Disabled-2 (Dab2) Mediates Transforming Growth Factor β (TGFβ)-stimulated Fibronectin Synthesis through TGFβ-activated Kinase 1 and Activation of the JNK Pathway*

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The multifunctional cytokine transforming growth factor β (TGFβ) exerts many of its effects through its regulation of extracellular matrix components, including fibronectin (FN). Although expression of both TGFβ and FN are essential for embryonic development and wound healing in the adult, overexpression leads to excessive deposition of extracellular matrix observed in many fibroproliferative disorders. We previously have demonstrated that TGFβ-stimulated FN induction requires activation of the c-Jun N-terminal kinase (JNK) pathway; however, the signaling molecules that link the TGFβ receptors to the JNK pathway remain unknown. We show here that the cytosolic adaptor protein disabled-2 (Dab2) directly stimulates JNK activity, whereas stable small interfering RNA-mediated ablation of Dab2 in NIH3T3 mouse fibroblasts and A10 rat aortic smooth muscle cells demonstrates that its expression is required for TGFβ-mediated FN induction. We demonstrate that TGFβ treatment stimulates the association of Dab2 with the mitogen-activated protein kinase kinase kinase, TAK1. Attenuation of cellular TAK1 levels by transient double-stranded RNA oligonucleotide transfection as well as overexpression of kinase-deficient TAK1 leads to abrogation of TGFβ-stimulated FN induction. Furthermore, cell migration, another JNK-dependent response, is attenuated in NIH3T3-siDab2-expressing clones. We, therefore, delineate a signaling pathway proceeding from the TGFβ receptors to Dab2 and TAK1, leading to TGFβ-stimulated JNK activation, FN expression, and cell migration.

Fibronectin (FN),¹ a widely expressed component of the extracellular matrix (ECM), is a dimeric glycoprotein whose ability to promote cell adhesion and migration plays a key role in signaling events that regulate cell growth and differentiation (1–3). Fibronectin expression is essential for embryogenesis and angiogenesis and modulates wound healing, host defense, and maintenance of tissue homeostasis in the adult (2, 4). The cytokine transforming growth factor β (TGFβ) controls cellular growth, proliferation, adhesion, and migration in part due to its ability to modulate expression of cell surface adhesion receptors and ECM protein expression, including FN (5, 6).

TGFβ, the founding member of the TGFβ superfamily, mediates its effects through two Ser/Thr kinase receptors, termed the TGFβ type I (TβRI) and II (TβRII) receptors (7). TGFβ ligand binding to TβRII triggers recruitment of TβRII, thereby forming an active receptor complex. Further propagation of TGFβ signaling can then be initiated through Smad-dependent or Smad-independent pathways, such as the mitogen-activated protein kinase (MAPK) pathway (8, 9). Signaling through the Smad family of proteins is triggered by phosphorylation of the receptor-activated Smads by TβRI. In the case of Smad2 and Smad3, the TGFβ- and activin-specific receptor-activated Smads, this phosphorylation can be facilitated by SARA (Smad anchor for receptor activation) (10) or Disabled-2 (Dab2) (11). After phosphorylation, receptor-activated Smads dimerize with the required co-Smad, Smad4, to translocate to the nucleus, and activate gene transcription by either directly binding to DNA or through cooperation with other DNA binding transcription factors such as FAST-1 (12), FAST-2 (13), c-Jun (14, 15), ATF-2 (16), and TFE3 (17).

Members of the MAPK family have also been shown to be activated after TGFβ stimulation, including the extracellular signal-regulated kinase (ERK) (18, 19), c-Jun N-terminal kinase (JNK) (20, 21), and the p38 kinase pathways (22). We previously have demonstrated that TGFβ-mediated induction of FN is mediated through a JNK-dependent, Smad-independent pathway (21). Consistent with this observation, mouse fibroblasts derived from Smad2-, Smad3-, and Smad4-deficient embryos retain the ability to induce FN synthesis after TGFβ stimulation (23, 24). Furthermore, efficient activation of the Smad pathway may require activation of JNK, in that fibroblasts that express antisense JNK oligonucleotides show decreased TGFβ-stimulated Smad2 phosphorylation (25), whereas JNK has been shown to directly phosphorylate Smad3, leading to its activation and nuclear accumulation (26).

Activation of JNK is triggered by dual phosphorylation of a TYP motif by the upstream MAPK kinases MKK7 and MKK4, whereas activation of these MAPK kinases can be mediated through multiple MAPK kinase kinases including members of the MAPK/extracellular signal-regulated kinase (MEK) kinase (MEKK), mixed-lineage kinase, and apoptosis signal-regulating kinase families,Tpl2, and TGFβ-activated kinase 1 (TAK1) (27). TAK1 was initially identified as a kinase whose

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1 The abbreviations used are: FN, fibronectin; ECM, extracellular matrix; TGF, transforming growth factor; TAK1, TGFβ-activated kinase 1; TAB1, TAK1-binding protein; PTB, phosphotyrosine binding domain; si-, small interfering; TAKKW, kinase-deficient form of TAK1; Dab2, Disabled-2; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; TβRI and TβRII, TGFβ type I and type II, respectively; HA, hemagglutinin; GST, glutathione S-transferase.

