Regulation of Platelet-derived Growth Factor Receptor Activation by Afadin through SHP-2

IMPPLICATIONS FOR CELLULAR MORPHOLOGY*

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Upon binding of platelet-derived growth factor (PDGF), PDGF receptor is autophosphorylated at tyrosine residues in its cytoplasmic region, which induces the activation of diverse intracellular signaling pathways such as those involving Ras-ERK, c-Src, and Rap1-Rac. Signaling through activated Ras-ERK promotes cell cycle and cell proliferation. The sequential activation of Rap1 and Rac affects cellular morphology and induces the formation of leading-edge structures, including lamellipodia, peripheral ruffles, and focal complexes, resulting in the enhancement of cell movement. In addition to the promotion of cell proliferation, the Ras-ERK signaling is involved in the regulation of cellular morphology. Here, we showed a novel role of afadin in the regulation of PDGF-induced intracellular signaling and cellular morphology in NIH3T3 cells. Afadin was originally identified as an actin filament-binding protein, which binds to a cell-cell adhesion molecule nectin and is involved in the formation of cell-cell junctions. When afadin was tyrosine-phosphorylated by c-Src activated in response to PDGF, afadin physically interacted with and increased the phosphatase activity of Src homology 2 domain-containing phosphatase-2 (SHP-2), a protein-tyrosine phosphatase that dephosphorylates PDGF receptor, leading to the prevention of hyperactivation of PDGF receptor and the Ras-ERK signaling. In contrast, knockdown of afadin or SHP-2 induced the hyperactivation of PDGF receptor and Ras-ERK signaling and consequently suppressed the formation of leading-edge structures. Thus, afadin plays a critical role in the proper regulation of the PDGF-induced activation of PDGF receptor and signaling by Ras-ERK. This effect, which is mediated by SHP-2, impacts cellular morphology.

Afadin is a nectin- and actin filament (F-actin)2-binding protein that connects nectin to the actin cytoskeleton (1). Nectin is a Ca2+-independent immunoglobulin-like molecule that first forms cell-cell adhesion and then assembles cadherin at the nectin-based cell-cell adhesion sites, resulting in the formation of adherens junctions (AJs) in epithelial cells and fibroblasts (2, 3). The nectin family of proteins comprises four members, nectin-1, -2, -3, and -4. A series of our studies revealed the roles and modes of action of nectin and afadin for the formation of AJs (2–10). Nectin initiates the formation of cell-cell adhesion and then induces the activation of Rap1, Cdc42, and Rac small G proteins through the activation of c-Src. Subsequently, Cdc42 and Rac bind to IQGAP1 and induce the reorganization of the actin cytoskeleton, thereby causing the accumulation of non-trans-interacting E-cadherin at the nectin-based cell-cell adhesion sites. On the other hand, afadin, which binds to Rap1 activated by the action of nectin, associates with p120ctn and inhibits the endocytosis of accumulated non-trans-interacting E-cadherin to facilitate the trans-interaction of E-cadherin. In parallel with these processes, Cdc42 increases the number of filopodia and cell-cell contact sites, whereas Rac induces the formation of lamellipodia, which efficiently zip the cell-cell adhesion between the filopodia, acting like a “zipper.” We recently found that nectin also interacts with integrin α3β3 and that the activation of integrin α3β3 is necessary for the nectin-induced signaling and formation of AJs (11).

Afadin furthermore plays an essential role in the recruitment of Claudins to the apical side of the nectin-based cell-cell adhesion sites to form tight junctions (10). We recently showed that cell-cell adhesion based on the nectin-afadin complex is indispensable for the formation of tight junctions but that the cadherin-based cell-cell adhesion is not always essential for it under certain conditions (12), although it had been believed from several lines of circumstantial evidence that cadherin-based cell-cell adhesion is required for the formation of tight junctions (13–16). Therefore, afadin critically functions in the formation of both AJs and tight junctions.

Platelet-derived growth factor (PDGF) is a family of growth factors consisting of three isoforms, PDGF-AA, -AB, and -BB (17). Binding of PDGF to its receptor causes the autophosphorylation of specific tyrosine residues in the intracellular parts of Eagle’s medium: Ab, antibody; pAb, polyclonal Ab; mAb, monoclonal Ab; BSA, bovine serum albumin; GST, glutathione S-transferase; MBP, maltose-binding protein; BrdUrd, bromodeoxyuridine; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; CA, constitutively active; GFP, green fluorescent protein; WT, wild-type; siRNA, small interfering RNA; LMW, low molecular weight.
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the receptor, leading to the activation of diverse intracellular signaling pathways such as Ras-ERK, c-Src, and Rap1-Rac signalings. It is well known that the Ras-ERK signaling enhances cell cycle progression and facilitates cell proliferation (18–20). c-Src activated in response to PDGF tyrosine-phosphorylates various proteins to control the PDGF receptor-mediated signal transduction (21). Moreover, we recently found that the sequential activation of Rap1 and Rac locally at the leading edge, where PDGF receptor, integrin αvβ5, and nectin-like molecule-5 are accumulated and clustered (22, 23), influences the formation of leading-edge structures including lamellipodia, peripheral ruffles, focal complexes, and focal adhesions, resulting in the enhancement of cell movement.4 In addition, it has been reported that the Ras-ERK signaling plays roles in cellular morphology as well as cell proliferation (24, 25).

