Pin1 Promotes Transforming Growth Factor-β-induced Migration and Invasion*

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Transforming growth factor-β (TGF-β) regulates a wide variety of biological activities. It induces potent growth-inhibitory responses in normal cells but promotes migration and invasion of cancer cells. Smads mediate the TGF-β responses. TGF-β binding to the cell surface receptors leads to the phosphorylation of Smad2/3 in their C terminus as well as in the proline-rich linker region. The serine/threonine phosphorylation sites in the linker region are followed by the proline residue. Pin1, a peptidyl-prolyl cis/trans isomerase, recognizes phosphorylated serine/threonine-proline motifs. Here we show that Smad2/3 interacts with Pin1 in a TGF-β-dependent manner. We further show that the phosphorylated threonine 179-proline motif in the Smad3 linker region is the major binding site for Pin1. Although epidermal growth factor also induces phosphorylation of threonine 179 and other residues in the Smad3 linker region, the same as TGF-β, Pin1 is unable to bind to the epidermal growth factor-stimulated Smad3. Further analysis suggests that phosphorylation of Smad3 in the C terminus is necessary for the interaction with Pin1. Depletion of Pin1 by small hairpin RNA does not significantly affect TGF-β-induced growth-inhibitory responses and a number of TGF-β-Smad target genes analyzed. In contrast, knockdown of Pin1 in human PC3 prostate cancer cells strongly inhibited TGF-β-mediated migration and invasion. Accordingly, TGF-β induction of N-cadherin, which plays an important role in migration and invasion, is markedly reduced when Pin1 is depleted in PC3 cells. Because Pin1 is overexpressed in many cancers, our findings highlight the importance of Pin1 in TGF-β-induced migration and invasion of cancer cells.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that controls various fundamental biological activities such as cell proliferation, differentiation, migration, adhesion, and apoptosis (1). Disruption of the TGF-β signaling pathways is associated with a number of human diseases, especially cancer (2–8). TGF-β has two opposite roles in cancer. It is a potent tumor suppressor during the early stages of tumorgenesis through its growth-inhibitory effects and apoptosis-promoting function (2–8). TGF-β promotes cancer progression and metastasis at later stages (2–8).

TGF-β signal transduction is mediated by two types of cell surface serine/threonine kinase receptors (TβRI and TβRII) and downstream effectors, the Smad family proteins (9–14). TGF-β binding induces the formation and activation of a receptor complex containing TβRI and TβRII. The activated TβRII directly phosphorylates Smad2 and Smad3 at the SSXS motif in their C-tails. The phosphorylated Smad2 and Smad3 then form a complex with Smad4 and together accumulate in the nucleus to regulate transcription of a wide variety of target genes, leading to distinct biological effects in a cell context-dependent manner (9–14).

In addition to the C-tail phosphorylation sites for the receptor kinase, Smad2 and Smad3 contain multiple serine/threonine phosphorylation sites in the proline-rich linker region that connects the N- and C-terminal domains. Among them, several phosphorylation sites are followed by the proline residue and can be phosphorylated by proline-directed kinases, such as the mitogen-activated protein kinase superfamily members that include ERK, c-Jun N-terminal kinase, and p38, the cyclin-dependent kinase family members, and glycogen synthase kinase-3 (11, 15–38).

We and others have recently shown that three sites Thr-179, Ser-204, and Ser-208 in the Smad3 linker region that connects the N- and C-terminal domains. Among them, several phosphorylation sites are followed by the proline residue and can be phosphorylated by proline-directed kinases, such as the mitogen-activated protein kinase superfamily members that include ERK, c-Jun N-terminal kinase, and p38, the cyclin-dependent kinase family members, and glycogen synthase kinase-3 (11, 15–38).

We and others have recently shown that three sites Thr-179, Ser-204, and Ser-208 in the Smad3 linker region are phosphorylated in response to TGF-β (30, 36–38) and that the cy-

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3 The abbreviations used are: TGF-β, transforming growth factor-β; TJβRI, TβRI type I receptor; TJβRII, TβRII type II receptor; EGF, epidermal growth factor; ERK, extracellular-signal regulated kinase; Smurf2, Smad ubiquitination regulatory factor 2; Pin1, protein interacting with NIMA (never in mitosis A); shRNA, small hairpin RNA; GST, glutathione S-transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum; EPSM, Erk/proline-directed kinase site mutant.
Pin1 Promotes TGF-β-induced Migration and Invasion

clin-dependent kinase family members and glycogen synthase kinase-3 are responsible for the phosphorylation (36–38). The Thr-179 and Ser-208 are phosphorylated by the cyclin-dependent kinase family members in response to TGF-β (36), whereas the Ser-204 is phosphorylated by glycogen synthase kinase-3 in response to TGF-β (36, 37). We have further shown that the C-tail phosphorylation is necessary for the linker phosphorylation in response to TGF-β (36). When the C-tail phosphorylation sites in Smad3 are mutated, the linker sites are not phosphorylated in response to TGF-β (36).