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activity was stimulated by TGFβ and BMP4, a TGFβ superfamily member, resulting in phosphorylation of MKK4 (28). TAK1 has subsequently been shown to be involved in interleukin-1- and Wnt-mediated signal transduction (29–31) and can activate the p38 pathway by phosphorylation of MKK3 and MKK6 (32). Activation of TAK1 requires interaction with TAB1 (TAK1-binding protein) (33), which also binds to the X-linked inhibitor of apoptosis (34). TAB1 has been shown to associate with the type I bone-morphogenetic protein receptor when the X-linked inhibitor of apoptosis is co-expressed, suggesting a possible link of the receptor complex to TAK1. However, this association may involve another interacting protein since yeast two-hybrid analysis failed to demonstrate a direct interaction between X-linked inhibitor of apoptosis and type I BMP receptor (34).

Dab2, a member of the Disabled gene family, is a widely expressed adaptor molecule shown to be involved in several receptor-mediated signaling pathways (11, 35, 36). Disabled family members possess a highly conserved N-terminal phosphotyrosine-interacting/phosphotyrosine binding domain (PID/PTB), recently renamed the DAB homology domain (37), linked to divergent C-terminal sequences. Dab2 directly binds to several members of the lipoprotein receptor family through its PTB domain (35, 36, 38), whereas its association with clathrin, the clathrin adaptor protein AP2, and myosin VI, mediated by sequences in the linker and C-terminal regions of Dab2, facilitates clathrin-coated pit assembly and receptor-mediated endocytosis (35, 39, 40). We have recently demonstrated that Dab2 serves as an adaptor molecule to link the TGFβ receptors to activation of the Smad pathway (11). Expression of Dab2 restored Smad-dependent responses to a TGFβ-signaling-deficient mutant cell line. In addition, Dab2 was found to augment TGFβ-stimulated FN induction, a JNK-dependent and Smad-independent response (11). We, therefore, wished to explore the possibility that Dab2 could directly mediate TGFβ-stimulated JNK activity, leading to induction of FN.

We report here that stable small interfering RNA (siRNA)-mediated silencing of Dab2 in NIH3T3 mouse fibroblasts leads to decreased TGFβ-stimulated FN induction, concomitant with decreased TGFβ-mediated JNK activity. Dab2 is shown to directly stimulate JNK activity, which is blocked by expression of a kinase-deficient form of TAK1, TAKKW. We also report that abrogation of TAK1 expression or TAKKW overexpression leads to decreased TGFβ-stimulated FN protein and mRNA induction, respectively. At a biological level we provide evidence suggesting that Dab2 is required for TGFβ-stimulated cellular migration, a JNK-dependent response. Dab2, thus, provides a link between the activated TGFβ receptors and TAK1, leading to activation of the JNK-signaling pathway.

MATERIALS AND METHODS

Reagents—Recombinant TGFβ2 was generously provided by Genzyme (Cambridge, MA). SP600125 (JNK Inhibitor II) and SB203580 were purchased from Calbiochem, and PD98059 was purchased from Alexis. The following primary antibodies were purchased from Santa Cruz Biotechnology: mouse α-HA (sc-7392), mouse α-myv (sc-40), rabbit α-TAK1 (sc-7162), mouse α-TAK1 (sc-7967), rabbit α-HSP 90 (sc-7947), and rabbit α-JNK1 (sc-474). Mouse monoclonal antibody to Diablated-2/p96 was purchased from BD Transduction Laboratories. The mouse α-FLAG-M2 antibody and gelatin-agarose was obtained from Sigma. Rabbit polyclonal phospho-c-Jun (Ser63) antibody II was purchased from Cell Signaling Technology, and rabbit α-crat FN antibody was from Invitrogen.

Plasmid Construction—Construction of the FLAG-tagged human Dab2 in the RK5 plasmid has been described previously (11). The various domain constructs of human Dab2 were generated by standard PCR methods using the full-length construct as template and inserted into the RK5 vector containing a FLAG epitope. Sequences used for PCR amplification are available upon request. For construction of the stable siDab2 construct, forward and complementary reverse primers corresponding to mouse p96 (5′-CAATAGGATGCTACCTCGT-3′) flanked by AA at the 5′-end and TT at the 3′-end were synthesized, annealed, and ligated into the pSUPER expression vector as described (41). The pFLAG-tagged JNK construct was provided by Roger Davis (42), and the HA-tagged wild-type TAK1, kinase-deficient TAK1 (TAKKK6M), constitutively active TAK1 (TAK1ΔN) (29), and Myc-tagged TAB1 (34) were provided by E. Nishida. The pPur vector was purchased from Clontech.

