Filamin Associates with Smads and Regulates Transforming Growth Factor-β Signaling*

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Members of the Smad proteins transmit signals triggered by the ligands of transforming growth factor (TGF)-β superfamily. Ligand-activated receptors induce phosphorylation of so-called receptor-regulated Smads, which then accumulate in the nucleus to participate in target gene transcription, in collaboration with Smad-interacting proteins. We performed yeast two-hybrid screening and identified filamin, a cytoskeletal actin-binding protein 280, as a Smad5-interacting protein. Filamin was found to be associated not only with Smad5 but also with other Smad proteins, including TGF-β/activin receptor-regulated Smad2. TGF-β signaling was defective in filamin-deficient human melanoma cells M2 compared with a filamin-transfected subline A7, as determined by TGF-β-responsive reporter gene activation and Smad2 nuclear accumulation. M2 cells restored TGF-β responsiveness following transient transfection of full-length filamin encoding vector. The defective TGF-β signaling in M2 cells seemed to be due to impaired receptor-induced serine phosphorylation of Smad2. These results suggest that filamin plays an important role in Smad-mediated signaling.

The transforming growth factor (TGF)β superfamily cytokines act on a wide variety of cells and organs to regulate development and homeostasis. Members of the Smad proteins play pivotal roles in the intracellular signal transduction of TGF-β family proteins. Activated type I TGF-β family receptors phosphorylate conserved serine residues at the C terminus of so-called receptor-regulated Smads, Smad1, -5, and -8, for receptors of bone morphogenetic proteins (BMP), or Smad2 and -3 for receptors of TGF-β and activin, respectively. The phosphorylated Smads are then translocated into the nucleus with their common partner Smad4, where they activate target genes in collaboration with other transcriptional partners (1–3).

Smad-mediated signaling is regulated or modified through a number of Smad-interacting proteins. It has recently been shown that in the cytoplasm, a FVYE domain protein, SARA, binds to both Smad2 and TGF-β receptor, and plays an important role in recruiting Smad2 to the receptor by controlling the subcellular localization of Smad (4). Microtubules bind to Smad2, -3, and -4, and negatively regulate signaling, controlling the rate of Smad2 association to receptor and subsequent phosphorylation (5).

In the nucleus, Smad proteins form complexes with transcriptional partners depending on target genes. Whereas Smad4 itself has a DNA binding activity (6–9), a winged-helix DNA-binding protein, FAST1, is required for Smad2/4 complexes to activate activin-inducible Mix.2 gene transcription (10). The general co-activator p300 associates with Smad3 and induces transcriptional activity synergistically (11, 12). In contrast, Ski family oncoproteins (13–15) and a homeodomain protein TGIF (16) bind to Smad proteins and repress transcription through recruiting histone deacetylase complex into TGF-β-activated Smad complexes. AP-1 enhances but oncoprotein Evi-1 represses the TGF-β-induced transcription through binding to Smad3 (17, 18). Moreover, Smad3 has been shown to associate with vitamin D receptor and to act as a co-activator for vitamin D receptor-mediated transcription, implicating a cross-talk between vitamin D and TGF-β signaling pathways (19).

It has recently been shown that Smad signaling is also negatively regulated by a ubiquitin-dependent degradation. The Hect family of E3 ubiquitin-protein ligase Smurf1 interacts with Smad1 and Smad5, and triggers their ubiquitinization and subsequent degradation independently of receptor activation (20). In contrast, ubiquitin conjugating enzymes bind to Smad2 in an activation dependent manner in the nucleus and cause its multi-ubiquitination and subsequent degradation (21).

Filamin 1 (also called ABP-280) is the major human non-muscle isoform of a protein family (22). It is a homodimer of 280 kDa containing N-terminal actin-binding domain and 24 tandem repeats of 96 amino acids. The last repeat, 24, represents the transforming growth factor (TGF)-β receptor, and plays an important role in recruiting Smad2 to the receptor by controlling the subcellular localization of Smad (4). Microtubules bind to Smad2, -3, and -4, and negatively regulate signaling, controlling the rate of Smad2 association to receptor and subsequent phosphorylation (5).

