phosphorylation of JSAP1-WT and JSAP1-ΔJBD was equally augmented by adhesion of cells to FN in place of FAK/c-Src expression, and only JSAP1-WT but not JSAP1-ΔJBD associated with JNK1 (Fig. 1D). Moreover, immunoprecipitation analysis using lysates from mouse brain tissue demonstrated the existence of endogenous JSAP1-FAK-JNK complex (Fig. 1E). These results suggest that JSAP1 forms a scaffold for the JNK pathway with FAK following formation of a FAK-Src complex, and thus the association of JSAP1 with JNK is facilitated under conditions that induce FAK-Src complex formation such as FN stimulation.

**JSAP1 Elevates FAK Activity and p130Cas Phosphorylation**—FAK is a critical regulator of FN-induced JNK activation and cell migration. FAK activity is negatively regulated by its N-terminal FERM domain (29, 30), which binds to JSAP1. We next investigated whether JSAP1 affects FAK activity. In vitro kinase assays showed that expression of JSAP1-WT or JSAP1-ΔJBD increased FAK activity by 3-fold (Fig. 2A). In contrast, JSAP1-Δ2, which fails to bind to FAK, had no effect. FAK activity was elevated 10-fold by co-expression of c-Src under the same conditions, indicating that activation by JSAP1 is substantial but not maximal. 

p130Cas is phosphorylated by a FAK-Src complex and mediates FAK-induced JNK activation by forming a complex with CrkII (3). The effects of JSAP1 expression on p130Cas phosphorylation and FAK activity were studied using deletion mutants of p130Cas (Fig. 2, B and C). p130Cas is phosphorylated by FAK-Src complex and mediates FAK-induced JNK activation by forming a complex with CrkII (3). The effects of JSAP1 expression on p130Cas phosphorylation and FAK activity were studied using deletion mutants of p130Cas (Fig. 2, B and C).
expressed by transfection of the gene was not tyrosine-phosphorylated, and co-expression of FAK slightly induced its phosphorylation. Although expression of JSAP1 alone did not affect p130Cas phosphorylation, co-expression of JSAP1 with FAK strongly stimulated it. This JSAP1-FAK-enhanced phosphorylation of p130Cas was not observed with the mutants p130CasΔSH3 or ΔSB, but p130CasΔSD was slightly phosphorylated under the same conditions. Because FAK is known to interact with the SH3 domain of p130Cas through its C-terminal proline-rich region and to phosphorylate the Src-binding domain of p130Cas, these results suggest that JSAP1 stimulates phosphorylation of p130Cas by Src at the substrate domain followed by phosphorylation of its Src-binding domain by FAK. In vitro kinase assays showed that FAK activity was slightly elevated by p130Cas expression, which was stimulated by co-expression of JSAP1. In contrast, p130Cas mutants had no effect. JSAP1 phosphorylation was also induced by co-expression of FAK with p130Cas. This is supported by the results presented in Fig. 2D showing that phosphorylation of both p130Cas and JSAP1 induced by co-expression of FAK was reduced by Src inhibitor PP2 treatment. These results suggest that complex formation of FAK with JSAP1 and p130Cas promotes p130Cas phosphorylation and FAK activation through Src.

JSAP1 but Not JIP1 Enhances JNK Activation—Although JSAP1 is a member of the JIP family, the primary structure of JSAP1 is different from the others (18). To compare JSAP1 with JIP1 in terms of association with FAK, JSAP1 or JIP1 tagged with FLAG epitope was co-expressed with FAK and c-Src, and immunoprecipitation analysis was carried out. As shown in Fig. 3A, only JSAP1 but not JIP1 co-precipitated FAK. JNK activation was faintly induced by cultivation of cells on FN or by JSAP1 expression when cultured on PLL (Fig. 3B). In contrast, consistent with its binding to FAK, the expression of JSAP1 but not JIP1 significantly enhanced the JNK activating signal from FN (Fig. 3B). To confirm the role of JSAP1 in JNK activation, U87MG cells co-transfected with either JSAP1-WT or JSAP1-ΔJBD and JNK1 were cultured on FN, and JNK phosphorylation was examined (Fig. 3C). JSAP1-WT expression augmented JNK phosphorylation in a dose-dependent manner, whereas JSAP1-ΔJBD expression did not affect it. As shown in Fig. 3D, the JNK phosphorylation enhanced by JSAP1 was suppressed by the Src inhibitor and a dominant negative form of p130Cas (ΔSD). These results suggest that JSAP1 expression promotes FN-induced JNK activation by facilitating the FAK/p130Cas pathway.