Interestingly, these three same sites, Thr-179, Ser-204, and Ser-208, in the Smad3 linker region are also phosphorylated by ERK in response to EGF treatment and Ser-208 is the best ERK phosphorylation site in Smad3 (29). The ERK phosphorylation does not require the C-tail phosphorylation. When the C-tail phosphorylation sites in Smad3 are mutated, ERK still phosphorylates these three sites in response to EGF treatment (36).

The Smad2 linker region is also phosphorylated in response to TGF-β or EGF (11, 15–17, 19–25, 30, 31, 36). However, due to the lack of specific phosphopeptide antibodies against each of the putative phosphorylation sites and the lack of mapping of the phosphorylation sites by other methods, the exact sites in the Smad2 linker region that are phosphorylated in response to TGF-β or EGF remain to be determined.

Pin1 is a peptidyl-prolyl cis/trans isomerase that recognizes the phosphorylated serine/threonine-proline motifs in certain proteins and catalyzes prolyl cis/trans isomerization (39–41). The prolyl isomerization induces conformational changes, leading to distinct effects in different target proteins, such as increased stability, increased turnover, alteration of the sensitivity to phosphatases, alteration in subcellular localizations, and altered enzymatic activities, and enabling protein-protein interactions (39–41). Since its discovery, a number of Pin1 targets have been identified, indicating the importance of Pin1 in the cellular physiology (39–41). The various studies also suggest that the effects of Pin1 are cell context-dependent (39–41). Pin1 is overexpressed in many cancers, such as in prostate, breast, lung, colon, and hepatocellular carcinoma (39–51). In prostate cancer, overexpression of PIN1 is correlated with a higher probability of tumor recurrence and a shorter period to tumor recurrence after radical prostatectomy (52). In addition to cancer, Pin1 is also linked with other diseases, such as Alzheimer disease and asthma (39, 41).

Because the Thr-179, Ser-204, and Ser-208 phosphorylation sites in Smad3 are followed by the proline residue, they constitute putative Pin1 binding sites. It is possible that certain or all of these sites serve as the Pin1-binding site(s) when they are phosphorylated. Recently, Nakano et al. (53) reported that Pin1 can associate with Smad2 and Smad3 to enhance their interaction with Smurf2 (Smad ubiquitination regulatory factor 2), a HECT domain E3 ubiquitin ligase, resulting in enhanced Smad ubiquitination and reduced Smad2/3 levels. We show in this report that Pin1 binds to Smad2/3 in a TGF-β-dependent manner and that the phosphorylated Thr-179-proline is the major binding site for Pin1 in Smad3 in response to TGF-β. We further show that knockdown of Pin1 does not have a significant effect on TGF-β-induced growth-inhibitory response, as analyzed in the human HaCaT keratinocytes. On the contrary, knockdown of Pin1 in human PC3 prostate cancer cells significantly inhibited TGF-β-induced cell migration and invasion. Our study uncovered an important role of Pin1 in TGF-β-mediated cancer cell migration and invasion.

EXPERIMENTAL PROCEDURES

Constructs, Antibodies, and Chemical Inhibitors—Mammalian expression plasmids for Smad3, its phosphorylation mutants, and TβRI were described previously (26, 36). Plasmids for GST-Pin1, pSUPER-puro-shRNA against Pin1 or the scrambled control were also described previously (54, 55). The Smad3-specific peptide antibody and the Smad2-specific peptide antibody were from Invitrogen. The Smad2/3 antibody that was raised against the full-length Smad3 and recognizes both Smad2 and Smad3, the Pin1 polyclonal antibody, the SIP1 polyclonal antibody, and the actin antibody was purchased from Santa Cruz Biotechnology, Inc. The N-cadherin antibody was purchased from Upstate Biotechnology. The Slug monoclonal antibody (clone 2B6) was obtained from the Millipore Corp. The Snail polyclonal antibody was from ABGENT. The E-cadherin monoclonal antibody (clone 67A4) was from Chemicon International. Smad3 phospho-specific antibodies were described previously (26, 36). The Smad3 (C-tail) phosphopeptide antibody was generously provided by Dr. Edward B. Leof (Mayo Clinic Cancer Center, Rochester, MN). Horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce or Chemicon International. ECL Western blot reagents were purchased from Roche Applied Science or Millipore Corp. PiB, diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzyl[mnm][3,8]phenan throline-2,7-di-acetate, a Pin1 inhibitor, was purchased from Merck.

Cell Culture, Transfection, and Retroviral Infection—Human PC3 prostate cancer cell line was cultured in RPMI1640 with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. Amphotropic Phoenix cells (Phoenix A) were grown in Dulbecco’s modified Eagle’s medium with 10% FBS, 1% penicillin/ streptomycin. Human HaCaT keratinocytes and human HEK293T cells were cultured in minimum essential medium, 10% FBS, 1% penicillin/streptomycin. L17, a TβRI-deficient cell line derived from the Mv1Lu murin lung epithelial cell line, was maintained in minimum essential medium without histidine but plus histidinol, 10% FBS, 1% penicillin/streptomycin. The L17 cells and the 293T cells were transfected by DEAE-dextran and Lipofectamine Plus reagent (Invitrogen), respectively, as previously described (26, 36). For shRNA retrovirus production, Phoenix A cells were transfected with pSUPER-puro-shRNA targeting Pin1 or a scrambled control by Lipofectamine Plus reagent. HaCaT and PC3 cells were infected several times with the retrovirus targeting Pin1 or the scrambled control. The infected cells were selected with 5 μg/ml puromycin.

