Activation of Mps1 Promotes Transforming Growth Factor-β-independent Smad Signaling*

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The primary intracellular mediators of TGF-β signaling are the Smad proteins. Phosphorylation of R-Smad at the C-terminal SSXS motif by the activated TGF-β type I receptor kinase triggers a conformation change in R-Smad and facilitates complex formation between R-Smad and Smad4, which shuttle into the nucleus where they interact with DNA and other transcription factors to regulate gene expression. In an attempt to identify proteins interacting with activated Smad signaling complex, we discovered that Mps1, a protein kinase that plays important roles in normal mitotic progression and mitotic checkpoint signaling, co-purifies with this complex. We demonstrated that Smad2 and Smad3 but not Smad4 are substrates of Mps1 in vitro and in vivo. Mps1 phosphorylates Smad2 and Smad3 at the SSXS motif in their C-terminal regions in vitro and in vivo. Disruption of microtubule networks by nocodazole activates Mps1 and promotes TGF-β-independent activation of Smad signaling. We found that Mps1 is involved in turning on Smad signaling by phosphorylating R-Smads. Our results reveal a novel functional link between Mps1 and Smads in a non-canonical Smad signaling pathway.

Cells adapt to their environment largely through the activities of signal transduction networks. Transforming growth factor (TGF)β regulates diverse aspects of cellular homeostasis including proliferation, differentiation, migration, and death (1, 2). The primary intracellular mediators of TGF-β signaling are the Smad proteins, which are classified functionally as the receptor-regulated Smads (R-Smads, includes isoforms 1, 2, 3, 5, and 8), the common mediator Smad (Co-Smad, isoform 4), and the inhibitory Smads (I-Smads, isoforms 6 and 7) (3, 4). TGF-β binds two receptor molecules, the type I and type II receptors (TβRI and TβRII, respectively), to form a heterotetrameric active receptor complex. The TβRII is a constitutively active kinase that transphosphorylates serines and threonines in the TβRI, which activates it. Signal flow through the TβRI is required for most TGF-β responses (5). TβRI phosphorylates Smad2 and Smad3 at the two distal serines that are part of the C-terminal SSXS motif (6–8), a process that may be facilitated by adaptor proteins such as SARA (9). Phosphorylation of Smad2 and Smad3 triggers a conformation change and induces complex formation with Smad4 (10). The resulting Smad complex translocates into nucleus where they interact with DNA and other transcription factors to regulate gene expression (11, 12). Phospho-Smad2/3 is continually dephosphorylated by the nuclear phosphatase PPM1A (13, 14). Unmodified R-Smads and unbound Smad4 can shuttle back to the cytoplasm, where the cycle persists pending continued receptor activity (14).

Whereas phosphorylation of R-Smad at the C-terminal serine residue generally positively regulates Smad activity, phosphorylation of other Ser/Thr residues in the linker region of Smad2 or Smad3 by Erk1/2 (15) or cyclin-dependent kinase (Cdk2/4) (16) or calcium-calmodulin-dependent protein kinase-II (17) suppresses Smad2/3 signaling activity. In the case of Erk1/2, it has been proposed that phosphorylation of the linker region of Smad1, -2, and -3 may attenuate the nuclear accumulation (15, 18). The extent of this effect is cell type-dependent and depends on the level of expression of the upstream effector proteins (15, 18, 19). In contrast, phosphorylation of the same region by Cdk2/4 appears to suppress the Smad3 transcriptional activation (16). Thus R-Smad phosphorylation modulates its signaling activity either positively or negatively depending on the sites of phosphorylation.

TGF-β is not the only physiological stimulus that causes Smad2/3 phosphorylation at the C-terminal SSXS motif. Activin A, a member of the TGF-β superfamily has also been shown to activate Smad2/3 phosphorylation by its cognate type I receptor kinase (20). Phosphorylation of the C-terminal SSXS motif also occurs in response to HGF treatment and this phosphorylation event appears to be independent of TGF-β type I receptor (21). Moreover, as the drug nocodazole, which causes disruption of microtubule networks, induces Smad2 phosphorylation at the receptor site (SSXS) and signaling activity (22). Whether nocodazole-induced Smad2 phosphorylation requires the activity of TGF-β type I receptor is still not clear. In either case, the kinase(s) responsible for phosphorylation of Smad2/3 has not been identified.