Because FAK, p130Cas, Crkl, and Rac1 are known to accumulate at the leading edge in migrating cells (31, 32), we next examined the localization of JSAP1 in U87MG cells cultured on FN. Immunofluorescence staining demonstrated the extension of tubulin-containing microtubules toward the cell periphery (Fig. 3E) and the well organized distribution of paxillin (Fig. 3F) and autophosphorylated FAK (Fig. 3G) at the
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Figure 3. JSAP1 but not JIP1 enhances basal JNK activation. A, 293T cells were co-transfected with 1 μg of FLAG-JSAP1 or 0.3 μg of FLAG-JIP1 and 0.5 μg of VSV-FAK and c-Src plasmids as indicated. At 36 h after transfection, the cells were lysed, and the cell lysates were immunoprecipitated with anti-FLAG antibody (IP: FLAG), and analyzed by immunoblotting with anti-VSV (Blot: VSV) and anti-FLAG (Blot: FLAG) antibodies. The WCL were immunoblotted with anti-VSV (Blot: VSV) and anti-Src antibodies. B, U87MG cells were seeded on either poly-lysine (PLL) or FN-coated dishes, cultured overnight, and co-transfected with 1 μg of FLAG-JSAP1 or 0.3 μg of FLAG-JIP1 and 0.3 μg of FLAG-JNK1 plasmids as indicated. The cells were cultured in complete medium for 36 h after transfection, and then lysed. The lysates were immunoprecipitated with anti-FLAG antibody (IP: FLAG). The precipitates were analyzed by immunoblotting with anti-active JNK (Blot: pJNK) and anti-JNK1 (Blot: pJNK) antibodies. The WCL were immunoblotted with anti-FLAG (Blot: FLAG) antibody. C, U87MG cells cultured overnight on FN-coated dishes were co-transfected with 0.2 or 1 μg of His-S-JSAP1 or -ΔJBD and 0.3 μg of FLAG-JNK1 plasmids as indicated. The cells were cultured in complete medium for 36 h after transfection, and then lysates were immunoprecipitated with anti-FLAG antibody (IP: FLAG). The precipitates were analyzed by immunoblotting with anti-active JNK (Blot: pJNK) and HRP-conjugated anti-FLAG (Blot: FLAG) antibodies. The WCL were immunoblotted with anti-His antibody (Blot: His). D, U87MG cells cultured overnight on FN-coated dishes were co-transfected with either 1 μg of His-S-JSAP1, 0.3 μg of FLAG-JNK1, or 2 μg of p130Cas-35D, plasmids as indicated. At 36 h after transfection, the cells were treated with 10 μM, or Me2SO (vehicle) for 1 h. The cells were lysed, and immunoprecipitates with anti-FLAG antibody (IP: FLAG) were analyzed by immunoblotting with anti-active JNK (Blot: pJNK) and HRP-conjugated anti-FLAG (Blot: FLAG) antibodies. The WCL were immunoblotted with anti-p130Cas-35D (Blot: p130Cas) and anti-His antibodies (Blot: His). E–G, U87MG cells transfected with myc-JSAP1 plasmids were replated onto FN-coated cover slipped for 6 h. The cells were fixed, permeabilized, and double-stained with anti-myc and anti-α-tubulin (E), anti-paxillin (F), or anti-FAK(pY397) (G) antibodies. Bar, 20 μm.

leading front of migrating U87MG cells. It has been reported that JSAP1 is transported to the tips of neurites through its interaction with the tetratricopeptide repeat domain of kinesin light chains (24, 33). We observed that JSAP1 is distributed diffusely in the cytoplasm but is also concentrated at the cell periphery where paxillin is well organized and microtubules have elongated. These results indicate that JSAP1 functions together with FAK scaffold at the leading edge.