Tyrosine-phosphorylated PDGF receptor is in turn dephosphorylated and inactivated by protein-tyrosine phosphatases (PTPs) such as Src homology 2 (SH2) domain-containing phosphatase-2 (SHP-2) (26, 27). SHP-2 is a widely expressed cytosolic non-transmembrane PTP containing two SH2 domains at the N terminus and a single central phosphatase domain. It is reported that when PDGF receptor is autophosphorylated by PDGF, SHP-2 binds to PDGF receptor, and its phosphatase activity is up-regulated to dephosphorylate PDGF receptor (28, 29).

We show here a novel role of afadin in the regulation of the activation of PDGF receptor and Ras-ERK signaling. When afadin is tyrosine-phosphorylated by c-Src activated in response to PDGF, it binds to SHP-2 and stimulates the phosphatase activity of SHP-2, resulting in dephosphorylation of PDGF receptor. This contributes to the fine tuning of the activation of PDGF receptor and Ras-ERK signaling. We also found that the afadin- and SHP-2-mediated regulation of PDGF receptor and Ras-ERK signaling are important for the formation of leading-edge structures necessary for directional cell movement.

EXPERIMENTAL PROCEDURES

Cell Culture, Expression Vectors, Transfection, and Knockdown Experiment—NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum. Full-length (amino acids 1–1829), ΔRA (amino acids 351–1829), ΔDIL (amino acids 1–234), Δ3 (amino acids 1122–1829), and Δ4 (amino acids 1–593), Δ3 (amino acids 1122–1829), and Δ4 (amino acids 1–1131), Δ1 (amino acids 1–1131), Δ2 (amino acids 982–1829), Δ3 (amino acids 1329–1829), and Δ4 (amino acids 1509–1829) of mouse SHP-2 cDNAs were subcloned into pCIneo-Myc (Promega). The full-length cDNA of Myc-tagged rat LMW-PTP and Myc-tagged constitutively active mutant of MEK1 (pSRα-Myc-MEK1-CA) were kindly supplied by Dr. T. Kondo (Nagasaki University, Nagasaki, Japan) and Dr. E. Nishida (Kyoto University, Kyoto, Japan), respectively. Expression vectors for constitutively active (pUSE-c-Src-CA) and kinase inactive (pUSE-Src (K297R)) mutants of c-Src was purchased from Upstate Biotechnology.

Afadin-knockdown NIH3T3 cells, in which afadin is stably knocked down, were generated as follows: the fragment containing the H1-RNA promoter and short hairpin RNA sequence against afadin was excised from the pBS-H1-afadin vector, which was previously created to transiently knock down afadin as described (10), and ligated into the EB virus-based vector, pEB, which was generated by deletion of the CAG promoter and DsRed gene from pRUBY-M2 kindly supplied by Dr. Y. Miwa (University of Tsukuba, Tsukuba, Japan) to construct the pEB-H1-afadin vector. NIH3T3 cells stably expressing the short hairpin RNA specific for afadin were generated by the transfection of pEB-H1-afadin into NIH3T3 cells followed by selection with 500 μg/ml G418 (Nacalai Tesque). Control NIH3T3 cells for afadin short hairpin RNA (shRNA) were similarly produced using scrambled shRNA sequence (5’-CCATCTCAATTCTTGGACG-3’). To knock down SHP-2, double-stranded 25-nucleotide RNA duplex (StealthTM RNA interference; Invitrogen) for SHP-2 (5’-CCACUUUGCU-GAACUGUACAGUAUA-3’) was transfected into NIH3T3 cells with HiPerFect transfection reagent (Qiagen). As a control for SHP-2 small interfering RNA (siRNA), the scrambled RNA duplex (5’-CCAGGUAGUCGUACUCUAGUA-3’) was also purchased from Invitrogen and transfected into NIH3T3 cells. For DNA transfection, the Lipofectamine 2000 or the Lipofectamine LTX reagent (Invitrogen) was used.

Antibodies and Reagents—A rabbit anti-afadin polyclonal Ab (pAb) and a mouse anti-afadin monoclonal Ab (mAb) were prepared as described (30). Abs listed below were purchased from commercial sources; a rabbit anti-GFP pAb was from MBL, a mouse anti-FLAG M2 mAb was from Sigma, a rabbit anti-phospho-PDGF receptor (Tyr716) pAb was from Upstate Biotechnology, a rabbit anti-phospho-ERK1/2 mAb was from Cell Signaling Technology, a rabbit anti-phospho-PDGFR receptor (Tyr716) pAb was from Upstate Biotechnology, a rabbit anti-phospho-ERK1/2 pAb was from Cell Signaling Technology, a rabbit anti-p-Src pAb was from Cell Signaling Technology, a rabbit anti-N-cadherin pAb was from Takara, and a rabbit anti-glyceraldehyde-3-phosphate dehydrogenase mAb was from Cell Signaling Technology. Hybridoma cells expressing a mouse anti-Myc mAb were purchased from the American Type Collection. Rhodamine phalloidin was purchased from Molecular Probes. Horseradish peroxidase-conjugated and fluorophore-conjugated secondary Abs were purchased from Amersham Biosciences and Chemicon.