Cell Culture and Generation of Cell Lines—NIH3T3 and COS7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum. A10 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. To generate cells that stably express the siDab2 construct, NIH3T3 and A10 cells in 100-mm-diameter plates were transfected with the siDab2 construct (10 μg) and pPur (2 μg) utilizing FuGENE 6 (Roche Diagnostics) or Lipofectamine PLUS (Invitrogen), respectively, as per the manufacturer’s instructions. Stable transfectants were selected in a medium containing Dulbecco’s modified Eagle’s medium supplemented with 1 μg/ml puromycin and maintained in the same media either as a pool (A10) or individual clones (NIH3T3). Expression levels of Dab2 were assessed by Western analysis utilizing the monoclonal α-p96 antibody. To generate the 3T3-TAKKW cell line, NIH3T3 cells were co-transfected with TAKKW (10 μg) and pPur (2 μg) constructs using FuGENE 6 (Roche Diagnostics) and maintained in 10% newborn calf serum and 1 μg/ml puromycin. Expression of the TAKKW construct was verified by Western analysis utilizing a monoclonal α-HA antibody.

Preparation of Cell Lysates, Immunoprecipitation, and Protein Kinase Assays—For immunoprecipitation and Western blot analysis, cells were lysed in buffer D (20 mM Tris, pH 7.5, 1% Triton X-100, 10% glycerol, 1% SDS, 2 mM EDTA, 150 mM β-glycerophosphate, 1 mM Na3VO4, and Complete EDTA-free protease inhibitor mixture; Roche Diagnostics), and immunoprecipitation was carried out as previously described (21). For JNK assays in COS7 cells, transient transfection of the indicated constructs was performed utilizing FuGENE 6 (Roche Diagnostics) according to the manufacturer’s protocols. After 48 h cells were lysed in buffer D. Equal amounts of cellular protein (250 μg) were co-precipitated with α-FLAG antibody and protein G-Sepharose beads. The beads were subsequently washed twice with buffer D followed by 1 wash in kinase assay buffer (25 mM HEPES, pH 7.5, 25 mM β-glycerophosphate, 25 mM MgCl2, 2 mM dithiothreitol, 0.1 mM Na3VO4, and Complete EDTA-free protease inhibitor mixture; Roche Diagnostics). Kinase reactions were performed in 30 μl of kinase buffer containing 1 μg GST-c-Jun (Stratagene), 50 μM ATP, and 2 μCi [γ-32P]ATP. Reactions were incubated at 30 °C for 20 min and terminated by the addition of an equal volume of 2× Laemmli sample buffer. Samples were resolved on 10% SDS/PAGE gels, dried, and visualized by autoradiography. The JNK assay in NIH3T3 and 3T3-siDab2 clones was performed as described previously (32) after stimulation with 5 ng/ml TGFβ for various times. Reaction products were resolved on 10% SDS/PAGE gels, transferred to Immobilon membranes, and subjected to Western blot analysis with α-phospho-c-Jun (Ser-63) antibody II.

Fibronectin Protein Assay—TGFB-stimulated FN induction was determined as previously described (21), with the exception that in some assays gelatin-agarose beads (Sigma) were used to precipitate 35S-labeled FN from the media of labeled cells. Samples were resolved on 6% SDS/PAGE gels, subjected to fluorography using Enhance (Amer sham Biosciences), and visualized by autoradiography. The inhibitors SP600125 (10 μM and 50 μM), SB203580 (10 μM), or PD98059 (10 μM) were added simultaneously with TGFβ (2 ng/ml) at the initiation and maintained throughout the FN assay.

Transient siRNA-mediated Silencing of TAK1—The negative control, TAK1 pool, and individual double-stranded RNA oligos to TAK1 were purchased from Dharmacon RNA Technologies (SMARTpool Plus, Q-004500-00-09) and resuspended in reagents provided by the manufacturer. NIH3T3 cells were transfected in 6-well plates (2×105 cells/well) using Oligofectamine reagent (Invitrogen) with 200 nM concentrations each of double-stranded RNA oligo per manufacturer’s instructions. After 48 h of siRNA treatment, the FN assay was initiated as described above. FN protein synthesis was analyzed from the media, whereas cell lysates from the same wells were analyzed for TAK1 expression by Western analysis.

Northern blot Analysis—Total RNA was isolated from NIH3T3, 3T3-siDab2 clone 8, and 3T3-TAKKW cells after treatment with 5 ng/ml TGFβ for the various times indicated using TRIzol reagent (Invitrogen) per the manufacturer’s instructions. Total RNA (40 μg) of each sample was resolved in a 1.2% formaldehyde/agarose gel, transferred to nitro-
Dab2 Mediates TGFβ-stimulated JNK Activation