JSAP1-deficient ES Cells Are Impaired in Lamellipodial Protrusion—ES cells provide a highly informative in vitro cell system to study the phenotypic effects of gene disruption at the cellular level. Jsap1−/− murine ES cell lines have been described elsewhere (Fig. 4A). Attachment of wild-type Jsap1+/+ ES cells to FN induced lamellipodial protrusion and membrane ruffling (Fig. 4, B and C). In contrast, Jsap1-null (Jsap1−/−) ES cells displayed a substantial decrease of the rate of lamellipodial protrusion and membrane ruffling (Fig. 4, B and D). Rac1 and p130Cas/Crk has been shown to induce lamellipodial protrusion and membrane ruffle formation associated with cell migration (5, 38). These observations suggest that JSAP1 is required for FN-induced lamellipodial protrusion and membrane ruffling through the p130Cas/Crk/Rac1 pathway, which led us to test the hypothesis that JSAP1 contributes to cell motility.

JSAP1 Expression Promotes Cell Migration—To explore whether JSAP1 is involved in cell migration, the distribution of JSAP1 and JNK1 in wound-edge cells was monitored. As shown in Fig. 5A, JNK1 was barely localized at the leading edges of cells at the edge of in vitro wounds in cell monolayers expressing JNK1 alone. In contrast, co-expression of JSAP1 with JNK1 induced accumulation of JNK1 at the leading edge, suggesting the recruitment of JNK1 to the leading edge where JSAP1 and FAK also accumulate. Next, we examined the effect of JSAP1 expression on cell migration induced by FN and serum. Express-
sion of JSAP1-WT but not JSAP1-DJBD enhanced U87MG migration by 18% compared with control cells \((p < 0.01, \text{Fig. 5B})\). The cell migration of mock and JSAP-WT-transfected cells was reduced to 44 and 42% of control cells by treatment with the JNK inhibitor SP600125. In parallel with suppression of cell migration, SP600125 treatment resulted in the loss of motile cell morphology with an increase in the number and size of focal adhesions and less-oriented actin stress fiber formation \((\text{Fig. 5C})\) and the suppression of JNK activity \((\text{Fig. 5D})\). These results indicate that activation of JNK by JSAP1 can contribute to cell migration.

**DISCUSSION**

JNK is activated in response to a variety of extracellular and intracellular stimuli, and it plays crucial roles in cellular processes that include cell proliferation, differentiation, apoptosis, and migration. However, the mechanisms determining specificity and efficiency of JNK activation remain to be clarified. In the present study, we have tested for functional cooperation between JSAP1 and FAK. Our novel findings are as follows: 1) the function of JSAP1 as a JNK scaffold is augmented by its binding to FAK; 2) JSAP1 and p130Cas function cooperatively to transmit signaling to JNK and to regulate FAK activity; 3) Jsap1-null ES cells are impaired in FN-induced lamellipodial protrusion formation and membrane ruffling; 4) JSAP1 expression stimulates FN-induced cell migration, and this stimulation depends on the ability of JSAP1 to promote JNK activation; and 5) JSAP1 mRNA expression correlates with the malignant phenotype of brain tumors. These studies define a signaling pathway that stimulates FN-induced JNK activation and cell migra-
tion that depends on mutual regulation and functional cooperation between JSAP1 and FAK.