GST Pulldown Assay and Coimmunoprecipitation Assay—GST and GST-Pin1 were expressed in bacteria strain BL21 (DE3). GSH-Sepharose beads containing GST or GST-Pin1 were prepared as described previously (26). For analysis of Smad interaction with GST-Pin1, HaCaT cells were serum-starved before the addition of EGF or TGF-β. Cells were then treated with either 50 ng/ml EGF for 15 min or 300 pM TGF-β for 1 h for maximal induction of Smad3 phosphorylation in the linker sites. Cells were lysed with the lysis buffer (10 mM Tris-
Pin1 Promotes TGF-β-induced Migration and Invasion

Cl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1× complete protease inhibitor mixture (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 1 μg/ml RNase A). Cell lysates (300 μg) were incubated with 10 μl of either GST or GST-Pin1 beads at 4 °C for 2 h. The beads were washed with the cell lysis buffer 5 times, and the bound proteins on the beads were eluted by SDS sample buffer. For analysis of Smad3 and its mutants for interaction with GST-Pin1, L17 cells were cotransfected with expression plasmids for wild type or a mutant Smad3 together with TβRI, and the cells were treated with or without TGF-β for 1 h. Alternatively, the HEK293T cells were transfected with the wild type Smad3 or a mutant Smad3 and treated with TGF-β for 1 h. The cell lysates were subjected to GST pulldown assays as described above.

For coimmunoprecipitation assays to detect Smad2/3 and Pin1 interaction at endogenous levels, HaCaT cells were treated with or without TGF-β for 1 h, and the cell lysates (800 μg) were precleared by incubation with 20 μl of protein A/G beads at 4 °C for 0.5 h. The precleared lysates were then incubated with 2.5 μg of either control IgG or the Smad2/3 antibody at 4 °C overnight. Protein A/G-beads were then added and incubated for 2 h. The beads were washed 5 times with the lysis buffer and eluted with SDS sample buffer. Proteins eluted from the beads were subjected to Western blot analysis using the polyclonal Pin1 antibody by fresh ECL reagents (Roche Applied Science).

**Thymidine Incorporation Assay—**[3H]Thymidine incorporation assay was performed as described previously (26). In brief, 2 × 10^5 shRNA-transduced HaCaT cells were seeded on 6-well plates in triplicate for 24 h and then treated with or without TGF-β at various concentrations for 24 h. 5 μCi of [3H]thymidine was added to each well during the last 4 h of the incubation. Cells were then washed 3 times with phosphate-buffered saline, fixed with 95% methanol, and extracted with 0.2 N NaOH. The extracts were counted for radioactivity. The results represent the average and S.D. of three independent experiments.

**RNA Preparation and Northern Blot Analysis—**shRNA-transduced HaCaT cells were treated with or without 300 pm TGF-β for 8 h. Poly(A)^+ RNA was prepared by the FAST track kit (Invitrogen). Northern blot was performed as previously described (56). Briefly, poly(A)^+ RNA was separated on a 1% formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with a random primed ^32P-labeled probe. The following probes were used: Pin1, p15, p21, Smad7, JunB, desmalin, p120-Catenin, and hybridized with an antibody against Smad3. As shown in Fig. 1A, Pin1 binds specifically to the TGF-β-stimulated Smad3. To confirm this result, we analyzed the precipitates by immunoblot with phospho-specific antibodies against Thr-179, Ser-204, and Ser-208 in Smad3. Although the phosphorylation of Thr-179, Ser-204, and Ser-208 in Smad3 was induced to a similar extent by EGF and TGF-β (Fig. 1A), the phosphorylated Thr-179, Ser-204, and Ser-208 were detected in GST-Pin1 beads only in response to TGF-β treatment (Fig. 1A). Very little of Thr(P)-179, Ser(P)-204, or Ser(P)-208 was detected in the GST-Pin1 beads in response to EGF treatment (Fig. 1A).

Because the linker region of Smad2 is also phosphorylated in response to EGF or TGF-β (11, 15–17, 19–25, 30, 31, 36), we analyzed whether Pin1 binds to Smad2 after EGF or TGF-β treatment. HaCaT cells were treated with EGF or TGF-β. The cell lysates were incubated with GST-Pin1 beads or the control GST beads followed by immunoblot with an antibody against Smad3. As shown in Fig. 1B, Pin1 binds to Smad2 in response to TGF-β but not in response to EGF.

To provide further evidence that Smad2/3 binds to Pin1 in response to TGF-β, we analyzed whether Smad2/3 interacts with Pin1 after TGF-β treatment at endogenous levels. HaCaT cells were treated with or without TGF-β. Cell lysates were then immunoprecipitated with a Smad2/3 antibody that was raised against the full-length Smad3 and recognizes both Smad3 and Smad2. The immunoprecipitates were then analyzed by immunoblot with a Pin1-specific antibody. As shown in Fig. 1C, Pin1 binds to Smad2/3 in response to TGF-β at endogenous levels.