RESULTS

Dab2 Expression Is Required for TGFβ-stimulated FN Induction—We have previously shown that TGFβ-stimulated FN induction is greatly abrogated in a TGFβ-signaling-deficient cell line, derived from the human HT1080 fibrosarcoma cell line, which expresses a mutant form of Dab2 (11, 21). We, therefore, wished to test whether Dab2 expression is required for TGFβ-mediated FN induction in two different cell types that respond to TGFβ stimulation, namely fibroblast and smooth muscle cells. To ablate the expression of Dab2, we chose to use stable expression of a siRNA specifically targeted to decrease expression of the p96 splice-form of Dab2, which we previously have shown to restore TGFβ signaling to a mutant cell line (11). We have previously characterized this siRNA-Dab2 vector in several cell lines with regard to its specificity for the p96 form of Dab2 (71). Stable introduction of the siDab2 construct into mouse fibroblasts NIH3T3 and rat A10 aortic smooth muscle cells was performed. As shown by Western analysis in Fig. 1A, the siDab2 construct was able to decrease the p96, but not p67, form of Dab2 expression in individual clones of NIH3T3 cells (left panel) and in a pool of A10 (right panel) cells to varying extents. Analysis of these clones for induction of FN synthesis after TGFβ stimulation reveals a dose dependence of Dab2 expression for both basal and TGFβ-mediated FN induction, in that clones that express the lowest levels of Dab2 (i.e. clone 8 and 9) also demonstrate the lowest basal and TGFβ-stimulated FN protein induction (Fig. 1B). FN protein expression was also assessed in wild-type and the siDab2-expressing A10 cells after stimulation with TGFβ for 4 and 24 h. Similarly to NIH3T3 cells, TGFβ-mediated FN induction was markedly attenuated in A10 cells with decreased Dab2 expression (Fig. 1C). These results, thus, demonstrate that Dab2 expression is required for TGFβ-mediated FN induction in two different cell types that can be physiologically stimulated to express FN after TGFβ treatment, which are fibroblast and smooth muscle cells.

Dab2 Directly Stimulates JNK Activity—We have previously shown that TGFβ stimulation of human HT1080 fibrosarcoma cells leads to activation of the JNK pathway and that TGFβ-stimulated induction of FN could be blocked by overexpression of dominant-negative JNK and MKK4 (21). We, therefore, wished to assess whether Dab2 could directly activate the JNK pathway, since we observed that TGFβ-stimulated FN induction was dependent on expression of Dab2. In addition, we wished to assess the contribution of the individual domains of Dab2 to JNK activation, because we have previously demonstrated that restoration of TGFβ signaling to a TGFβ-signaling-deficient cell line requires both the N-terminal PTB and C-terminal proline-rich domains of Dab2 (11). To assess this, COS7 cells were transiently transfected with JNK along with full-length Dab2 or various constructs containing the different domains of Dab2 (Fig. 2, A and C). As shown in Fig. 2B, expression of full-length Dab2 is capable of efficiently stimulating JNK activity that was assessed by the ability of JNK to phosphorylate GST-c-Jun in an in vitro kinase assay. Furthermore, the construct bearing only the C-terminal portion of Dab2, designated #323 consisting of amino acids 323–770, stimulates JNK activity to the same extent as full-length (Fig. 2B). Expression of a construct bearing the proline-rich domain of Dab2, #534, consisting of amino acids 534–770, however, is not sufficient to stimulate JNK activity even though it is expressed at the same level as full-length Dab2 (Fig. 2C). These results demonstrate that Dab2 can directly mediate JNK activation and that this activity can be localized to the C-terminal domain of Dab2.

TGFβ-stimulated FN Induction Requires JNK Activity—The results presented above demonstrate the requirement of Dab2 in TGFβ-stimulated FN induction and suggest that Dab2 can modulate JNK activity. We next wished to assess whether TGFβ-stimulated JNK activation is affected by the level of Dab2 expression. To determine this, parental NIH3T3 and the siDab2 expressing clone #9 were stimulated with TGFβ for various times followed by assessment of JNK activation using GST-c-Jun as a substrate. As depicted in Fig. 3A, JNK activation in response to TGFβ is observed within 5 min in the

cellulose, and subjected to Northern analysis utilizing probes to FN (45) and cyclophilin (1B15) (43) as previously described (21).

Wound Closure Assay—For the wound closure assay, confluent monolayer cells in 60-mm plates were wounded by manual scraping with a pipette tip. Plates were washed several times to remove non-adherent cells and placed in complete media with or without TGFβ (5 ng/ml). Assessment of cell migration into the wounded area was performed by microscopy after 24 h. Cell migration of NIH3T3 and 3T3-siDab2 cells was quantitated utilizing the QCM™ 24-well colorimetric cell migration assay (Chemicon International) as suggested by the manufacturer. Briefly, 2×10⁵ cells resuspended in serum-free media were added to the upper chamber and allowed to migrate through a membrane insert with an 8-μm pore size for 4 h toward 10% fetal bovine serum-containing media in the absence or presence of 5 ng/ml TGFβ. After the incubation period, the cells on the upper surface of the membrane were removed, cells that had migrated through the membrane were stained, and 5 random fields were counted by microscopy at 250× magnification.