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Filamin 1 (also called ABP-280) is the major human non-muscle isoform of a protein family (22). It is a homodimer of 280 kDa containing N-terminal actin-binding domain and 24 tandem repeats of 96 amino acids. The last repeat, 24, represents self-association domain of the molecule. Filamins are multifunctional proteins (23–28). They efficiently cross-link actin filaments, connect cortical actin filament networks to cell membrane receptors, and act as a scaffold for intracellular proteins involved in signal transduction.

Here we report the identification of filamin as a Smad-binding protein. Filamin-deficient melanoma cells showed impaired TGF-β signaling activity compared with filamin-supplemented cells, evidenced by a decreased signal-dependent Smad2 phosphorylation. The interaction of filamin with Smad family proteins may represent a potential regulatory mechanism in TGF-β superfamily signaling.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—To construct a bait plasmid (pAS-Smad5) for yeast two-hybrid system, a mouse Smad5 cDNA fragment, corresponding to amino acids 5–378 and lacking approximately half of
the MH2 C-terminal domain, was inserted in frame into pAS2–1 GAL4 DNA-binding vector (CLONTECH, Palo Alto, CA). The same cDNA fragment was subcloned in frame into pGBK9 to generate pGBK9-Smad5. A yeast strain, Y190, was co-transformed with pAS-Smad5 and human chondrocyte cDNA library constructed in pACT2 (CLONTECH). The colonies grown on the selection media were screened for LacZ gene transactivation by β-galactosidase activity in filter assay. pLM5 encoding GALA DNA-binding domain/human lamin C fusion protein was used as a negative control. Prey plasmids were recovered from β-galactosidase-positive colonies, and DNA sequence analysis was performed with ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Construction of Expression Vectors—A C-terminal fragment of Smad5 corresponding to amino acids 2163–2647 (ABP-C) was obtained from the positive clones. Various deletion mutants of Smad5 were generated by polymerase chain reaction and subcloned in frame into pGEX-5X-1 (Amersham Pharmacia Biotech, Upsalla, Sweden) or pCDNA3 (InVitrogen, Carlsbad, CA) with HA epitope at N terminus. cDNAs for full-length Smad1, -2, -4, -5, and -6 and deletion mutants of Smad5 were generated by polymerase chain reaction and were subcloned into pCDNA3 with FLAG epitope at the N terminus. All constructs were fully sequenced.

ABP-C, a phosphorylated form of filament, an expression vector for a truncated filament that lacks most of the N-terminal actin-binding domain and repeat 1–13, was generated by excision of Sall/BamHI fragment from pEFBOS-filamin1 (an expression vector for full-length human filament) and self-ligation with an oligonucleotide spacer for adjusting reading frame.

Cell Culture—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 9% fetal bovine serum (FBS). M2, a human melanoma cell line lacking filament, and its subline, A7, transfected stably with a full-length filament cDNA, were cultured in MEM (Life Technologies, Inc.) supplemented with 8% newborn calf serum and 2% FBS (23). A7 cells were cultured in the presence of 0.3 mg/ml G418 (Life Technologies, Inc.) to maintain filament expression.

GST Pull-down Assay—GST and GST fusion proteins were expressed in DH5α induced by 0.25 mM isopropyl-1-thio- β-D-galactopyranoside and purified by affinity chromatography using glutathione-Sepharose beads (Amersham Pharmacia Biotech). [35S]Methionine-labeled Smad5 and its deletion mutants were generated by in vitro transcription/translation system (Promega, Madison, WI). Aliquots were mixed with the GST or GST-ABP-C-conjugated glutathione-Sepharose beads in binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) and then incubated with glutathione-Sepharose beads. Bound proteins were eluted from beads by boiling in SDS sample buffer, separated by SDS-PAGE, and visualized by autoradiography.