FAK autophosphorylation at Tyr-397 following adhesion depends on its association with Src family kinases, leading to the formation of multimolecular signaling complexes in which FAK serves as a scaffold (1, 6, 34). FAK scaffolding is thought to function in integrin-mediated signal transduction (1, 6). Among various signaling pathways, the FAK/p130Cas pathway is believed to be essential for FN-induced JNK activation and cell migration (2, 4, 5, 7–9, 15), even though the precise mechanism by which JNK pathway constituents associate with the FAK scaffold is largely unknown. We have previously shown that the association of JSAP1 with FAK is enhanced by binding of c-Src to FAK, and adhesion of cells to FN induces FAK/c-Src/JSAP1 complex formation (20). Biochemical and gene disruption studies indicate that JSAP1 functions as a scaffold for JNK signaling (17–19, 35). In the present study, we showed that the association of JSAP1 with JNK and subsequent JNK phosphorylation were enhanced by either FAK/c-Src expression or attachment of cells to FN. In contrast, JIP1, which associates with JNK-pathway components but not with FAK, and JSAP1ΔJBD, which associates with FAK but not with JNK, both failed to augment FN-induced JNK activation. These results indicate that JSAP1 but not JIP1 serves as a specific scaffold for FN-induced JNK activation, supporting the importance of the association with FAK for this JIP protein to function as a scaffold.

Phosphorylation of p130Cas is initiated by FAK, followed by interaction of the SH3 domain of p130Cas with the C-terminal proline-rich region of FAK and hyperphosphorylation by Src. Hyperphosphorylated p130Cas is critical for FAK-mediated JNK activation by binding to CrkII (25). We find that complex formation between JSAP1 and FAK induces activation of FAK and phosphorylation of both JSAP1 and p130Cas; this process requires p130Cas hyperphosphorylation and is abolished by inhibition of Src (Fig. 2). FAK activity is known to be negatively regulated by its N-terminal FERM domain (29, 30), which binds to JSAP1, and thus binding of JSAP1 to the N terminus of FAK might abrogate the

FIGURE 5. JSAP1 expression promotes cell migration via JNK. A, U87MG cells were transfected with FLAG-JNK1 alone or together with myc-JSAP1 plasmids, and replated onto FN-coated coverslips for 6 h. The cell layer was wounded by scraping, and 4 h after wounding, the cells were triple-stained with rhodamine-phalloidin (F-actin), anti-FLAG, and anti-myc antibodies. Bar, 20 μm. B, U87MG cells were co-transfected with His-S-JSAP1 or ΔJBD and GFP plus plha262pur plasmids, and transfectants were selected by puromycin for 48 h as described under “Experimental Procedures.” The puromycin-selected cells were pretreated with either SP600125 or Me2SO (vehicle) for 1 h. Cell movements were monitored for 4 h by time-lapse video microscopy. Error bars indicate ±S.D. for at least 60 cells per condition (*, p < 0.01; **, p < 0.001 versus control). C, U87MG cells were co-transfected with His-S-JSAP1 and plha262pur plasmids, and transfectants were selected by puromycin. The selected cells were replated onto FN-coated coverslips with either SP600125 or Me2SO carrier for 4 h. The cells were double-stained with rhodamine-phalloidin (F-actin) and anti-paxillin antibodies. Bar, 20 μm. D, U87MG cells expressing FLAG-JSAP1 were cultured on FN for 12 h, and then were incubated with or without SP600125 for 1 h. JNK activity was measured as described under “Experimental Procedures.” The precipitates with GST-c-Jun (Pull down: GST-c-Jun) were incubated in the presence of ATP for 30 min at 30 °C, and then were analyzed by immunoblotting with anti-phospho-c-Jun (Ser 63) (Blot: pc-Jun) and anti-GST (Blot: GST) antibodies. The WCL were analyzed by immunoblotting with anti-FLAG (Blot: FLAG) and anti-tubulin (Blot: Tubulin) antibodies.
autoinhibitory interaction of FAK. Our demonstration that FAK activity is significantly elevated by co-expression of JSAP1 with p130Cas and is reduced by Src inhibition suggests that release from autoinhibition by JSAP1 cooperatively enhances FAK activity with the p130Cas/CrkII complex. This interpretation is supported by the fact that FAK autophosphorylation and JNK activation are both attenuated in JSAP1-deficient mice (22) and that complex formation of p130Cas with CrkII followed by integrin engagement promotes FAK autophosphorylation, probably through Src family kinases (36). The stimulation of FN-induced JNK activation by JSAP1 was suppressed by expression of p130Cas-ΔSD and by inhibiting Src, indicating essential roles for p130Cas and Src (Fig. 3). Thus, the cooperation between JSAP1 and p130Cas not only functions to mediate FAK-mediated JNK activation, but it may also affect the activation of FAK as an upstream regulator. We speculate that JSAP1 plays an important role in the spatial regulation of JNK pathway players together with the FAK scaffold in response to FN stimulation.