3 H. Amano, W. Ikeda, S. Kawano, M. Kajita, Y. Tamaru, N. Inoue, Y. Minami, A. Yamada, and Y. Takai, unpublished observation.

4 M. Takahashi, Y. Rikitake, Y. Nagamatsu, T. Hara, W. Ikeda, K. Hirata, and Y. Takai, unpublished observation.
respectively. PDGF-BB and fatty acid-free bovine serum albumin (BSA) were purchased from PEPROTECH and Sigma, respectively. Vitronectin was purified from human plasma (Kohjinbio) as described (31).

Phosphorylation of PDGF Receptor and ERK and Activation of Ras—To examine the level of the phosphorylation of PDGF receptor and ERK in each kind of NIH3T3 cells after the PDGF stimulation, cells were serum-starved for 16 h and treated with 15 ng/ml PDGF at 25 °C for the indicated periods of time. After being washed with ice-cold PBS, cells were harvested using pre-warmed Laemmli buffer (32) containing 1 mM Na3VO4, 10 mM NaF, and a phosphatase inhibitor cocktail I (Sigma), boiled for 5 min, and sonicated 3 times for 10 s with 20-s cooling periods. Protein concentrations of the samples were determined using an RC DC protein assay kit (Bio-Rad) with BSA as a reference protein. The samples were subjected to SDS-PAGE followed by Western blotting using the indicated phospho-specific Abs.

To assess the activation of Ras, the pulldown assay was performed as described previously (33). Briefly, after the treatment with PDGF, cells were lysed with Buffer A (50 mM Tris-HCl at pH 7.5, 200 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 10% glycerol, 10 μM p-aminophenylmethanesulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM Na3VO4) containing 10 μg of glutathione S-transferase (GST)-Raf-RBD+CRD (the Ras binding and cysteine-rich domains of Raf-1 fused to GST) and incubated at 2 °C for 30 min. The cell extract was obtained by centrifugation at 20,000 × g at 0 °C for 5 min and incubated with 50 μl of glutathione-agarose beads (Amersham Biosciences) at 2 °C for 1 h. After the beads were washed with Buffer A, proteins bound to the beads were eluted with Laemmli buffer and subjected to SDS-PAGE followed by Western blotting using the anti-Ras mAb. Band intensity was determined by densitometer FluorchemTM (Alpha Innotech Corp.). The $K_d$ value was calculated by Scatchard analysis.

Phosphatase Assay for SHP-2—Control NIH3T3 or afadin-knockdown NIH3T3 cells were seeded at density of 2.5 × 103 cells/cm², cultured for 18 h, starved of serum with DMEM containing 0.5% BSA for 16 h, and then stimulated with DMEM containing 0.5% BSA and 15 ng/ml PDGF at 25 °C for 0 or 2 min. After stimulation, the cells were lysed with Buffer D (25 mM HEPES at pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100). The cell lysates were rotated for 30 min and subjected to centrifugation at 12,000 × g for 20 min. The supernatant was then incubated with the anti-FLAG mAb or the anti-Myc mAb at 4 °C for 2 h followed by incubation with the protein G-Sepharose beads at 4 °C for 2 h. After the beads were extensively washed with Buffer B, the bound proteins were eluted from the beads by boiling with the SDS sample buffer. The samples were then subjected to SDS-PAGE followed by Western blotting using the anti-SHP-2 mAb or control mouse IgG by incubation with protein G-Sepharose beads. The immunoprecipitated samples were then analyzed by Western blotting.

Biotinylation Assay for Internalization of PDGF Receptor upon PDGF Stimulation—After stimulation with 15 ng/ml PDGF for the indicated periods of time, cells were incubated with 0.2 mg/ml sulfo-SU6656 (Sigma) dissolved in 0.2% Me2SO, 10 μM PP2 (Calbiochem-Novabiochem) dissolved in 0.2% Me2SO, 10 μM SU6656 (Sigma) dissolved in 0.2% Me2SO, or 0.2% Me2SO as a negative control in DMEM for 4 h and lysed with Buffer B, and the cell lysates were incubated with the anti-SHP-2 mAb or control mouse IgG by incubation with protein G-Sepharose beads. The immunoprecipitated samples were then analyzed by Western blotting.