Immunoprecipitation and Immunoblotting—HA epitope-tagged ABP-C expression vector (pHA-ABP-C) and FLAG epitope-tagged expression vectors for Smad1–6 (pF-Smad1–6) were co-transfected transiently into HEK293 cells using LipofectAMINE reagent (Life Technologies, Inc.). At 24 h after transfection, cells were lysed with radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) supplemented with a mixture of proteinase inhibitors (Complete™, Roche Molecular Biochemicals, Mannheim, Germany). Pre-cleared cell lysates were subjected to immunoprecipitation with anti-FLAG M2 antibody (Sigma) or anti-HA 3F10 antibody (Roche Biochemicals) following adsorption to protein G-Sepharose beads (Amersham Pharmacia Biotech). In some experiments, agarose-conjugated anti-FLAG antibody and FLAG peptide as a competitor (Sigma) were used. Bound proteins were eluted from beads by boiling in SDS sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Immunoblotting was performed using anti-FLAG M5 antibody (Sigma) or anti-HA 3F10 antibody, and visualized by ECL Plus luminescent imaging system (Amersham Pharmacia Biotech).

Reporter Gene Assay—Co-transfection with the plasmids pCMV-Rluc, Renilla luciferase expression vector (Promega), was used as an internal control for normalization of transfection efficiency. Cells were lysed in Passive Lysis Buffer (Promega) and assayed for firefly and Renilla luciferase activities (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany). Each assay was carried out at least in triplicate.

Immunofluorescence Microscopic Examination—M2 and A7 cells were plated on chamber slides and cultured in MEM with 0.5% FBS for 24 h. Cells were treated with 10 ng/ml human recombinant TGF-β1 (Roche Molecular Biochemicals) for 90 min, fixed with 4% paraformaldehyde, and then permeabilized with methanol. Smad2 proteins were detected by indirect immunofluorescence technique using anti-Smad2 monoclonal antibody (Transduction Laboratories, Lexington, KY) and FITC-conjugated anti-mouse IgG (Sigma). Cells were counterstained with 4,6-diamidino-2-phenylindole to visualize nuclei. Microscopic examination was performed (Axiovert S100, Carl Zeiss, Jena, Germany) and visualized with an image analysis system (Spot Software version 2.2, Diagnostic Instruments, Inc., Sterling Heights, MI).

In Vivo Phosphorylation—M2 and A7 cells were co-transfected with pF-Smad2 and pALK5-TD or pALK5-KR in serum-free medium containing 0.1% BSA. At 24 h after transfection, cells were lysed and immunoprecipitation was performed using antibodies against phosphoserine antibody (Zymed Laboratories Inc., San Francisco, CA). The cell lysates were immunoprecipitated with anti-FLAG antibody, and the precipitates were subjected to immunoblotting as described above. The phosphorylated proteins were detected with anti-phosphoserine antibody (Zymed Laboratories Inc., San Francisco, CA).

RESULTS

Identification of Filamin as a Smad5-interacting Protein—Three million transformants of yeast were screened by interaction trap, and 110 β-galactosidase-positive colonies were obtained using Smad5 as a bait. The prey plasmids were recovered from 47 positive clones and sequenced, resulting in isolation of 4 independent clones encoding the C-terminal portion of filamin. In addition to filamin, clones encoding ABP1, a homologue of filament (29), were isolated. At least two clones encoding Smad4 were also detected (data not shown). These positive clones were re-introduced into yeast with pGBT9-Smad5 to confirm specific interaction. Only pGBT9-Smad5, but not pAS2–1 or pLAM5 showed transactivation of HIS3 and lacZ with these prey plasmids encoding filamin, ABP1, and Smad4 (data not shown).

Sequence analysis of the positive clones revealed that all of the clones encoding filamin started at repeat 20 (Fig. 1), it is reasonable to assume that this region contains a Smad-binding site. The shared domain, encoded by clone 22, was herein termed ABP-C.