**Pin1 Binding Requires Smad3 Phosphorylation in the C-tail and at the Thr-179 Site in the Linker Region—**As described above, although EGF and TGF-β induces the phosphorylation of the same sites in the Smad3 linker region, Pin1 binds only to TGF-β-induced Smad3. We reasoned that there should be

RESULTS

**Pin1 Binds to Smad2/3 Specifically in a TGF-β-dependent Manner—**We previously showed that phosphorylation of three serine/threonine residues (Thr-179, Ser-204, and Ser-208) in the proline-rich linker region of Smad3 is induced by both EGF and TGF-β (29, 36). These phosphorylation sites are followed by the proline residue, thus constituting putative Pin1 binding sites. We therefore examined the possibility of whether Smad3 binds to Pin1 in response to EGF or TGF-β. We treated human HaCaT keratinocytes with either EGF or TGF-β. The cell lysates were then subjected to GST pulldown assays with GST-Pin1 beads or the control GST beads followed by immunoblot with an antibody against Smad3. As shown in Fig. 1A, Pin1 binds specifically to the TGF-β-stimulated Smad3. To confirm this result, we analyzed the precipitates by immunoblot with phospho-specific antibodies against Thr-179, Ser-204, and Ser-208 in Smad3.

**Pin1 Promotes TGF-β-induced Migration and Invasion—**We previously showed that phosphorylation of three serine/threonine residues (Thr-179, Ser-204, and Ser-208) in the proline-rich linker region of Smad3 is induced by both EGF and TGF-β (29, 36). These phosphorylation sites are followed by the proline residue, thus constituting putative Pin1 binding sites. We therefore examined the possibility of whether Smad3 binds to Pin1 in response to EGF or TGF-β. We treated human HaCaT keratinocytes with either EGF or TGF-β. The cell lysates were then subjected to GST pulldown assays with GST-Pin1 beads or the control GST beads followed by immunoblot with an antibody against Smad3. As shown in Fig. 1A, Pin1 binds specifically to the TGF-β-stimulated Smad3. To confirm this result, we analyzed the precipitates by immunoblot with phospho-specific antibodies against Thr-179, Ser-204, and Ser-208 in Smad3. Although the phosphorylation of Thr-179, Ser-204, and Ser-208 in Smad3 was induced to a similar extent by EGF and TGF-β (Fig. 1A), the phosphorylated Thr-179, Ser-204, and Ser-208 were detected in GST-Pin1 beads only in response to TGF-β treatment (Fig. 1A). Very little of Thr(P)-179, Ser(P)-204, or Ser(P)-208 was detected in the GST-Pin1 beads in response to EGF treatment (Fig. 1A).

Because the linker region of Smad2 is also phosphorylated in response to EGF or TGF-β (11, 15–17, 19–25, 30, 31, 36), we analyzed whether Pin1 binds to Smad2 after EGF or TGF-β treatment. HaCaT cells were treated with EGF or TGF-β. The cell lysates were incubated with GST-Pin1 beads or the GST control beads. The precipitates were then analyzed by immunoblot with an antibody against Smad2. As shown in Fig. 1B, Pin1 binds to Smad2 in response to TGF-β but not in response to EGF.

To provide further evidence that Smad2/3 binds to Pin1 in response to TGF-β, we analyzed whether Smad2/3 interacts with Pin1 after TGF-β treatment at endogenous levels. HaCaT cells were treated with or without TGF-β. Cell lysates were then immunoprecipitated with a Smad2/3 antibody that was raised against the full-length Smad3 and recognizes both Smad3 and Smad2. The immunoprecipitates were then analyzed by immunoblot with a Pin1-specific antibody. As shown in Fig. 1C, Pin1 binds to Smad2/3 in response to TGF-β at endogenous levels.
other components in Smad3 that contributes to this specificity. In addition to the linker sites, TGF-β induces phosphorylation of the C-tail of Smad3. Because this phosphorylation was absent in EGF-stimulated Smad3, we tested whether mutation of the C-tail of Smad3. Because this phosphorylation was absent in EGF-stimulated Smad3 phosphorylation in the linker region. The cell lysates were incubated with either GST or GST-Pin1 beads. The bound proteins were analyzed by immunoblot with an antibody against Smad3. The bound proteins were also analyzed by immunoblot with specific phosphopeptide antibodies against the phosphorylated Thr-179, Ser-204, and Ser-208 in the Smad3 linker region. β, TGF-β, but not EGF, induces Pin1-Smad2 interaction. HaCaT cells were treated with TGF-β or EGF as described in A. The cell lysates were incubated with GST or GST-Pin1 beads. The bound proteins were analyzed by immunoblot with an antibody against Smad2. The Smad2 levels in the cell lysates were also analyzed as a control. C. TGF-β induces Pin1-Smad2/3 interactions at endogenous protein levels. HaCaT cells were treated with or without TGF-β for 1 h. The cell lysates were immunoprecipitated with an antibody that was raised against the full-length Smad3 and recognizes both Smad3 and Smad2. The precipitates were analyzed by immunoblot with an antibody against Pin1. The expression levels of Pin1, Smad2, Smad3, and actin in the cell lysates were also analyzed as controls.

by the receptor kinase is necessary for Smad3 binding to Pin1. Although EGF and TGF-β induce phosphorylation at the same linker sites, the structure of Smad3 at the C termini is different under the two conditions. Pin1 binding may require a discrete C termini structure, which results from phosphorylation of the SSXS motif (10). Taken together, our result suggests that Pin1 binding requires the phosphorylation of both the C-tail and the linker.