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Afadin and SHP-2 in PDGF Receptor Signaling—Recombinant GST-fused full-length SHP-2 (GST-SHP-2) was prepared as described (34). Recombinant maltose-binding protein (MBP)-fused full-length afadin (MBP-afadin) was prepared as described (6). Briefly, High Five insect cells were infected with the baculovirus bearing the afadin cDNA, cultured for 64 h, treated with or without 0.1 mM pervanadate for 8 h, and then purified by the use of TALON metal affinity beads (Clontech). Pervanadate, which strongly inhibits tyrosine dephosphorylation and, thus, retains tyrosine phosphorylation, was prepared as described (35). The tyrosine phosphorylation of purified MBP-afadin was confirmed by Western blotting. To examine the affinities of SHP-2 for afadin, GST-SHP-2 or GST was incubated with MBP-afadin (20 pmol) immobilized on 20 μl of amylase resin beads in 400 μl of Buffer C (20 mM Tris-HCl at pH 8.0, 27.5 mM NaCl, 25 mM KCl, and 0.1% Triton X-100) at 4 °C for 2 h. After the beads were extensively washed with Buffer C, the bound proteins were eluted by boiling in the SDS sample buffer. The samples were then subjected to SDS-PAGE followed by staining with Coomassie Brilliant Blue. The amount of bound GST-SHP-2 was determined by comparing the band intensity of various amounts of BSA using a densitometer FluorchemTM (Alpha Innotech Corp.). The $K_d$ value was calculated by Scatchard analysis.
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room temperature for 15 min. Absorbances were read at 620 nm with a microplate reader. The absorbance was compared with a phosphate standard curve to determine the release of phosphate in picomoles. For the quantities of inorganic phosphate released, the absorbance values were in the middle of the optical density range where the absorbance curve is linear. To control the expression levels of SHP-2 in the immune complex in the assays, aliquots of the immune complexes were Western blotted using the anti-SHP-2 mAb. Data are expressed as the means ± S.E. of three independent experiments.
experiments. Paired Student’s t test was performed for statistical analysis.

Cell Proliferation and Directional Stimulation by PDGF—Cell proliferation assessed by BrdUrd incorporation was performed as described previously (36). Briefly, control and afadin-knockdown and SHP-2-knockdown NIH3T3 cells were starved of serum with DMEM containing 0.5% BSA for 24 h and then stimulated by 30 ng/ml PDGF and 10 μg/ml insulin for the indicated periods of time. Cells were incubated with BrdUrd for 2 h before the end of the stimulation. For directional stimulation of cells with PDGF, the μ-Slide VI flow (Ibidi), which has six parallel channels and was coated with 5 μg/ml vitronectin, was used to generate a concentration gradient of PDGF as described previously (23). The concentration gradient of PDGF was made using DMEM containing 30 ng/ml PDGF and 0.5% BSA according to the manufacturer’s protocol. Cells were seeded at a density of 5 × 10^3 cells/cm^2, cultured for 18 h, and starved of serum with DMEM containing 0.5% BSA in the presence or absence of 10 μM U0126 (Calbiochem-Novabiochem). After 30 min of directional stimulation by PDGF, cells were fixed with 3.7% formaldehyde in PBS for 15 min, permeabilized with 0.2% Triton X-100 for 15 min, incubated with 0.2% Triton X-100 for 15 min, incubated with 1% BSA in PBS for 30 min, and stained with rhodamine-phalloidin for F-actin and immunostained with the anti-Myc mAb for detection of Myc-MEK1-CA-transfected cells. The samples were observed by LSM510 META confocal microscope (Carl Zeiss). The number of cells with the leading edge was determined, and paired Student’s t tests were performed for statistical analysis.

RESULTS

Prolongation of the PDGF-induced Autophosphorylation of PDGF Receptor and Activation of Ras-ERK Signaling by Knockdown of Afadin—Binding of PDGF to its receptor initiates autophosphoryla-
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...tion on a number of tyrosine residues of PDGF receptor, resulting in the modulation of several signal cascades including the Ras-ERK pathway (17, 20, 37, 38). We first confirmed these earlier observations in NIH3T3 cells. PDGF induced the phosphorylation of Tyr716 and Tyr1009 residues (Tyr(P)-716 and Tyr(P)-1009) of PDGF receptor in a time-dependent manner in control NIH3T3 cells (28, 39), which were sparsely cultured and hardly formed cell-cell contacts (Fig. 1A). In this assay, the peak of phosphorylation of PDGF receptor was observed at 10 min after the treatment with PDGF. The PDGF-induced activation of Ras and ERK also peaked at 10 min after the treatment with PDGF (Fig. 1, B and C). In afadin-knockdown NIH3T3 cells, in which the amount of afadin was reduced to ~20% compared with that in control cells (Fig. 1D), the phosphorylation of PDGF receptor and activation of Ras and ERK occurred more rapidly and were more prolonged than that in control cells after the treatment with PDGF (Fig. 1, A–C). In addition, internalized PDGF receptor upon treatment with PDGF was the same in both afadin-knockdown and control NIH3T3 cells (Fig. 1E). Essentially the same results were obtained using two other afadin-knockdown and control NIH3T3 cell lines (data not shown). Collectively, these results indicate that afadin regulates the activation of PDGF receptor and Ras-ERK signaling. Because knockdown of afadin prolonged the phosphorylation of PDGF receptor, the data might be interpreted to suggest that afadin may regulate the activation of PTP(s) that dephosphorylates PDGF receptor.