Smad5 Binds to Filamin in Vitro—To examine whether Smad5 protein directly binds to filamin, GST pull-down assay was performed using GST-fused ABP-C protein and 35S-labeled Smad5. As shown in Fig. 2A, 35S-labeled Smad5 was precipitated with GST-ABP-C, but not with GST, indicating that the binding of Smad5 is specific to ABP-C. The interacting site in Smad5 was then determined by GST pull-down assay with Smad5 deletion mutants. Smad5-ΔC lacking the MH2 domain bound to GST-ABP-C stronger than full-length Smad5 and Smad5-ΔN, Smad5-MH1, Smad5-ΔN, and Smad5-ΔC bound only weakly to GST-ABP-C and Smad5-MH2 did not (Fig. 2A). The results of binding analysis with Smad5 deletion mutants are summarized in Fig. 2C. Based on these assays, it is suggested...
that a filamin-binding site is located within the MH1 domain and the linker portion of Smad5, which is consistent with the result of yeast two-hybrid screening.

A Smad5-binding site in filamin was also determined using deletion mutants of ABP-C. In GST pull-down assay, GST-ABP-D, which lacks the 24th repeat of filamin, bound to Smad5-DC efficiently or even stronger than GST-ABP-C (Fig. 2), indicating that the repeat is not essential for binding to Smad5. Almost identical results were obtained by yeast two-hybrid system (data not shown). The results of binding analysis with ABP-C deletion mutants are summarized in Fig. 2D.

FIG. 1. Identification of cDNA clones encoding filamin (ABP-280) in yeast two-hybrid screening. Schematic diagram of filamin structure (upper) and four filamin-encoding cDNA clones obtained in yeast two-hybrid screening (lower). The first amino acid residue of filamin in each clone is indicated as one-letter symbol. The C-terminal portion of filamin encoded by clone 22 was termed ABP-C.

Filamin (ABP-280)

A

GST pull-down

clone 19 2142E

clone 15 2159V

clone 22 1663L

clone 38 2173D

B

GST pull-down

10% input

Binding to HA-ABP-C

F-Smad5

ΔN

lN

MH2

ΔC

lC

MH1

I

pAS2 / Smad5

(bait)

D

filamin

repeat1314

20 21 22 23 24

Binding to F-Smad5

GST Yeast pull-down hybrid

APB-C

Δ1

Δ2

Δ3

Δ4

Δ5

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Filamin Binds to Smad Proteins

Filamin (Fig. 3 and 4) with constitutive active type I TGF-β induction of p3TP-lux reporter activity when co-transfected with Smad2 protein per cell was comparable between A7 and M2 cells following TGF-β treatment. The extracts from A7 cells co-transfected with pBIND-Smad5 (GAL4-Smad5 fusion construct) and pG5-luc (GAL4-dependent luciferase reporter construct) showed ~2.5-fold increase in the luciferase activity, compared with those transfected with pBIND control vector and pG5-luc. Whereas M2 cells did not show such increased luciferase activity (data not shown). Although this may suggest that BMP signaling activity was reduced in filamin-defective cells, both M2 and A7 cells failed to enhance ligand-dependent luciferase activity with co-transfection of the constitutive active type I BMP receptor or with BMP4 treatment (data not shown). We therefore switched to TGF-β-responsive p3TP-lux reporter system, because filamin was also shown to be associated with TGF-β-activin-specific Smad2 (Fig. 3D). As shown in Fig. 4A, A7 showed ~8-fold induction of p3TP-lux reporter activity when co-transfected with constitutive active type I TGF-β receptor plasmid, whereas M2 exhibited impaired response (~3-fold). A7 cells also showed ~6-fold induction of p3TP-lux reporter activity in response to TGF-β1 (data not shown).

To rule out the possibility that the difference in the responsiveness to TGF-β was due to the clonal difference between M2 and A7 cells, an expression vector for full-length human filamin, pEFBOS-filamin1, was transfected transiently into filamin-deficient M2 cells, and the reporter gene assay was performed using p3TP-lux. M2 cells restored the response to the constitutive active receptor, dependently on the amounts of transfected pEFBOS-filamin1 (Fig. 4B). M2 cells also restored the response following transfection of N-terminal truncated filamin, suggesting that the actin-binding domain is dispensable for TGF-β signaling.