In the Smad3 linker region there are four putative Pin1 binding sites. These include the Thr-179, Ser-204, and Ser-208. In addition, it includes the Ser-213 site, which is phosphorylated by cyclin-dependent kinase at the basal state (26). We mutated these four sites individually. GST pulldown assays with these mutants revealed that Thr-179 is the major binding site in Smad3 for Pin1 binding to Smad3 requires both the linker and the C-tail phosphorylation, and the Thr-179 is the major binding site for Pin1. A. Phosphorylation of both the linker and the C-tail in Smad3 is necessary for interaction with Pin1 in response to TGF-β. L17 cells were cotransfected with TβRI along with the wild type (WT) Smad3, a linker phosphorylation mutant (EPSM), or a C-tail phosphorylation mutant (C-tail 3A). Cells were then treated with or without 300 pm TGF-β for 1 h. The cell lysates were incubated with GST-Pin1 beads. The bound proteins were analyzed by immunoblot with the Smad3 antibody. B. Thr-179 is the major binding site in Smad3 for interaction with Pin1 in response to TGF-β. HEK293T cells were transfected with the wild type Smad3, a linker phosphorylation mutant Smad3, or a control plasmid that encodes GFP. Cells were treated with 300 pm TGF-β for 1 h. The cell lysates were incubated with the GST-Pin1 beads. The bound proteins were analyzed by immunoblot with the Smad3 antibody. The expression levels of the various Smad3 proteins were also analyzed as a control.

Depletion of Pin1 Does Not Have a Significant Effect on the Smad2/3 Levels, on TGF-β-induced Smad3 Linker Phosphorylation, or on TGF-β-induced Smad2/3 Nuclear Accumulation—To determine the consequence of Pin1 binding in TGF-β signaling, we generated stable cell lines in HaCaT cells that knock down the expression of Pin1 or a scrambled control. As shown in Fig. 3A, Pin1 is very effectively depleted by shRNA against Pin1. The depletion of Pin1 has little effect on Smad2 or Smad3 levels in HaCaT cells (Fig. 3A).

Because Pin1 binding to Smad3 may regulate TGF-β signaling by affecting the phosphorylation levels at Thr-179 and potentially also at the Ser-204 and Ser-208 sites, we analyzed
Pin1 Promotes TGF-β-induced Migration and Invasion

![Diagram](image)

**FIGURE 3.** Knockdown of Pin1 does not have a significant effect on Smad2/3 levels, on TGF-β-induced linker and C-tail phosphorylation of Smad3, or on TGF-β-induced Smad2/3 nuclear accumulation. A, depletion of Pin1 has little effect on Smad2/3 levels in HaCaT cells. Stable HaCaT cell lines with an shRNA targeting Pin1 or the scrambled control were generated. The cell lysates were analyzed by immunoblot for Pin1, Smad2, and Smad3 levels. Actin levels were also analyzed as a control. B, depletion of Pin1 does not have a significant effect on TGF-β-induced linker and C-tail phosphorylation of Smad3 in HaCaT cells. The Pin1 knockdown HaCaT cells and the scrambled control HaCaT cells were treated with or without TGF-β for 1 h. The cell lysates were analyzed by immunoblot with phosphopeptide antibodies against the phosphorylated Thr-179, Ser-204, Ser-208, and the C-tail. Smad3 and Pin1 expression levels were also analyzed as controls. C, depletion of Pin1 does not affect TGF-β-induced Smad2/3 nuclear accumulation in HaCaT cells. The Pin1 knockdown HaCaT cells and the scrambled control HaCaT cells were treated with or without TGF-β for 1 h. Cells were then harvested and fractionated into the cytoplasmic (C) and nuclear (N) fractions. The same amount of proteins from the cytoplasmic fraction and nuclear fraction was analyzed for Smad2, Smad3, and Pin1 levels by immunoblot. GAPDH and proliferating cell nuclear antigen (PCNA) serve as cytoplasmic and nuclear markers, respectively.

whether Pin1 knockdown affected the phosphorylation levels of Thr-179, Ser-204, or Ser-208 in response to TGF-β. As shown in Fig. 3B, depletion of Pin1 did not have a significant effect on the TGF-β-induced phosphorylation levels at these three sites or a significant effect on the Smad3 C-tail phosphorylation in response to TGF-β (Fig. 3B).