**Binding of Afadin to SHP-2**—PTPs that are known to interact with and dephosphorylate PDGF receptor include SHP-2, LMW-PTP, PTP-1B, PTP-PEST, and TC-PTP (26, 27). Of these phosphatases, SHP-2 and LMW-PTP have been reported to be highly expressed in NIH3T3 cells (26, 40). We examined by the co-immunoprecipitation assay whether afadin associates with SHP-2 and/or LMW-PTP. FLAG-tagged afadin (FLAG-afadin) and Myc-tagged SHP-2 (Myc-SHP-2) or Myc-tagged LMW-PTP (Myc-LMW-PTP) were co-expressed in HEK293 cells. When FLAG-afadin was immunoprecipitated with the anti-FLAG mAb, Myc-SHP-2, but not Myc-LMW-PTP, was co-immunoprecipitated with FLAG-afadin (Fig. 2A). Similarly, when Myc-SHP-2 was immunoprecipitated with the anti-Myc mAb, FLAG-afadin was co-immunoprecipitated with Myc-SHP-2 (see also Fig. 4Ab). We also investigated the association of endogenous afadin with SHP-2 in the co-immunoprecipitation assay. In control NIH3T3 cells, afadin as well as PDGF receptor was actually co-immunoprecipitated with SHP-2 when SHP-2 was immunoprecipitated with the anti-SHP-2 mAb but not with control IgG (Fig. 2B). In contrast, neither afadin nor PDGF receptor was co-immunoprecipitated with SHP-2 in afadin-knockdown NIH3T3 cells, providing an important notion that afadin is necessary for the interaction of SHP-2 with PDGF receptor.

To further examine the direct binding of SHP-2 to afadin, we performed in vitro binding assay using the pure recombinant proteins of MBP-afadin and GST-SHP-2. When MBP-afadin, which was simply purified from baculovirus and was not phosphorylated, was immobilized on amylose resin beads and GST-SHP-2 was incubated with these beads, GST-SHP-2 did not bind to MBP-afadin (data not shown). On the other hand, we obtained tyrosine-phosphorylated MBP-afadin by treatment with pervanadate before and during the purification process and found that GST-SHP-2 bound to phosphorylated MBP-
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results indicate that afadin directly interacts with SHP-2 and is essential for both the binding of SHP-2 to PDGF receptor and the tyrosine phosphatase activity of SHP-2, leading to the enhancement of dephosphorylation of PDGF receptor.

c-Src-catalyzed Tyrosine Phosphorylation of Afadin—Because it is known that SHP-2 interacts with tyrosine-phosphorylated molecules through its SH2 domains and that c-Src is activated in response to PDGF and tyrosine-phosphorylates many proteins (42, 43), we next examined whether afadin is tyrosine-phosphorylated by c-Src and whether the phosphorylation of afadin is indeed necessary for its binding to SHP-2 endogenously. GFP-tagged afadin (GFP-afadin) was co-expressed with either a constitutively active (CA) or a kinase inactive (K297R) mutant of c-Src in HEK293 cells. GFP-afadin was tyrosine-phosphorylated when co-transfected with c-Src-CA but not c-Src-K297R (Fig. 3A). GFP itself was not phosphorylated by c-Src-CA. The c-Src-catalyzed tyrosine phosphorylation of afadin was inhibited by PP2, an inhibitor of Src family kinases, but not PP3, an inactive analogue of PP2, or Me2SO (Fig. 3B).

To determine the site(s) of afadin that is tyrosine-phosphorylated by c-Src, various truncated mutants of afadin were co-expressed with c-Src-CA, and the tyrosine phosphorylation of each mutant was analyzed as described above. Only GFP-afadinΔ2 was tyrosine-phosphorylated by c-Src-CA similar to GFP-tagged wild-type afadin (GFP-afadin-WT) (Fig. 3C). This result indicates that some tyrosine residues within amino acids 1132–1328 of afadin is phosphorylated by c-Src-CA. The 1132–1328 region contains eight tyrosine residues. Each of these tyrosine residues in GFP-afadinΔ2 was replaced with phenylalanine by mutagenesis. The level of tyrosine phosphorylation of GFP-afadinΔ2-Y1237F was markedly reduced but not that of GFP-afadinΔ2-Y1259F, GFP-afadinΔ2-Y1292F, or the other GFP-afadin point mutants (Fig. 3D and data not shown). This result indicates that the Tyr1237 residue of afadin is at least a major phosphorylation site by c-Src.

We further examined whether the interaction of endogenous afadin with SHP-2 is dependent on the PDGF-induced activation of c-Src in NIH3T3 cells. Co-immunoprecipitation of afadin with SHP-2 after treatment with PDGF is suppressed in the

afadin (Fig. 2Ca). The binding of GST-SHP-2 to MBP-afadin was dose-dependent and saturable (Fig. 2Cb). Scatchard analysis demonstrated a single class of the binding site with a $K_d$ value of about 0.1 μM. These results indicate that SHP-2 directly binds to tyrosine-phosphorylated afadin.