TGF-β-induced nuclear translocation of Smad2 was also impaired in M2 cells. As shown in Fig. 4C, nuclear accumulation of endogenous Smad2 in response to TGF-β1 was observed in A7 cells, whereas the nuclear accumulation was not seen in M2 cells following TGF-β1 treatment. The expression level of Smad2 protein per cell was comparable between A7 and M2 cells in immunoblotting analysis (data not shown). To examine whether the distribution of filamin may change upon TGF-β treatment, we observed filamin by anti-filamin antibody and fluorescein-labeled secondary antibody in A7 cells with or without TGF-β1 treatment. Filamin was distributed throughout the cytoplasm, occasionally concentrated at filopodia and lamellipodia, with no change in distribution upon TGF-β1 treatment (data not shown).

Impaired Smad2 Phosphorylation in Filamin-deficient Cells—The result of impaired nuclear translocation in M2 cells suggests that Smad2-mediated signaling is impaired at cytoplasmic level in filamin-deficient M2 cells. We therefore compared Smad2 phosphorylation in M2 and A7 cells after stimulation with constitutive active TGF-β receptor. As shown in Fig. 5A, F-Smad2 was serine-phosphorylated in A7 cells following co-transfection with pALK5-TD, whereas phosphorylation of F-Smad2 was impaired in M2 cells.

Next, we investigated phosphorylation of endogenous Smad2 in response to TGF-β1 treatment. The anti-Smad2 immunoprecipitates from [32P]orthophosphate-labeled cells were examined by SDS-PAGE to detect phosphorylation of Smad2. As shown in Fig. 5B, A7 cells increased Smad2 phosphorylation in response to TGF-β1 within 20 min, whereas M2 cells did not.

To investigate whether M2 cells restore Smad2 phosphorylation, the full-length and a truncated filamin lacking N-terminal actin-binding domain were transiently transfected to M2 cells, and the Smad2 phosphorylation were assessed as described above. In agreement with the results of reporter gene assay (Fig. 4B), both the full-length and the N-terminal truncated filamin augmented Smad2 serine phosphorylation in M2 cells upon stimulation with constitutive active TGF-β receptor (Fig. 5C).

DISCUSSION

Subcellular localization, phosphorylation, nuclear translocation, transcriptional activity, and turnover of Smad proteins are thought to be regulated by Smad-interacting molecules. In
the present study we have identified filamin, a cytoskeletal protein, as a Smad-interacting protein, and demonstrated the involvement of filamin in Smad2-mediated TGF-β signaling in the cytoplasm.

Interaction of Smad family proteins with filamin suggested the involvement of filamin in TGF-β superfamily signaling. Filamin-deficient M2 cells showed impaired TGF-β signaling compared with filamin-supplemented A7 cells (Fig. 4, A and C) and the defect in M2 cells was shown to be due to decreased Smad2 phosphorylation in response to TGF-β stimulation (Fig. 5, A and B). The reduced TGF-β signaling activity was restored following supplement of filamin by transfection (Figs. 4B and 5C). The N-terminal truncated filamin also rescued TGF-β responsiveness in M2 cells, indicating that the binding of filamin to actin with subsequent actin network reorganization and stabilization is dispensable for the function of filamin at least in TGF-β signaling. Since phosphorylation of Smad is recognized as a trigger for Smad-mediated signaling, interaction with filamin may be essential for effective Smad phosphorylation in TGF-β superfamily signaling pathway. However, it is also possible that nuclear translocation of Smads is impaired in M2 cells, through distinct mechanism from phosphorylation of Smad protein.