Here we describe the biochemical characterization of the Smad4 complex in a colon cancer cell line. Unexpectedly, we discovered that Mps1, a component of the mitotic spindle checkpoint, co-purifies with the Smad signaling complex. Smad2 and Smad3 but not Smad4 are substrates of Mps1 in

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The abbreviations used are: TGF, transforming growth factor; cdk, cyclin-dependent kinase; GST, glutathione S-transferase; GFP, green fluorescent protein; MALDI, matrix-assisted laser desorption/ionization; KD, kinase dead; ERK, extracellular signal-regulated kinase; YFP, yellow fluorescent protein; EGF, epidermal growth factor.
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vitro and in vivo. Activation of Mps1 by nocodazole treatment leads to Smad2 and Smad3 phosphorylation at the SSXS motif and promotes TGF-β-independent Smad signaling activity. Our results implicate Mps1 in a non-canonical Smad signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Transfection—All mammalian cell lines were cultured at 37 °C in a 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen), penicillin, and streptomycin (100 international units/ml and 100 mg/ml, respectively). For DNA and siRNA duplex transfection, FuGENE 6 (Roche Applied Science) and Genesilencer (Gene Therapy Systems, Inc.) transfection reagents were used, respectively. The 21-nucleotide RNA duplexes targeting Mps1, Smad2, and Smad3 were purchased from Dharmacon Research, Inc. Antibodies against Mps1 and Smad4 are from Santa Cruz Biotechnology. Anti-Smad2 and anti-Smad3 antibodies were purchased from Zymed Laboratories Inc.. Anti-phospho-Smad2 is a gift of Drs. Ten Djike, Hel-din, and Moustakas. Anti-phospho-Smad3 was kindly provided by Dr. Leof (Mayo Clinic). Nocodazole and SB-431542 were from Sigma-Aldrich and Tocris, respectively.

DNA Manipulation, Mammalian Expression Constructs—The cDNA encoding human Mps1 (MGC865) was purchased from ATCC. Mps1 was subcloned into mammalian expression vector pcDNA3 or pRK5 by PCR. Smad2, Smad3, and Smad4 expression vectors have been described previously (8, 23). Catalytically inactive hMps1 with a change at codon 663 (D663A) was constructed using the QuikChange mutagenesis kit (Stratagene) (24). Details for construction of pRex-TAP-Smad4-IRES-GFP are available upon request. All constructs and mutations were confirmed by DNA sequencing.

Generation of Stable Cell Lines—Retroviral expression construct pRex-TAP-Smad4-IRES-GFP was transfected into 293T cells with the pCL amphotropic helper plasmid (25). 48 h after transfection, virus-containing supernatant was collected and used to infect SW480 cells as described previously (8). Cells expressing a defined level of GFP were isolated using a MoFlo cell sorter (DakoCytomation) as described (8).

Purification of Smad4 Signaling Complexes—SW480M4 cells were grown to 70% confluency on 15-cm culture dishes and harvested by trypsinization. The cell suspension was washed with phosphate-buffered saline and then pelleted by centrifugation. The pellet was re-suspended in lysis buffer I (50 mM Tris, pH 7.4, 430 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40, and 15% glycerol) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.7 µg/ml pepstatin A, 2 µg/ml aprotonin, 2 mM NaF, and 1 mM Na3VO4). Lysates were passed through a self-packed column containing 100 µl of anti-FLAG M2 affinity gel resin (Sigma-Aldrich), and the column was washed three times with 10 ml of PBS to remove proteins bound to resin. Then, 500 µl of 1 mg/ml FLAG peptide (Sigma-Aldrich) was added to elute Smad4. The eluted proteins were applied to a Ni-NTA column (100-µl volume) and fractionated on a SMART gel filtration column (PC3.2) installed in the SMART SYSTEM gel filtration column (GE Healthcare).

Mass Spectrometry Protein Sequencing Analysis—A small portion of each fraction off of the gel filtration column was separated by a 20% SDS-PAGE gradient gel and analyzed by silver staining or immunoblotting with anti-Smad4. Fractions containing Smad4 were pooled together. A portion of the samples was digested with trypsin, and the resulting peptides were fractionated by a microcapillary reverse phase HPLC column directly coupled to an electron ion-spray mass spectrometer (Applied Biosystems/MDS Sciex QSTAR Pulsar). The collected data files were analyzed by Mascot or Protein Prospector programs. The remainder of the fractions was also separated by a 20% SDS-PAGE gradient gel and stained with a zinc staining protocol. Visible bands were excised and processed for MALDI-Q-TOF analysis as described previously (26).