We further examined the effect of afadin on the tyrosine phosphatase activity of SHP-2. The endogenous sample of SHP-2 was immunoprecipitated from control and afadin-knockdown NIH3T3 cells before and after PDGF stimulation. The tyrosine phosphatase activity of SHP-2 was measured with a small phosphopeptide, TSTEPQpYQPGENL (pY is phosphotyrosine), as a model substrate (41). After the treatment with PDGF, the phosphatase activity was increased in both types of cells, but this increase was significantly suppressed in afadin-knockdown NIH3T3 cells (Fig. 2D). Taken together, these
presence of Src inhibitors PP2 or SU6656 but not PP3 or Me2SO as a negative control (Fig. 3E). In parallel with this, co-immunoprecipitation of SHP-2 with PDGF receptor is attenuated by treatment with PP2 or SU6656. Thus, the interaction of afadin with SHP-2 and the afadin-mediated interaction of SHP-2 with PDGF receptor rely on the PDGF-induced activation of c-Src endogenously.

Regions Necessary for Binding between Afadin and SHP-2—We then determined the binding regions of afadin and SHP-2. We co-expressed various truncated mutants of Myc-SHP-2 shown in Fig. 4Aa and FLAG-afadin in HEK293 cells and immunoprecipitated Myc-SHP-2 with the anti-Myc mAb. Afadin was co-immunoprecipitated with the C-terminal-truncated mutant SHP-2Δ1 containing two SH2 domains but not the N-terminal truncated mutant SHP2Δ4 containing the phosphatase domain (Fig. 4Ab). In addition, afadin was co-immunoprecipitated with the SHP2Δ3 containing the C-terminal SH2 domain, but not SHP2Δ2 containing the N-terminal SH2 domain (Fig. 4Ac), indicating that the C-terminal SH2 domain of SHP-2 is responsible for the binding to afadin.

We also examined whether the Tyr1237 residue of afadin is critical for the direct binding of SHP-2 and afadin because SHP-2 directly bound to tyrosine-phosphorylated afadin and the Tyr1237 residue of afadin was phosphorylated by c-Src. For this purpose, Myc-SHP-2 and FLAG-afadin-WT or FLAG-afadin-Y1237F were co-transfected into HEK293 cells. When Myc-SHP-2 was immunoprecipitated Myc-SHP-2 with the anti-Myc mAb, FLAG-afadin-WT was co-immunoprecipitated with Myc-SHP-2, whereas FLAG-afadin-Y1237F was hardly co-immunoprecipitated (Fig. 4B). This result indicates that the Tyr1237 residue of afadin is critically implicated in the physical association of afadin with SHP-2.

We further examined whether re-expression of afadin in afadin-knockdown NIH3T3 cells restores
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Involvement of the enhanced Ras-ERK signaling by knockdown of afadin or SHP-2 in cell morphology. A, assessment of DNA synthesis by BrdUrd incorporation. After the serum starvation for 24 h, control, afadin-knockdown, and SHP-2-knockdown NIH3T3 cells were cultured in the presence of PDGF for the indicated periods of time. BrdUrd was added in the medium at 2 h before each end point. Cells were stained with the anti-BrdUrd (BrdU) mAb (red) and counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). Plots in the graph represent the mean percentage ± S.E. of BrdUrd-positive cells. Scale bars, 50 μm. B, formation of leading edges to the higher concentration of PDGF. At 1 h after the serum starvation, NIH3T3 cells were directionally stimulated with 30 ng/ml PDGF from the lower side for 30 min. F-actin was stained with rhodamine-phalloidin. C, no polarization by treatment with PDGF in afadin-knockdown, SHP-2-knockdown, or Myc-MEK1-CA-transfected NIH3T3 cells. These cells were treated as described in B, and then F-actin was stained with rhodamine-phalloidin. Myc-MEK1-CA-transfected cells were determined by immunostaining with the anti-Myc mAb (not depicted). D, rescue of cellular phenotype by inhibition of ERK in afadin-knockdown and SHP-2-knockdown NIH3T3 cells. After a 4-h pretreatment with 10 μM U0126, the cells were treated as described in A, and then F-actin was stained with rhodamine-phalloidin. Arrows indicate leading edges. Scale bars, 10 μm. The results shown in this figure are representative of three independent experiments. E, quantification of formation of the leading edge. Bars in the graph represent the mean percentage of cells forming the leading edge of the total cells counted (n = 100) in each type of cells in three independent experiments. Error bars indicate S.E. *p < 0.05 versus NIH3T3 cells without U0126 pretreatment.