How does filamin work in the phosphorylation of Smad? There are several possible explanations for the potential function of filamin in Smad pathway (Fig. 5D). First, filamin may serve as an anchor protein, like SARA, to control localization of Smad proteins near the cell surface receptors. Supporting this idea is the observation that filamin is concentrated in cortical regions of the cytoplasm tethered by certain cell surface molecules (25, 27, 30). Second, filamin-associated Smad proteins may keep their conformation suitable for receptor-mediated phosphorylation. MH1 domains of Smad2 and Smad4 can interact with their corresponding MH2 domains to form inactive conformation (31), and association with filamin may interrupt this self-association to promote phosphorylation or subsequent
Filamin Binds to Smad Proteins

hetero-oligomerization. There is a possibility that filamin protects Smad proteins from ubiquitin-dependent degradation by masking a ubiquitin ligase-binding site of Smads. However, the expression level of Smad2 in both M2 and A7 cells did not change following treatment with a proteasome inhibitor, MG132 (data not shown), which suggest that absence of filamin did not cause an increased turnover of Smad2 protein in M2 cells.

It was recently reported that cytoskeletal microtubules bind to Smad proteins, and negatively regulate the signaling (5). Our study strongly suggests that filamin, which is localized in the juxtamembrane region where receptors and their signaling mediators interact, work positively on Smad phosphorylation. Although it remains a possibility that cytoplasmic filamin far from cell surface membrane may negatively regulate Smad signaling like microtubules, filamin is essential for maximal function of Smad-mediated signaling, evidenced by the results from the experiments using filamin-deficient cells. It is suggested that the mode of the regulation in Smad signaling is different between these cytoskeletal elements, filamins and tubulins, depending on their cytoplasmic organization and localization.

A variety of proteins have been reported to interact with filamin, including cell surface protein integrin β chains (24, 30, 32), glycoprotein Ibα (33), immunoglobulin G Fc receptor I (34), tissue factor (25), presenilin-1 (35), processing enzyme furin (28), cytoplasmic MAPK protein SEK1 (36), the small GTPase RALA (27), and tumor necrosis factor receptor-associated factor 2 (37). Among these molecules, integrin β chains, presenilin-1, and SEK1 bind to a portion of filamin similar to the Smad-binding site identified in current study, namely from repeat 20 to repeat 23 or 24.

Accumulating evidence suggests that MAPK cascade closely correlates with TGF-β superfamily signaling. The extracellular signal-regulated kinase subfamily of MAPK phosphorylates specific sites in the linker region of Smads, thereby inhibiting (38, 39), or activating (40) nuclear translocation. MAPK cascade is also included by TGF-β signaling itself. It was reported that c-Jun N-terminal kinase is also activated in a Smad-independent manner (41, 42). p38 is activated by TGF-β superfamily stimulation in rat pheochromocytoma cells (43), human gingival fibroblasts (44), and developing Drosophila wing (45), possibly via TAK1-MKK6/3-p38 cascade (46). c-Jun N-terminal kinase-activated AP-1 complex and p38-activated ATF-2 work synergistically with Smad complex in TGF-β-induced gene transcription (47, 48). Interestingly, SEK1, which binds to the C-terminal region of filamin, is activated by TAK1 (49). Therefore, it is possible that filamin mediate cross-talk between Smad and MAPK pathways.

On the other hand, cross-talk between integrins and TGF-β signaling system has also been reported. Expression of TGF-β receptors is controlled by ligand-activated integrins in breast cancer (50) and osteoblastic (51, 52) cells. In bleomycin-induced pulmonary fibrosis, integrin αvβ6 binds to latency-associated peptide and induces TGF-β activation to induce pulmonary inflammation (53). MAPK is activated by integrin stimulation in fibroblasts (54) and osteoblasts (55) through focal adhesion protein-tyrosine kinase activation. Since MAPK phosphorylates linker portion of Smads and regulates their nuclear trans-