FIG. 1. Dab2 is required for TGFβ-stimulated FN induction. A, the siDab2 construct silences expression of the p96 form of Dab2. NIH3T3 (left panel) and A10 (right panel) cells were transfected with the stable siDab2 construct followed by selection of individual clones (3T3) or pool of clones (A10). Lysates were examined for expression of Dab2 by Western analysis using a monoclonal antibody to Dab2. The p96 and p67 isoforms of Dab2 are indicated by the arrows. B and C, decreased Dab2 expression leads to decreased TGFβ-stimulated FN induction. FN induction after TGFβ stimulation was assayed in NIH3T3, individual 3T3-siDab2 clones (B), A10, and A10-siDab2 cells (C) by immunoprecipitation of 35S-labeled FN from the media of metabolically labeled cells. Immuno-complexes were resolved on 6% SDS/PAGE gels, subjected to fluorography, and visualized by autoradiography. The position of FN is indicated by the arrow. FN assays were performed a minimum of three times for each cell line; shown is a representative experiment.
NIH3T3 cells, with maximal activation occurring at 2 h, and a secondary wave of activity persisting through 24 h. In contrast, JNK activation in response to TGFβ/H9252 is greatly attenuated in cells expressing the siDab2 construct (3T3-siDab2#9). This lack of JNK activation in cells deficient in Dab2 expression cannot be attributed to a decrease in expression of the p54 and p46 forms of JNK, which are equivalent in both the wild-type and siDab2-expressing cell lines (Fig. 3B).

Because TGFβ has been shown to activate all three branches of the MAPK family in various cell lines, we wished to confirm the requirement for TGFβ-mediated JNK activation in the induction of FN synthesis. To address this, we utilized pharmacological inhibitors that have been shown to specifically inhibit JNK (SP600125 (44)), p38 (SB203580 (45)), or MEK1 (PD98059 (46)), the upstream activator of extracellular signal-regulated kinase, in FN assays. As shown in Fig. 3C, treatment of NIH3T3 cells with the specific JNK inhibitor SP600125 significantly abrogates TGFβ-stimulated FN induction in a dose-dependent manner. In contrast, neither the p38-specific nor the MEK1-specific inhibitor had any significant effect on TGFβ-mediated FN induction (Fig. 3C). These results demonstrate that Dab2 expression is required for TGFβ-mediated JNK activation that in turn modulates induction of FN expression.

**TAK1 Associates with Dab2 and Mediates Dab2-stimulated JNK Activation**—We previously have demonstrated the involvement of MKK4 as an upstream activator of JNK in modulation of TGFβ-stimulated FN induction (21); however, the upstream MAPK kinase kinase that activates MKK4 has not been determined. TAK1 is a potential candidate to mediate activation of MKK4, as TAK1 is activated by TGFβ treatment and has been shown to phosphorylate MKK4 both in vitro and in vivo (29, 47). To investigate if TAK1 is involved in mediating JNK activation stimulated by Dab2, we transiently transfected COS7 cells with JNK and either wild-type (WT) or kinase-deficient (KW) forms of TAK1 in the presence or absence of Dab2 and assessed for activation of the JNK pathway. As seen in Fig. 4A, transfection of Dab2 leads to JNK activation as monitored by phosphorylation of GST-c-Jun in an in vitro JNK assay. Expression of wild-type TAK1 by itself is seen to lead to efficient JNK activation, which is further increased by the co-expression of Dab2. In contrast, transfection of the kinase-deficient form of TAK1, TAKKW, eliminates activation of JNK stimulated by Dab2 (Fig. 4A). In addition, we observe that Dab2 exists as a doublet when co-transfected with JNK, which collapses to a single band when TAKKW is co-expressed (Fig. 4B), suggesting that Dab2 itself may be phosphorylated by JNK.

To extend the observation that TAK1 is able to modulate Dab2-mediated JNK activation, we asked whether TAK1 and Dab2 associate in vivo. NIH3T3 cells were stimulated with TGFβ for the indicated times followed by immunoprecipitation with α-TAK1 and subsequent Western analysis with α-Dab2 antibody. As shown in Fig. 4C, TGFβ treatment results in a time-dependent association of endogenous Dab2 and TAK1. These results, thus, demonstrate a time-dependent TGFβ-stimulated association between endogenous TAK1 and Dab2 and TGFβ-stimulated JNK activation.

**Fig. 2.** Dab2 directly stimulates JNK activity. A, diagrammatic representation of the various constructs of Dab2. Depicted are the full-length and deletion constructs of Dab2 containing the N-terminal PTB domain and the C-terminal proline-rich domain. FL, full-length. B, Dab2-induced JNK activation is mediated by its C-terminal domain. COS7 cells were transiently co-transfected with FLAG-tagged JNK (FL) and the various FLAG-tagged expression constructs of Dab2 as indicated. Lysates were immunoprecipitated with α-FLAG antibody and subjected to JNK kinase assays utilizing GST-c-Jun as substrate. Phosphorylated GST-c-Jun was detected by autoradiography and is indicated by the arrow. The JNK assay was performed six independent times; shown is a representative experiment. C, the expression of JNK and the various Dab2 constructs was confirmed by Western analysis utilizing an α-FLAG antibody. The positions of JNK and Dab2 constructs are indicated by the arrows.
suggest that this interaction may mediate TGFβ-stimulated JNK activation (Fig. 3).