Recombinant Protein Production—Baculoviruses encoding human Mps1, its kinase-dead version (Mps1_KD) and constitutively active human TGF-β type I receptor kinase cytoplasmic domain (TβRI_T204D) were constructed by cloning the corresponding cDNAs into the pFAST-GTH vector, a derivative of pFAST (Invitrogen) in which a tandem affinity-tagged (GST-TEV-6XHis) was inserted into the polylinker region. Baculoviruses were generated and amplified twice in SF9 cells following the manufacturer’s instructions (Invitrogen). Hi-Five cells were infected with the indicated viruses and harvested after 3 days. Extracts of Hi-Five cells were collected, and the corresponding fusion proteins were purified by binding to glutathione-Sepharose beads (GE Healthcare). Where indicated, a second purification was performed using Ni-NTA beads and the elution performed with either thrombin or TEV.

Luciferase Reporter Gene Assay—SW480 or SW480M4 cells were seeded in triplicate at 1 × 10⁵ cells/well in a 12-well plate and grown for 24 h before they were transfected with 3TP-Lux and pCMV-β-gal. 24 h after the transfection of the luciferase construct, the cells were treated with drug or vehicle for 16 h in Dulbecco’s modified Eagle’s medium/0.5% fetal bovine serum. The luciferase activity was measured using the luciferase assay system (Promega) with a Dynex luminometer. The luciferase activity was normalized by total protein concentration as determined by BCA assay (Pierce).

In Vitro Kinase Assay—The kinase reaction typically includes 0.2–0.4 µg of purified recombinant GST-tagged Mps1 or GST-tagged constitutively active TGF-β type I receptor kinase, 5 µg of MBP (myelin basic protein, Sigma) or the desired amount of specific substrates in 1× kinase reaction buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 10 µM ATP, and 1 µCi of [γ-³²P]ATP. Reactions were incubated at room temperature for 15 min and stopped by adding SDS loading buffer. Reaction mixtures were resolved by SDS-PAGE and analyzed by PhosphorImaging.

RESULTS

Purification and Identification of Mps1 as a Protein Associated with the Smad4 Signaling Complex—To identify partners of the Smad signaling complex in colon cancer cells, we expressed a tandem affinity-tagged Smad4 (TAP-Smad4) in the
FIGURE 1. Mps1 associates with the Smad signaling complex. A, expression of a TAP-tagged Smad4 in the SW480 colon cancer cell line. Schematic diagram of the bicistronic retroviral expression vector containing the tandem affinity-tagged Smad4 used in the generation of the SW480M4 cell line. SW480 cells were infected with a recombinant retrovirus encoding the TAP-Smad4 as illustrated. Cells expressing TAP-tagged Smad4 (SW480M4) were obtained by FACS as described previously (8). B, levels of Smad2, Smad3, and Smad4 in SW480 and SW480M4 cells were determined by immunoblotting analysis. Note that SW480 cells express no detectable Smad4 because of a homozygous deletion. The presence of TAP-Smad4 did not affect the levels of endogenous Smad2 or Smad3. C, SW480 or SW480M4 cells were transiently transfected with a Smad/TGF-β responsive reporter gene (p3TP-Lux). 24 hours after transfection, cells were treated with or without TGF-β (100 pM) for 18 h, harvested, and assayed for luciferase activity. The luciferase activity was normalized by protein concentration measurements. Error bars indicate standard deviation from triplicate samples. D, protein complexes isolated from tandem affinity purification were analyzed by SDS-PAGE and silver staining. Proteins identified by MALDI and LC/MS/MS are indicated on the left. E, immunoblotting analysis confirming the presence of Mps1 in the tandem affinity-purified Smad4-associated protein complexes. Lanes 1 and 2, cell lysates prepared from 2 x 10^7 (1) or 4 x 10^7 (2) SW480M4 cells. Lanes 3 and 4, eluates after tandem affinity purification of TAP-Smad4 from the equivalent 2 x 10^7 (1) or 6 x 10^7 (3) of SW480M4 cells. F, TAP purification using parental SW480 and derivative SW480M4 cell lines. 2 x 10^7 cells from respective cell lines were lysed and subjected to two-step tandem affinity purification as described. Eluates from the final step of purification were immunoblotted with an anti-Mps1 antibody. Lanes 1 and 3, whole cell lysates from SW480M4 and SW480 cells. Lanes 3 and 4, eluates from TAP purification. G, list of Mps1/TTK peptides identified by MS after submitting TIC dataset to the Mascot search engine. Results from two separate runs are presented.
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A