Fig. 5. Defective Smad2 phosphorylation in filamin-deficient M2 cells. A, M2 and A7 cells were co-transfected with pF-Smad2 and pALK5-TD (TD), pALK5-KR (KR), or pcDNA3 (C). 24 h after transfection, cells were lysed, immunoprecipitated (IP) with anti-FLAG antibody, followed by immunoblotting with anti-phosphoserine antibody (upper panel) or anti-FLAG antibody (lower panel). B, M2 and A7 cells were labeled with [32P]orthophosphate for 3 h and treated with TGF-β1 (10 ng/ml) for 20 min. Cell lysates were immunoprecipitated with anti-Smad2 antibody and resolved by SDS-PAGE (upper panel). Lysates of unlabeled cells treated with TGF-β1 in the same way were immunoprecipitated and immunoblotted with anti-Smad2 antibody to evaluate the amounts of immunoprecipitated Smad2 protein (lower panel). C, M2 and A7 cells were co-transfected with pF-Smad2 and pALK5-TD, lysed, immunoprecipitated, and immunoblotted in the same way as A. M2 cells were also co-transfected with pEFBOS-filamin1 (F), pEFBOS-filamin-ΔN (ΔN), or pcDNA3 (C). D, schematic representation of a potential function of filamin in Smad phosphorylation. Filamin may serve as an anchor protein to recruit Smad proteins to control its localization near the cell surface receptors (a). Alternatively, filamin may prevent Smad proteins to undergo inactive conformation induced by self-association, and thereby help phosphorylation or subsequent hetero-oligomerization (b).
location, it is possible that activated integrin modulates Smad-mediated signaling via MAPK pathway. It has been recently shown that periventricular heterotopia (PH), a human X-linked dominant disorder, is caused by a mutation of the filamin1 gene (55). Most patients of PH suffer from seizures because a subset of neuron cells fail to migrate into developing cerebral cortex and persist as nodules of neurons lining the ventricular surface. PH patients also show shortened digits, syndactyly, and clinodactyly. These anomalies have also been observed in impairment of TGF-β superfamily protein (56, 57), and defective Smad-mediated signaling due to mutation of the gene (55). Most patients of PH suffer from seizures because a subset of neuron cells fail to migrate into developing cerebral cortex and persist as nodules of neurons lining the ventricular surface.

In conclusion, we have identified a cytoskeletal protein, filamin, as a Smad-associating protein, and demonstrated this interaction plays an important role in receptor-mediated phosphorylation of Smad. In view of its association with various mediators of intracellular signaling, filamin may provide a scaffold of cross-talk between TGF-β superfamily and other signal transduction pathways.

Acknowledgments—We thank Dr. Kohei Miyazono (University of Tokyo, Tokyo, Japan) for providing p7TP-lux, pALK5-TD, and pALK5-KR, and Miho Kamiya (National Institute for Longevity Sciences, Aichi, Japan) for technical assistance.