The activation levels of PDGF receptor, Ras, and ERK. When FLAG-afadin-WT, which was resistant to afadin RNA interference, was expressed in afadin-knockdown NIH3T3 cells, the phosphorylation of PDGF receptor and activation of Ras and ERK after treatment of PDGF was reduced to a similar extent to those observed in control NIH3T3 cells (Fig. 4C). However, the re-expression of RNA interference-resistant FLAG-afadin-Y1237F in afadin-knockdown NIH3T3 cells did not affect the phosphorylation or activation level of PDGF receptor, Ras, and ERK. These results indicate that the formation of the afadin-SHP-2 complex is necessary for the regulated activation of PDGF receptor and Ras-ERK signaling in response to PDGF.

Prolongation of the PDGF-induced Activation of PDGF Receptor, Ras, and ERK by Knockdown of SHP-2—Because the complex formation of afadin and SHP-2 is necessary for the dephosphorylation of PDGF receptor, we examined whether knockdown of SHP-2 also enhances the tyrosine phosphorylation of PDGF receptor and the Ras-ERK signaling similar to those observed in afadin-knockdown NIH3T3 cells. When SHP-2 was knocked down in NIH3T3 cells using siRNA method, the amount of SHP-2 was reduced to ~20% as compared with control scrambled siRNA-transfected NIH3T3 cells, but the amounts of other proteins tested remained unchanged (Fig. 5A). Consistent with the results in afadin-knockdown NIH3T3 cells, the phosphorylation of PDGF receptor and activation of Ras and ERK in SHP-2-knockdown NIH3T3 cells was more rapidly induced and prolonged for a longer time than that observed in control NIH3T3 cells (Fig. 5, B–D). These results confirm that SHP-2 and afadin cooperatively mediate the PDGF-induced activation of the receptor and Ras-ERK signaling.

Inhibition of the Formation of Leading Edges by Hyperactivation of Ras-ERK Signaling Induced by Knockdown of Afadin or SHP-2—Although the Ras-ERK signaling is well known to contribute to cell cycle progression and cell proliferation, we could not detect a significant difference among control and afadin-knockdown and SHP-2-knockdown NIH3T3 cells in DNA synthesis necessary for cell proliferation by measuring BrdUrd incorporation (Fig. 6A). Then we investigated whether the up-regulated Ras-ERK signaling by knockdown of afadin or SHP-2 is responsible for the morphological change in NIH3T3 cells because the Ras-ERK signaling also plays a key role in the regulation of cytoskeletal dynamics (24, 25). To test this, NIH3T3 cells were sparsely plated on μ-slide VI flow dishes precoated with vitronectin, starved of serum, and directionally stimulated by PDGF. Control NIH3T3 cells became polarized in shape and formed leading edges to the direction of higher concentrations of PDGF. F-actin staining showed a significant increase in the formation of cell protrusions such as lamellipodia at the leading edge after the PDGF stimulation (Fig. 6, B and E). However, afadin- or SHP-2-knockdown NIH3T3 cells adopted thin and angular shapes without polarization, and the formation of leading edges to the direction of higher concentrations of PDGF was markedly impaired in these types of cells (Fig. 6, C and E). The signal for F-actin showed the
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formation of stress fibers in these cells. Such phenotypes were also observed in NIH3T3 cells expressing the constitutively active mutant of MEK1. In contrast, a MEK inhibitor U0126 reversed the phenotypes of SHP-2- or afadin-knockdown NIH3T3 cells, although control NIH3T3 cells pretreated with U0126 did not show any response to PDGF nor form leading edges (Fig. 6, D and E). These results indicate that hyperactivation of Ras-ERK signaling by knockdown of afadin or SHP-2 causes abnormality in cell shape. Thus, tightly regulated activation of the Ras-ERK signaling induced by PDGF is essentially required for maintenance of normal cellular morphology.

DISCUSSION

We demonstrated here that afadin regulates the PDGF-induced tyrosine phosphorylation of PDGF receptor and activation of Ras-ERK signaling through SHP-2 in NIH3T3 cells. These results are consistent with recent observations that termination of the PDGF-induced activation of PDGF receptor is preferentially caused by the activation of PTPs rather than the PDGF receptor down-regulation in NIH3T3 cells (44) because afadin did not affect the PDGF receptor down-regulation after the treatment with PDGF. In this study we clearly revealed that afadin has two important roles in the function of SHP-2; first, the direct binding of afadin to SHP-2 is critical for the interaction of SHP-2 with PDGF receptor, and second, afadin is essentially involved in the regulation of the phosphatase activity of SHP-2. In addition, the direct binding of afadin to SHP-2 and the activation of PDGF receptor, Ras, and ERK are keenly dependent on the phosphorylation at Tyr1237 of afadin, which is induced by activated c-Src in response to PDGF. The c-Src-mediated phosphorylation of afadin is also shown by another group (45).