B

C

D

E

F

G
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SW480 colon cancer cell line, which harbors a homozygous deletion of endogenous Smad4 (27). Stable expression of TAP-Smad4 was achieved by infecting SW480 cells with recombinant retrovirus produced from a bicistronic retroviral vector in which TAP-Smad4 was placed upstream of IRES-GFP (Fig. 1A) (26, 28). Cells expressing a defined level of GFP were collected. Because retroviruses integrate randomly into the host genome, the resulting cell pools (SW480M4) after sorting are equivalent to a mixture of multiple independently generated lines. The TAP-tagged Smad4 expressed well in the infected cells, whereas the expression levels of Smad2 and Smad3 were unaltered (Fig. 1B). Smad4 was expressed at a level about 2–3-fold higher than the endogenous level of Smad4 in the mink lung epithelial cell line CCL64, which contains ∼100,000 molecules/cell (29). To demonstrate that TAP-Smad4 is functional and able to restore TGF-β signaling, SW480 and SW480M4 were transfected with p3TP-Lux, a TGF-β-responsive luciferase reporter gene (30). TGF-β treatment had no effect on luciferase reporter activity in SW480 cells but led to an increase in luciferase activity in SW480M4 (Fig. 1C). This suggested that a Smad4-dependent pathway was restored and that the TAP affinity tag does not appear to alter its function.

Smad4 and its associating proteins were purified from SW480M4 cells using sequential affinity chromatography followed by gel filtration chromatography as described under “Experimental Procedures.” Fractions containing the Smad4 complex were pooled, subjected to SDS-PAGE, and analyzed by silver staining (Fig. 1D). Both ESI- LC/MS/MS and MALDI-Q-TOF mass spectrometry were used to reveal the identity of proteins that were associated with Smad4. For MALDI-Q-TOF analysis, proteins bound to the column were eluted by incubation with highly purified recombinant His₆-tagged TEV protease prior to SDS-PAGE. For the LC/MS/MS experiment, TEV cleavage step was omitted because the TEV protease was His₆-tagged, and excessive amounts of TEV complicate LC/MS/MS analysis and often suppress the signals from low abundance peptides. The 70-, 60-, and 55-kDa bands were readily identified as Smad4, Smad2, and Smad3, respectively by both LC/MS/MS and MALDI-Q-TOF experiments (Fig. 1D). The 100-kDa protein was identified as Mps1, because more than three different peptides of this protein were definitively sequenced and unequivocally assigned to Mps1 (Fig. 1G). Other proteins identified were SARA and Dab-2, which have been previously demonstrated to associate with the Smad complex (9, 31). To further confirm that the 100-kDa protein interacting with Smad4 was indeed Mps1, a portion of the eluates used for mass spectrometry analysis was separated by SDS-PAGE and immunoblotted with an anti-Mps1-specific antiserum (Fig. 1E). Mps1 was absent when the TAP purification was also performed using the parental SW480 line, suggesting that TAP purification of Mps1 is Smad4-dependent (Fig. 1F). Thus, Mps1 copurifies with Smad4 in colon cancer cells.

Association of Mps1 with Smad4 in Vivo and in Vitro—To further characterize Smad4-Mps1 association in vivo, we performed a reciprocal coimmunoprecipitation experiment with SW480M4 cells in the presence or absence of TGF-β and/or the microtubule depolymerization drug nocodazole, which triggers the mitotic checkpoint and activates Mps1 (32–34). As shown in Fig. 2A, TAP-Smad4 in SW480M4 associates with Mps1, and there is a slight increase in association between Mps1 and Smad4 in the presence of nocodazole. Association between endogenous Smad4 and Mps1 was also observed in HeLa cells (Fig. 2B). The observed interaction is specific, as Smad4 did not coimmunoprecipitate with an antibody raised against another protein kinase, Erk5 (Fig. 2C). In addition, Smad4–Mps1 association was also observed by coimmunoprecipitation with an anti-Smad4 antibody (Fig. 2B, bottom two panels). In contrast, Bub1, another component of the mitotic spindle checkpoint, was not coimmunoprecipitated with the Smad4 antibody (Fig. 2D). These data suggest that Smad4 associates with Mps1 specifically in vivo.