Given the requirement of Dab2 expression for TGFβ-stimulated FN induction and the ability of TAKKW to block Dab2-mediated JNK activation, we hypothesized that TAK1 may also be required for TGFβ stimulation of FN synthesis. To test this hypothesis, we transiently transfected NIH3T3 cells with double-stranded RNA oligonucleotides targeted to specifically abrogate basal FN from the media of metabolically labeled cells. FN is indicated by the arrow. The experiment was performed four times with similar results; depicted is an individual experiment.

FIG. 3. Decreased Dab2 expression leads to attenuated JNK activation by TGFβ. A, 3T3 siDab2 clones exhibit altered TGFβ-stimulated JNK activation. NIH3T3 cells and 3T3 siDab2 clone #9 were treated with 5 ng/ml TGFβ for the various times indicated. Lysates were prepared, and JNK kinase assays were performed utilizing GST-c-Jun as substrate. Reaction products were resolved on 10% SDS/PAGE gels, transferred to Immobilon membranes, and subjected to Western analysis utilizing a phospho-specific antibody to c-Jun (Ser-63). The assay was performed three times with similar results. Shown is a representative experiment. B, the p54 and p46 isoforms of JNK are expressed equally in NIH3T3 cells and 3T3 siDab2 clone #9 as determined by immunoblotting with α-JNK antibody. C, JNK activity is required for stimulation of FN synthesis by TGFβ. NIH3T3 cells were untreated or treated with SP600125 (SP, 10 and 50 μM), SB203580 (SB, 10 μM), or PD98059 (PD, 10 μM) in the absence or presence of TGFβ. The ability to induce FN after stimulation with TGFβ was assessed by immunoprecipitation of 35S-labeled FN from the media of metabolically labeled cells. FN is indicated by the arrow. The experiment was performed four times with similar results; depicted is an individual experiment.

Given the requirement of Dab2 expression for TGFβ-stimulated FN induction and the ability of TAKKW to block Dab2-mediated JNK activation, we hypothesized that TAK1 may also be required for TGFβ stimulation of FN synthesis. To test this hypothesis, we transiently transfected NIH3T3 cells with double-stranded RNA oligonucleotides targeted to specifically abrogate basal FN expression. Analysis of these cells for their ability to induce FN synthesis after TGFβ stimulation reveals that TAK1 oligonucleotides, but not a negative control pool, could attenuate basal FN as well as TGFβ-stimulated FN expression (Fig. 5A, left panel). To further confirm these observations, we tested a stable pool of NIH3T3 cells that overexpresses the kinase-deficient form of TAK1, TAKKW (Fig. 5B). As analyzed by Western blot (Fig. 5B), this pool of cells (3T3-TAKKW) expresses similar levels of Dab2 to the wild-type NIH3T3 cells but expresses very high levels of the mutant form of TAK1 (Fig. 5B). The induction of FN mRNA was then assessed in NIH3T3, 3T3 siDab2#8, and 3T3-TAKKW cells after TGFβ stimulation for 0, 8, and 24 h by Northern analysis. Consistent with previous results, we observe that TGFβ induces FN mRNA in a time-dependent fashion in NIH3T3 cells (Fig. 5C). In contrast, both basal and TGFβ-stimulated FN mRNA levels are greatly abrogated in 3T3 siDab2#8 and 3T3 TAKKW cells (Fig. 5C). These data suggest that, similarly to Dab2, TAK1 is required for TGFβ stimulation of FN synthesis.

Expression of Dab2 Alters Cell Migration—By mediating the phosphorylation of c-Jun, JNK plays an essential role in activation of AP-1 complexes leading to gene induction; however, a key role of JNK in controlling cell migration, independent of
gene transcription, has recently been demonstrated (48). We wished, therefore, to test whether Dab2 plays a role in mediating cell migration stimulated by TGFβ, since decreased Dab2 expression leads to decreased TGFβ-stimulated JNK activation. To address this, we performed a wounding assay on monolayer cells, which examines the ability of cells to migrate into an area denuded by scraping. We investigated the ability of TGFβ to stimulate migration in parental NIH3T3 and the 3T3-siDab2#8 clone. After a 24-h treatment with TGFβ, we observed that, although NIH3T3 cells efficiently migrate into the wounded area, the 3T3-siDab2#8 clone exhibited a decreased migratory capability (Fig. 6A). To obtain a more quantitative measure of cell motility, we assessed the ability of parental NIH3T3 and 3T3-siDab2#8 cells to migrate through a membrane containing 8-μm pores in the absence or presence of TGFβ. Cell migration was assessed by microscopy after 24 h. The wounding assay was performed four times with similar results. Depicted is a representative experiment. B, quantitative measurement of cell migration was assessed in the absence and presence of TGFβ utilizing the QCM™ 24-well colorimetric cell migration assay as described under "Materials and Methods." Plotted are the mean values ± S.D. of a representative experiment performed in triplicate.