REFERENCES

1. Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) Nature 390, 465–471
2. Whitman, M. (1998) Genes Dev. 12, 2445–2462
3. Derynck, R., Zhang, Y., and Feng, X. H. (1998) Cell 95, 737–749
4. Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L., and Wrana, J. L. (1998) Cell 95, 779–790
5. Dong, C., Li, Z., Alvarez, R., Jr., Feng, X. H., and Goldschmidt-Clermont, P. J. (2000) Mol. Cell 5, 27–34
6. Yingling, J. M., Datta, M. B., Wong, C., Frederick, J. P., Liberati, N. T., and Wang, X. F. (1997) Mol. Cell. Biol. 17, 7019–7028
7. Zawel, L., Dai, J. L., Buckhaults, P., Zhou, S., Kinzler, K. W., Vogelstein, B., and Kern, S. E. (1998) Mol. Cell 1, 611–617
8. Song, C. Z., Siok, T. E., and Gelehrter, T. D. (1998) J. Biol. Chem. 273, 29287–29290
9. Denli, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) EMBO J. 17, 3091–3100
10. Chen, X., Rubock, M. J., and Whitman, M. (1996) Nature 383, 691–696
11. Feng, X. H., Zhang, Y., Wu, R. Y., and Derynck, R. (1998) Genes Dev. 12, 2153–2163
12. Janknecht, R., Wells, N. J., and Hunter, T. (1998) Genes Dev. 12, 2114–2119
13. Luo, K., Stroschein, S. L., Wang, W., Chen, D., Martens, E., Zhou, S., and Zhou, Q. (1999) Genes Dev. 13, 2196–2206
14. Sun, Y., Liu, X., Eaton, E. N., Lane, W. S., Lodish, H. F., and Weinberg, R. A. (1999) Mol. Cell 4, 499–509
15. Stroschein, S. L., Wang, W., Zhou, S., Zhou, Q., and Luo, K. (1999) Science 286, 771–774
16. Wotton, D., Lo, R. S., Lee, S., and Massague, J. (1999) Cell 97, 29–39
17. Zhang, Y., Feng, X. H., and Derynck, R. (1998) Nature 394, 909–913
18. Kurokawa, M., Mitani, K., Irie, K., Matsuyama, T., Takahashi, T., Chiba, S., Yazaki, Y., Matsumoto, K., and Hirai, H. (1998) Nature 394, 92–96
19. Yanagisawa, J., Yanagi, Y., Masuhira, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., Toriyabe, T., Kawahata, M., Miyazono, K., and Kato, S. (1999) Science 283, 1317–1321
20. Zhu, H., Kavas, P., Abdullah, S., Wranja, L. J., and Thomsen, G. H. (1999) Nature 400, 687–693
21. Lo, R. S., and Massague, J. (1999) Nat. Cell Biol. 1, 472–477
22. Gorlin, J. B., Yamin, R., Egan, S., Stewart, M., Stossel, T. P., Kwaikowski, D. J., and Hartwig, J. H. (1990) J. Biol. Chem. 265, 1089–1105
23. Cunningham, C. C., Gorlin, J. B., Kwaikowski, D. J., Hartwig, J. H., Janney, P. A., Byers, H. R., and Stossel, T. P. (1992) Science 255, 325–327
24. Glogauer, M., Arora, P., Chou, D., Janney, P. A., Downey, G. P., and McCulloch, C. A. (1998) J. Biol. Chem. 273, 1689–1698
25. Ott, I., Fischer, E. G., Miyagi, Y., Mueller, B. M., and Ruf, W. (1998) J. Cell Biol. 140, 1241–1253
26. Derynck, R., Zhang, Y., and Feng, X. H. (1998) Cell 95, 737–749
27. Thomas, J. T., Lin, K., Nandedkar, M., Camargo, M., Cervenka, J., and Luyten, P. F. (1996) Nat. Genet. 12, 315–317
28. Thomas, J. T., Kilpatrick, M. W., Lin, K., Eralcher, L., Lembessis, P., Costa, T., Tsipouras, P., and Luyten, P. F. (1997) Nat. Genet. 17, 58–64

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24. Glogauer, M., Arora, P., Chou, D., Janney, P. A., Downey, G. P., and McCulloch, C. A. (1998) J. Biol. Chem. 273, 1689–1698
25. Ott, I., Fischer, E. G., Miyagi, Y., Mueller, B. M., and Ruf, W. (1998) J. Cell Biol. 140, 1241–1253
26. Derynck, R., Zhang, Y., and Feng, X. H. (1998) Cell 95, 737–749
27. Thomas, J. T., Lin, K., Nandedkar, M., Camargo, M., Cervenka, J., and Luyten, P. F. (1996) Nat. Genet. 12, 315–317
28. Thomas, J. T., Kilpatrick, M. W., Lin, K., Eralcher, L., Lembessis, P., Costa, T., Tsipouras, P., and Luyten, P. F. (1997) Nat. Genet. 17, 58–64

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17877

24. Glogauer, M., Arora, P., Chou, D., Janney, P. A., Downey, G. P., and McCulloch, C. A. (1998) J. Biol. Chem. 273, 1689–1698
25. Ott, I., Fischer, E. G., Miyagi, Y., Mueller, B. M., and Ruf, W. (1998) J. Cell Biol. 140, 1241–1253
26. Derynck, R., Zhang, Y., and Feng, X. H. (1998) Cell 95, 737–749
27. Thomas, J. T., Lin, K., Nandedkar, M., Camargo, M., Cervenka, J., and Luyten, P. F. (1996) Nat. Genet. 12, 315–317
28. Thomas, J. T., Kilpatrick, M. W., Lin, K., Eralcher, L., Lembessis, P., Costa, T., Tsipouras, P., and Luyten, P. F. (1997) Nat. Genet. 17, 58–64