We also analyzed whether depletion of Pin1 affects TGF-β-induced nuclear accumulation of Smad2 and Smad3. The Pin1 knockdown HaCaT cells and the scrambled control HaCaT cells were treated with or without TGF-β for 1 h. Cells were then harvested and fractionated into the cytoplasmic and nuclear fractions. As shown in Fig. 3C, knockdown of Pin1 does not affect Smad2 or Smad3 nuclear accumulation in response to TGF-β. Pin1 is predominantly localized in the nucleus as previously reported (59), and its subcellular localization was not affected by TGF-β treatment (Fig. 3C). The GAPDH serves as a marker for cytoplasmic localization, whereas the proliferating cell nuclear antigen serves as a marker for nuclear localization.

**Depletion of Pin1 Does Not Have a Significant Effect on TGF-β-induced Growth-inhibitory Effects**—One of the major functions of the TGF-β/Smad pathway is to induce growth inhibition. We therefore analyzed whether Pin1 knockdown affected TGF-β/Smad-mediated growth inhibition in HaCaT cells, which are strongly inhibited by TGF-β. The Pin1 knockdown HaCaT cells and the scrambled control HaCaT cells were treated with or without TGF-β at various doses and then subjected to [3H]thymidine incorporation assay. As shown in Fig. 4A, the scrambled control HaCaT cells are highly sensitive to TGF-β, and the Pin1 knockdown HaCaT cells are overall similar to the scrambled control in terms of TGF-β sensitivity. At 5 pM TGF-β, the Pin1 knockdown cells are a little more sensitive to TGF-β than the control cells (Fig. 4A). At a higher concentration of TGF-β, the control HaCaT cells are slightly more sensitive to TGF-β (Fig. 4A). Although we don’t understand why there is a subtle difference in TGF-β sensitivity at different concentrations between the control cells and the Pin1 knockdown cells, the results clearly indicate that knockdown of Pin1 does not have a significant effect on TGF-β-induced growth inhibition.

Previous studies have shown that TGF-β/Smad-mediated growth inhibition resulted from changes in the expression of cell cycle-related genes, such as induction of the expression of the cyclin-dependent kinase inhibitors p15 and p21 (2, 14, 60–65). We therefore analyzed whether knockdown of Pin1 affected several TGF-β/Smad target genes. The control HaCaT cells and the Pin1 knockdown HaCaT cells were treated with or without TGF-β. Poly(A)+ RNA were isolated from the cells and then subjected to Northern blot analysis. As a control, the Northern blot confirmed that Pin1 expression was essentially abolished in the knockdown cells (Fig. 4B). Consistent with the [3H]thymidine incorporation assay above, TGF-β induction of p15 and p21 was similar between the control cells and the Pin1 knockdown cells (Fig. 4, B and C). We also analyzed several other TGF-β/Smad target genes, including Smad7, JunB, and PAI-1 (2, 14, 56, 66–69). As shown in Fig. 4, B and C, TGF-β induction of Smad7, JunB, and PAI-1 was overall similar between the control cells and the Pin1 knockdown cells. For JunB, the basal levels and TGF-β-induced levels are modestly higher in Pin1 knockdown cells than in control cells. For PAI-1, the basal levels and TGF-β-induced levels are slightly higher in the control cells than in the Pin1 knockdown cells. We also analyzed the expression of Bcl2, which is reduced in response to
Knockdown of Pin1 does not have a significant effect on TGF-β-induced growth-inhibitory effects. Pin1 knockdown HaCaT cells and the scrambled control HaCaT cells were treated with or without TGF-β at various concentrations and then subjected to [3H]thymidine incorporation assay. The results represent the average of three independent experiments. B, knockdown of Pin1 does not have a significant effect on several TGF-β/Smad target genes. Pin1 knockdown HaCaT cells and the scrambled control HaCaT cells were treated with or without TGF-β for 8 h. The expression levels of Pin1 and several TGF-β/Smad target genes were analyzed by Northern blot analysis as indicated. GAPDH expression levels were also analyzed as a loading control. C, the mRNA levels of p15, p21, Smad7, JunB, PAI-1, Bcl-2, and Bub1 in B were quantified by densitometer and normalized to GAPDH mRNA levels. The bar graphs represent the average of results from 2 μg of poly(A)+ RNA and 4 μg of poly(A)+ RNA.
As shown in Fig. 4, B and C, the Bcl2 levels are little affected by TGF-β/H9252 treatment in the control HaCaT or Pin1 knockdown HaCaT cells, and the Bcl2 levels are very similar between the control cells and the Pin1 knockdown cells. Our previous unpublished results from a subtractive screen suggested that the expression level of Bub1, which regulates the spindle checkpoint function, was slightly reduced in response to TGF-β. We therefore also analyzed Bub1 in the Northern blot analysis. As shown in Fig. 4, B and C, the expression of Bub1 was slightly reduced in response to TGF-β in both the control cells and the Pin1 knockdown cells. The Bub1 levels at the basal state as well as after TGF-β treatment were modestly reduced in the Pin1 knockdown cells compared with the control cells. The GAPDH levels were also analyzed as a loading control (Fig. 4B). Taken together, the results in Fig. 4, B and C, support the notion that Pin1 does not have a significant effect on TGF-β-mediated growth-inhibitory responses.