FIG. 5. TAK1 is required for TGFβ-stimulated induction of FN. A, siRNA-mediated silencing of TAK1 expression attenuates TGFβ-stimulated induction of FN protein synthesis. NIH3T3 cells were transiently transfected with either negative control oligo pool (Control) or TAK1 double-stranded RNA oligo pool (TAK siRNA). Lysates were examined by immunoblot analysis to determine TAK1 and HSP90 expression (A, left panel). After 48 h of transfection, the ability of TGFβ stimulation to induce FN protein synthesis was evaluated by immunoprecipitation of 35S-labeled FN from the media (A, right panel). The experiment was performed two times in duplicate with similar results. Shown is an individual experiment. B, expression of Dab2 in NIH3T3, 3T3-siDab2 clone #8, and 3T3-TAKKW cells was determined by Western analysis using α-Dab2 antibody (top panel). Expression of endogenous TAK1 and the HA-tagged TAKKW construct was examined by immunoblot analysis utilizing α-TAK1 and α-HA antibodies, respectively (middle and lower panels). C, TGFβ-stimulated FN mRNA induction is abrogated in siDab2- and TAKKW-expressing NIH3T3 cells. NIH3T3, 3T3-siDab2 clone #8, and 3T3-TAKKW cells were stimulated with 5 ng/ml TGFβ for the indicated times. Total RNA was prepared, and 40 μg of each sample was resolved on a 1.2% formaldehyde/agarose gel, transferred to nitrocellulose, and subjected to Northern analysis to determine FN expression (top panel). The filter was stripped and reprobed with a cyclophilin (1B15) probe (lower panel). Positions of FN and 1B15 messages are indicated by arrows.

FIG. 6. Dab2 levels affect cell migration. A, decreased Dab2 expression results in decreased migratory capability after wounding. NIH3T3 cells in 60-mm plates were allowed to reach confluence followed by wounding with a pipette tip. Plates were washed to remove non-adherent cells and placed in media in the absence or presence of 5 ng/ml TGFβ. Cell migration was assessed by microscopy after 24 h. The wounding assay was performed four times with similar results. Depicted is a representative experiment. B, quantitative measurement of cell migration was assessed in the absence and presence of TGFβ utilizing the QCM™ 24-well colorimetric cell migration assay as described under "Materials and Methods." Plotted are the mean values ± S.D. of a representative experiment performed in triplicate.

DISCUSSION
Efficient tissue repair after wounding is essential for maintenance of tissue homeostasis. After injury, underlying fibroblasts proliferate, migrate into the wound site, and synthesize...
that stable siRNA-mediated silencing of Dab2 results in loss of muscle cells with a specific pharmacological inhibitor of JNK, elicited induction of FN (21), and here we show that treatment of treatment, whereas expression of constitutively active TAK1 yuked induction of FN synthesis and fibroblast migration.

TGFβ has previously been shown to potently stimulate FN protein synthesis, acting at both the level of gene transcription and at the level of mRNA stabilization (49–51). We report here that stable siRNA-mediated silencing of Dab2 results in loss of TGFβ-stimulated induction of both FN mRNA and protein levels (Fig. 1B and Fig. 5C), suggesting that Dab2 plays a role in regulation of FN gene transcription. The FN promoter contains three CRE elements (52), one of which has previously been shown to be occupied by a c-Jun/ATF-2 heterodimer (53). The JNK-signaling pathway, thus, is implicated in TGFβ-mediated up-regulation of FN, since JNK can phosphorylate both c-Jun and ATF-2, leading to their transcriptional activation (27). Indeed, we have previously demonstrated that expression of dominant-negative JNK and MKK4 abolishes TGFβ-mediated induction of FN (21), and here we show that treatment of both NIH3T3 mouse fibroblasts and A10 rat aortic smooth muscle cells with a specific pharmacological inhibitor of JNK, SP600125 (44), leads to decreased TGFβ-stimulated FN synthesis (Fig. 3C). In addition, we show that Dab2 can directly stimulate JNK activity and demonstrate that ablation of Dab2 expression in NIH3T3 cells leads to decreased TGFβ-stimulated JNK activation.

TAK1 was initially identified in a screen to identify potential MAPK kinase kinases that could mediate activation of the yeast pheromone-induced MAPK-signaling pathway (28). TAK1 activity was shown to be stimulated by TGFβ and BMP4 treatment, whereas expression of constitutively active TAK1 resulted in transactivation of the TGFβ-responsive PAI-1 promoter (28). TAK1 can also be stimulated by treatment with TNFα, interleukin 1, ceramide, and lipopolysaccharide and has been shown to be involved in the JNK and p38 activation elicited by these agents (47, 54). Although TAK1 was found to activate both p38 and JNK in 293T cells, activation of JNK was greater and found to be dependent on MKK4 (55). Studies in Drosophila also demonstrate that TAK1 participates in JNK, but not p38 activation during development (56, 57). Here we show that TAK1 and Dab2 can be found in association in vivo after treatment with TGFβ, with similar kinetics observed for TGFβ-stimulated JNK activation (Fig. 4C), suggesting a direct involvement of TAK1 in mediation of Dab2-stimulated JNK activation. In addition, down-regulation of TAK1 expression by double-stranded RNA oligonucleotides or overexpression of TAKKW in NIH3T3 cells (Fig. 5) attenuates TGFβ-stimulated FN protein and mRNA up-regulation, respectively. These results are in agreement with a previous study, which demonstrated that overexpression of the TAB1 binding domain of TAK1 led to decreased TGFβ-stimulated induction of the matrix components PAI-1, type I and type IV collagen, and FN (58). Collectively, our results suggest that TGFβ-mediated FN induction proceeds through Dab2 to TAK1, leading to MKK4 and subsequent JNK activation.