The phosphorylation at Tyr1009 of PDGF receptor promotes the direct binding of the N-terminal SH2 domain of SHP-2 to PDGF receptor (28, 46). Several studies reported that the interaction of SH2 domains of SHP-2 with its target proteins, especially simultaneous occupancy of both SH2 domains by phosphopeptides, is required for full activation of phosphatase activity of SHP-2 (29, 47–49). However, little is known about the target molecules against the C-terminal SH2 domain of SHP-2. We confirmed here that Tyr1009 of PDGF receptor is phosphorylated in response to PDGF. In addition, our analyses revealed that afadin associates with the C-terminal SH2 domain of SHP-2 when afadin is tyrosine-phosphorylated. Thus, it is likely that afadin is one of the target proteins against the C-terminal SH2 domain of SHP-2 and regulates its phosphatase activity.

We further showed here that the PDGF-induced activation of Ras-ERK signaling by knockdown of afadin or SHP-2 is involved in cell morphology. We previously showed that Madin-Darby canine kidney cells (MDCK) cells expressing a dominant negative mutant of SHP-2 markedly increase the formation of stress fibers and focal adhesions (50), whereas MDCK cells expressing a constitutively active mutant of SHP-2 markedly spread and increase the formation of lamellipodia and ruffles with the disappearance of peripheral actin bundles (51). In addition, fibroblasts derived from SHP-2 knock-out mice exhibit the increased formation of stress fibers and focal adhesions and the impaired cell migration on fibronectin (52). Consistent with these earlier observations, we found here that SHP-2- or afadin-knockdown NIH3T3 cells exhibit thin and angular shapes with well developed stress fibers, suggesting that afadin and SHP-2 tightly regulate the activation of Rho small G protein. More importantly, these cells do not form leading-edge structures to the direction of higher concentration of PDGF. In contrast, the suppression by a MEK inhibitor U0126 of hyperactivation of ERK in afadin- or SHP-2-knockdown NIH3T3 cells shows the reformation of leading-edge structures in response to PDGF. Taken together, it is likely that the fine tuning of activation of PDGF receptor and the Ras-ERK signaling mediated by afadin and SHP-2 is necessary for the dynamic formation of leading-edge structures.

We did not show a significant difference in the PDGF-induced cell proliferation process among control, afadin-knockdown, and SHP-2-knockdown NIH3T3 cells, although the enhanced activation of Ras-ERK signaling is usually correlated with the up-regulation of cell proliferation. It is known that activated ERK translocates from the cytosol to the nucleus and promotes G1- to S-phase progression by induction of positive regulators of cell cycle, such as cyclins, and inhibition of anti-proliferative genes (53). In contrast, too strong ERK signaling is in turn reported to arrest cell cycle (53). Although the precise molecular mechanism why the hyperactivation of ERK by knockdown of afadin or SHP-2 did not enhance cell proliferation in NIH3T3 cells remains to be elucidated, such hyperactivation of ERK may cause the up-regulation of both accelerative and suppressive mechanisms of cell cycle progression simultaneously, resulting in no alteration of cell proliferation as a whole.

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REFERENCES

1. Mandai, K., Nakanishi, H., Satoh, A., Obaishi, H., Wada, M., Nishioka, H., Itoh, M., Mizoguchi, A., Aoki, T., Fujimoto, T., Matsuda, Y., Tsukita, S., and Takai, Y. (1997) J. Cell Biol. 139, 517–528
2. Takai, Y., Irie, K., Shimizu, K., Sakisaka, T., and Ikeda, W. (2003) Cancer Sci. 94, 655–667
3. Takai, Y., and Nakanishi, H. (2003) J. Cell Sci. 116, 17–27
4. Fukushima, T., Shimizu, K., Kawakatsu, T., Fukuyama, T., Minami, Y., Honda, T., Hoshino, T., Yamada, T., Ogita, H., Okada, M., and Takai, Y. (2004) J. Cell Biol. 166, 393–405
5. Fukuyama, T., Ogita, H., Kawakatsu, T., Fukushima, T., Yamada, T., Sato, T., Shimizu, K., Nakamura, T., Matsuda, M., and Takai, Y. (2005) J. Biol. Chem. 280, 815–825
6. Hoshino, T., Sakisaka, T., Baba, T., Yamada, T., Kimura, T., and Takai, Y. (2005) J. Biol. Chem. 280, 24095–24103
7. Izumi, G., Sakisaka, T., Baba, T., Tanaka, S., Morimoto, K., and Takai, Y. (2004) J. Cell Biol. 166, 237–248
8. Kawakatsu, T., Ogita, H., Fukushima, T., Fukuyama, T., Minami, Y., Shimizu, K., and Takai, Y. (2005) J. Biol. Chem. 280, 4940–4947
9. Kawakatsu, T., Shimizu, K., Honda, T., Fukushima, T., Hoshino, T., and Takai, Y. (2002) J. Biol. Chem. 277, 50749–50755
10. Sato, T., Fujita, N., Yamada, A., Ooshio, T., Okamoto, R., Irie, K., and Takai, Y. (2006) J. Biol. Chem. 281, 5288–5299
11. Sakamoto, Y., Ogita, H., Hirota, T., Kawakatsu, T., Fukuyama, T., Yasumi, M., Kanazaki, N., Ozaki, M., and Takai, Y. (2006) J. Biol. Chem. 281, 19631–19644