**Pin1 Promotes TGF-β-mediated Migration and Invasion**—Pin1 expression is highly elevated in many cancers (39–51). Based on this notion, we next sought the possible role of Pin1 in TGF-β signaling in cancer cells. TGF-β often promotes cancer cell migration and invasion. We transduced human PC3 prostate cancer cell line with the same shRNA against Pin1 or the scrambled control as used for HaCaT cells. As shown in Fig. 5A, Pin1 depletion had little effect on Smad2 or Smad3 protein levels (Fig. 5A). TGF-β-mediated growth-inhibitory response was lost in this cell line (data not shown). On the other hand, TGF-β treatment of the scrambled control PC3 cells greatly stimulated their motility as analyzed in the migration assay (Fig. 5B). In the same assay, however, the TGF-β induction of cell motility was significantly reduced when Pin1 was depleted (Fig. 5B). Similarly, the...
TGF-β-induced invasion was also significantly reduced in the Pin1 knockdown cells compared with the scrambled control cells (Fig. 5C). Thus, Pin1 promotes TGF-β-mediated migratory and invasive responses in the prostate cancer cells. Similar results were also obtained in the MDA-MB-231 breast cancer cells (data not shown). Concomitant with the TGF-β-stimulated migratory and invasive responses in the control PC3 cells, N-cadherin, an important factor for cell migration and invasion (71–77), was up-regulated by TGF-β (Fig. 5D). In the Pin1 depleted cells, this up-regulation was greatly reduced (Fig. 5D). Thus, the significantly diminished TGF-β-induced cell migration and invasion in the Pin1 knockdown cells was correlated with the greatly reduced expression of N-cadherin.

We also analyzed whether knockdown of Pin1 affects the expression levels of several epithelial mesenchymal transi-
tion-related proteins, including Slug, Snail, SIP, and E-cadherin (78–80). As shown in Fig. 5D, the expression of Slug and Snail was slightly increased in the presence of TGF-β. Pin1 knockdown reduced the expression of Slug and Snail. However, this effect was not dependent on TGF-β. The expression of SIP1 was slightly increased in the presence of TGF-β (Fig. 5D); knockdown of Pin1 had no effect on SIP1 levels in the absence or presence of TGF-β (Fig. 5D). The expression of E-cadherin was slightly reduced in the presence of TGF-β (Fig. 5D); depletion of Pin1 had no effect on E-cadherin levels in the absence or presence of TGF-β (Fig. 5D). These results suggest that these four proteins may not be responsible for the effect of Pin1 in TGF-β-induced migration and invasion.

The Catalytic Activity of Pin1 Is Necessary for TGF-β-induced Migration and Invasion—We next analyzed whether the peptidyl-prolyl cis/trans isomerase activity of Pin1 is necessary for TGF-β-mediated migration and invasion. PiB is a small molecule chemical inhibitor that potently and selectively inhibits Pin1/Par14 isomerase activity through competitively binding to the active site (81–83). PC3 cell migration and invasion assays were performed in the presence of 1 µM PiB or in the presence of the vehicle DMSO, which was used to dissolve PiB. Cells were treated with or without TGF-β. As shown in Fig. 6A, PiB significantly inhibited TGF-β-induced migration. Similarly, PiB also significantly inhibited TGF-β-mediated invasion (Fig. 6B). Thus, we conclude that the catalytic activity of Pin1 is necessary for its effect in TGF-β-induced migration and invasion.

**DISCUSSION**

Pin1 is a peptidyl-prolyl cis/trans isomerase that recognizes a small subset of the phosphorylated serine/threonine-proline motifs (39–41). We show in this report that Pin1 binds to Smad2/3 in response to TGF-β. Depletion of Pin1 does not have a significant effect on the Smad2/3 levels as analyzed in the human HaCaT keratinocytes and the human PC3 prostate cancer cells. We further show that the phosphorylated Thr-179-proline motif in the Smad3 linker region is the major binding site for Pin1 after TGF-β treatment and suggest that C-tail
phosphorylation is necessary for association with Pin1. Pin1 binding does not have a significant effect on the phosphorylation levels of Thr-179 or the other sites in the linker region of Smad3 in response to TGF-β. Depletion of Pin1 does not have a significant effect on the TGF-β-induced growth-inhibitory responses. In contrast, depletion of Pin1 markedly reduces TGF-β-induced prostate cancer cell migration and invasion. Similar results were also obtained in the MDA-MB-231 breast cancer cells (data not shown). We conclude that Pin1 promotes TGF-β-mediated migration and invasion.

A recent study by Nakano et al. (53) reported that Pin1 enhances Smurf2 interaction with Smad2/3 and leads to decreased levels of Smad2/3. This study showed that knockdown of Pin1 increased Smad2/3 protein levels in the MDA-MB-231 breast cancer cell line (53). Overall, the effect of Pin1 on Smad2/3 protein levels was relatively modest, as quantified in this study. We detected little difference in the Smad2/3 levels in the HaCaT keratinocytes and the PC3 prostate cancer cells when Pin1 was very effectively depleted. One possibility is that the relatively modest Pin1- and Smurf2-dependent degradation of Smad2/3 is cell-type-dependent. Previous studies have shown that although Smurf2 binds to Smad3, it does not degrade Smad3 (84, 85). These observations are consistent with the idea of cell-type specificity.