The Smads are a family of transcription factors that display relatively low affinity for their cognate DNA binding elements, and thus, interactions between the Smads and other DNA-binding proteins are necessary to achieve specific, high affinity interactions with DNA (59). The Smads have been shown to interact with c-Jun and c-Fos (15), thereby providing the potential for synergistic as well as antagonistic cross-talk between the Smad- and MAPK-signaling pathways. The interaction of Smad3 with c-Jun, which occurs off-DNA, was found to antagonize transcription of the Smad-dependent COL7A1 and PAI-1 promoters; however, this interaction proved to be synergistic at AP-1-dependent promoter sites (60). Some promoters regulated by TGFβ contain both Smad and AP-1 binding sites, such as the c-Jun gene itself. Although TGFβ could still mediate a 2-fold activation of the c-Jun promoter that contained a mutated Smad binding site, mutation of the AP-1/CRE site eliminated all promoter activation (61). We have previously shown that TGFβ-mediated induction of FN was independent of Smad4 but dependent on JNK activation (21). Subsequent studies in mouse embryo fibroblasts that lack Smad2, Smad3, and Smad4 have corroborated the Smad independence of TGFβ-stimulated FN up-regulation (23, 24). Moreover, activation of the JNK pathway may actually be required for activation of the Smad pathway, since JNK phosphorylation of Smad3 was found to facilitate the subsequent TgfRI-mediated phosphorylation of Smad3, which led to enhanced nuclear translocation and transcriptional activity (26). In addition, fibroblasts transfected with antisense oligonucleotides to JNK1 and JNK2 exhibited loss of TGFβ-stimulated Smad2 phosphorylation (25). We previously demonstrated that the expression of Dab2 restored both Smad-dependent and Smad-independent responses to a TGFβ-signaling-deficient cell line (11). Dab2 may, thus, play the role of a scaffold molecule to bridge activation of the JNK pathway, mediated by its C-terminal domain, to activation of the Smad pathway, mediated through its N-terminal PTB domain.

Although the study of JNK signaling in mammalian cells has previously focused on transcriptional regulation of AP-1 activity, the role of JNK in mediating cell migration has recently gained attention (48). In Drosophila, the JNK pathway is responsible for the morphogenetic movement of lateral dorsal epithelia toward the dorsal midline, resulting in dorsal closure (62). This involves the JNK-mediated up-regulation of dpp, a TGFβ superfamily member, which then acts in a paracrine fashion to promote migration of the leading edge cells (63). In mice that are deficient in MEKK1 expression or compound Jnk1−/−/Jnk2−−/− mutant mice, an eye-open at birth phenotype is observed (64, 65). This process is also dependent on the movement of epithelial cells and, thus, resembles dorsal closure in Drosophila. The eye-open at birth phenotype is also observed in mice deficient in activin βB (66), again implicating the TGFβ superfamily in regulation of cell migration. Because the process of dorsal closure is morphologically similar to mammalian wound repair, JNK has been postulated to play a role in the cell migration required for efficient wound healing (67). We demonstrate here that loss of Dab2 expression in NIH3T3 mouse fibroblasts results in decreased cell migration assayed either by a wound healing assay or a quantitative cell migration assay (Fig. 6). A previous study has recently demonstrated that mouse fibroblasts deficient in JNK expression exhibit lower basal JNK activity and show a similar failure to migrate in a wound assay (68). Although MEKK1 deficiency leads to the migratory defect in epithelial cells observed in the eye-open at birth phenotype, MEKK1 expression is low or absent in eyelid dermis, thus suggesting that JNK-mediated cell migration in fibroblasts is regulated by another MAPK kinase kinase (48, 65). We demonstrate here that TAKKW expression blocks Dab2-mediated JNK activation, whereas studies in Drosophila also implicate the Drosophila homolog of TAK (dTAK) in JNK-mediated processes of dorsal closure and planar cell polarity.
(56, 57). Additionally, the defect we observe in cell migration may be due in part to the lack of basal and TGFβ-stimulated FN induction, which by itself has been shown to stimulate JNK activity and cell migration (69, 70). Taken together, our studies delineate a signaling pathway dependent on Dab2 expression and FN induction, which by itself has been shown to stimulate JNK (69). We also thank Drs. Bruce Pratt and Steve Ledbetter at Genzyme Inc. for generous provision of TGFβ.

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Disabled-2 (Dab2) Mediates Transforming Growth Factor β (TGFβ)-stimulated Fibronectin Synthesis through TGF β-activated Kinase I and Activation of the JNK Pathway

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