JSAP1 is transported to the tips of neurites along microtubules (24, 33), which are known to associate with JNK, FAK, MEKK1, paxillin, and JSAP1, and to regulate cell migration (37–39). Our observations showing that JSAP1 co-localizes with autophosphorylated FAK and JNK at the leading edge (Fig. 5) suggests that a JSAP1-FAK scaffold may cooperatively enhance FAK and JNK activation at the leading edge. In fact, JSAP1 expression promoted FN-induced cell migration, which depended on its JNK binding domain and was suppressed by a JNK inhibitor accompanied by an increase in the number and size of focal adhesions and less-oriented actin stress fiber formation (Fig. 5). FAK, p130Cas, CrkII, and Rac1 accumulate at the leading edge in migrating cells (31, 32). Translocation of JSAP1 and FAK to the leading edge may place them at an appropriate site for facilitating FN-induced JNK activation and subsequent cell migration (Fig. 7).

JIP1-deficient mice are developmentally normal and viable (40, 41), whereas JSAP1 null mice exhibit severe developmental defects (22, 33). JSAP1-deficient mice show various developmental deficits in the brain, including axon guidance defects of the corpus callosum, suggesting importance of JSAP1 in mice development. The present study has shown that JSAP1 but not JIP1 associates with FAK and facilitates JNK activation and cell migration and that jasp1-null ES cells are impaired in the formation of lamellipodial protrusions and membrane ruffling.

![FIGURE 6. mRNA expression of JIP family members in astrocytic tumors.](image URL) Quantitative reverse transcription-PCR for mRNA expression of JIP1 (A), JIP2 (B), and JSAP1 (C) in clinical samples was performed as described under "Experimental Procedures." NB, normal brain; LGA, low grade astrocytomas; AA, anaplastic astrocytomas; GB, glioblastomas. *, p < 0.05; **, p < 0.01.
JSAP1 is different from other JIP family members in its primary structure (18), consistent with a distinct role from that of JIP1. Transgenic expression of JIP1 in JSAP1-deficient mice only partially rescues the Jasp1 deficiency-induced developmental defects in parallel with partial restoration of JNK activation (22). From this study, an explanation for these findings is that even though JIP1 may partially compensate for JSAP1 by scaffolding JNK pathway components, the processes of JNK activation, FAK autophosphorylation, and cell migration induced by integrin-mediated signaling are not restored by JIP1 expression. The cooperative spatial regulation of JNK pathway components by JSAP1 and FAK may thus play critical roles in specific cellular and developmental processes.

Increased FAK expression and tyrosine phosphorylation are observed in many malignant tumors (42). We report here that the mRNA expression of JSAP1 is elevated, but expression of JIP1 and JIP2 is diminished in malignant brain tumors (Fig. 6), suggesting that JSAP1 may serve predominantly as a JNK scaffold in advanced brain tumors. Intriguingly, the expression of RhoA and its effectors, which regulate focal adhesion turnover, microtubule and actin stress fiber formation, and cell migration, as well as affecting FAK and JNK activation, are reduced in JSAP1-deficient mice (33). Taken together with our result that JSAP1 facilitates JNK activation and cell migration together with FAK, it is possible that JSAP1 regulates integrin-mediated JNK activation and cell migration by controlling both the FAK and Rho pathways and that JSAP1 may thereby contribute to the acquisition of malignancy in brain tumors.

The data presented here provide the first evidence that JSAP1 serves as a scaffold for the efficient activation of JNK in response to FN stimulation, which is required to promote FN-induced cell migration and depends on mutual regulation and functional cooperation between JSAP1 and FAK. We conclude that cooperation between JSAP1 and FAK can assemble the elements of signaling modules that can include focal adhesion components, JNK pathway players, and microtubules, and that it can modulate FAK-mediated cell migration by regulating JNK activation.

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