We have shown in this study that Smad2/3 interaction with Pin1 is specific in response to TGF-β. Although EGF induces phosphorylation of the same linker sites as TGF-β to a similar extent, EGF has little effect in inducing Pin1 interaction with Smad2/3. Analysis of the Smad3 mutant at the C-tail phosphorylation sites suggests that the C-tail phosphorylation is necessary for Pin1 binding. The study by Nakano et al. (53) showed that a constitutively activated Ras, which leads to Smad2/3 phosphorylation in the linker region, can also induce Smad2/3 binding to Pin1. In addition to activating ERK, which phosphorylates Smad2/3 in the linker region, Ras may have other functions in terms of regulating Smad2/3 phosphorylation. A previous study has shown that Mps1, a dual specificity protein kinase for spindle checkpoint function, can phosphorylate Smad2/3 at the C-tail SSXS motif independent of TGF-β signaling (86). Interestingly, a study showed that constitutively activated B-Raf increased Mps1 protein level and activity (87). Thus, it is possible that the constitutively activated Ras can increase the Mps1 activity, leading to the C-tail phosphorylation of Smad2/3. Both the C-tail and linker phosphorylated Smad2/3 then interact with Pin1. In any case, Pin1 binds to Smad2/3 to a much greater extent in response to TGF-β than in response to the activated Ras (53), indicating the importance of TGF-β in regulating the interaction between Smad2/3 and Pin1.

We have shown in this study that the Thr-179 in the Smad3 linker region plays a major role in the interaction with Pin1 in response to TGF-β. When the Thr-179 site was mutated, Smad3 association with Pin1 was dramatically reduced. This result suggests that Pin1 and Smad3 interact at 1:1 ratio. The recent study by Nakano et al. (53) showed that mutation of all four sites in the Smad3 linker region was necessary to disrupt Smad3-Pin1 interaction. Careful analysis of their data, taken into the consideration of the input protein levels, would also suggest that Thr-179 is the major binding site for Smad3 to interact with Pin1.

We analyzed several TGF-β-mediated responses in our studies. Depletion of Pin1 does not have a significant effect on the TGF-β growth-inhibitory responses. We have also analyzed whether depletion of Pin1 affected TGF-β-induced epithelial mesenchymal transition in the PC3 prostate cancer cells, and our studies suggest that depletion of Pin1 modestly reduced TGF-β-mediated epithelial mesenchymal transition in the PC3 cells (data not shown). Thus, Pin1 has a specific role in promoting migration and invasion in response to TGF-β.

The effect of Pin1 in TGF-β-induced migration and invasion is accompanied with the regulation of the expression of N-cadherin, which plays an important role in migration and invasion (71–77), including in the PC3 prostate cancer cells (75). N-cadherin protein levels are induced by TGF-β treatment. When Pin1 is depleted, the TGF-β induction of N-cadherin is dramatically reduced. Analysis of the N-cadherin promoter sequence in silico indicates that it contains a consensus Smad binding element, suggesting that Smad proteins may directly bind to its promoter and regulate its expression. Pin1 can induce conformational changes of a number of its substrates (39–41). This can lead to increased protein stability, decreased protein stability, altered transcriptional activity, or other effects (39–41). The effect of Pin1 on TGF-β/Smad target genes varies. For example, Pin1 essentially has no effect on the p15, p21, and Smad7 levels, has a modest inhibitory effect on JunB, has a modest stimulatory effect on PAI-1, and has a significant stimulatory effect on N-cadherin. Thus, the effect of Pin1 on the TGF-β/Smad target genes appears to depend on the promoter context.

Pin1 is overexpressed in many cancers (39–51). Among 60 different tumor types analyzed, 38 tumor types have Pin1 overexpression in 10% to nearly 100% of the cases analyzed, such as prostate, breast, lung, colon, ovary, cervical, brain tumors, and melanoma (44). Thus, Pin1 overexpression is a specific and prevalent event in human cancers. Previous studies have shown that Pin1 is an excellent prognostic marker in prostate cancer (52), the most common cancer in men in the United States. Patients with higher expression of Pin1 have a significantly higher probability of recurrence than patients with low expression of Pin1. In addition, patients with a high expression of Pin1 have almost four times the risk of having earlier recurrence than those patients with low expression of Pin1. Furthermore, patients with a very high level of Pin1 have more than eight times the risk of having earlier recurrence than the patients with a low level of Pin1 (52). TGF-β promotes cancer progression and metastasis. We have shown in this report that Pin1 mediates TGF-β-induced migration and invasion of prostate cancer cells. Pin1 can also promote migration and invasion at basal state (55). Our findings highlight the importance of Pin1 in cancer progression and metastasis, especially for prostate cancer. Furthermore, our findings highly suggest that Pin1 is an important therapeutic target for prostate cancer, breast cancer, and some other types of cancers.

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Pin1 Promotes TGF-β-induced Migration and Invasion

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