Because Mps1 co-purifies with the Smad signaling complex, we tested whether Smad2, Smad3, and Smad4 can bind Mps1 in vitro. Recombinant GST–Smad2, Smad3 and Smad4 were incubated with [35S]Met-labeled Mps1 or Bub1 prepared by in vitro translation. Binding between Smad proteins and Mps1 was determined by the GST-pull-down assay. As shown in Fig. 2E, all three Smads bind Mps1 but not Bub1, suggesting that association between Smads and Mps1 is specific. Essentially the same result was obtained with reciprocal binding experiment using GST–Mps1KD (Fig. 2F). To determine which domains of Smad may be involved in Mps1 binding, we performed a GST pull-down assay by incubating various deletion mutants of Smad4 with kinase-deficient Mps1 (GST–Mps1KD). MH1 but not MH2 domain of Smad4 appears to be required for Mps1 association in vitro (Fig. 2G). Taken together, our results suggest that Mps1 associates with Smad4 in vitro and in vivo.

*FIGURE 2.* In vivo association between Mps1 and Smad4. A, co-immunoprecipitation experiment was performed using cell lysates prepared from synchronized SW480M4 cells treated with Me₂SO vehicle, TGF-β, nocodazole, or TGF-β and nocodazole for 12 h with the antibodies indicated. B, endogenous Smad4 associates with Mps1 in HeLa cells. Proliferating HeLa cells were treated with Me₂SO vehicle, TGF-β, nocodazole, or TGF-β and nocodazole for 18 h prior to harvesting. Lysates from 6 × 10⁶ treated and untreated cells were immunoprecipitated with anti-Smad4 monoclonal antibody (B8) or anti-Mps1 antibody. Equal amounts of cell lysates (10% of input) were used as judged by the immunoblot of the input cell lysates with respective antibodies. C, Smad4 does not associate with Erk5 kinase in vivo. Control co-immunoprecipitation experiment was performed using HeLa cell lysates as described in B except that a rabbit polyclonal antibody against Erk5 was used for immunoprecipitation. D, Bub1 does not associate with Smad4 in vitro. A co-immunoprecipitation experiment was performed using HeLa cell lysates as described in B except that anti-Bub1 antibody was used for immunoblotting analysis. E, Mps1 but not Bub1 interacts with Smad2, Smad3, and Mps1 in vitro. [35S]Met-labeled Mps1 and Bub1 were synthesized by in vitro transcription and translation in a reticulocyte lysate system. Recombinant Smad proteins illustrated in the top panel were expressed as GST fusion proteins in bacteria. Each recombinant protein along with the GST control was incubated with radiolabeled Mps1 or Bub1, and the binding assay was performed as described. The amount of Mps1 that binds Smads was determined by the GST-pull-down assay. As shown in Fig. 2E, all three Smads bind Mps1 but not Bub1, suggesting that association between Smads and Mps1 is specific. Essentially the same result was obtained with reciprocal binding experiment using GST–Mps1KD (Fig. 2F). To determine which domains of Smad may be involved in Mps1 binding, we performed a GST pull-down assay by incubating various deletion mutants of Smad4 with kinase-deficient Mps1 (GST–Mps1KD). MH1 but not MH2 domain of Smad4 appears to be required for Mps1 association in vitro (Fig. 2G). Taken together, our results suggest that Mps1 associates with Smad4 in vitro and in vivo.
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**FIGURE 3.** Mps1 phosphorylates Smad2 and Smad3 but not Smad4 in vitro. **A,** left panel, Coomassie Blue staining of recombinant GST-Mps1 and GST-Mps1_KD (kinase dead) proteins purified from insect cells. 0.5, 1, and 2 μg of bovine serum albumin were loaded on the gel for quantification of the recombinant proteins. Right panel, Coomassie Blue staining of recombinant constitutively active TGF-β receptor type 1 (TβRI_T204D) and Mps1 kinase purified from insect cells and recombinant GST-Smad2, Smad3, Smad4, and Smad3A from bacterial cells. 0.5–1 μg of protein was loaded. **B,** phosphorylation of recombinant Smad2 or Smad3 by GST-Mps1 or GST-Mps1_KD. One microgram of GST-Smad2 or Smad3 was incubated with 0.5 μg of GST-Mps1 or GST-Mps1_KD at room temperature for 15 min in the presence of [γ-32P]ATP. The reactions were terminated by boiling in SDS sample buffer and then analyzed by SDS-PAGE. GST-Mps1_KD neither autophosphorylates nor phosphorylates Smad2 and Smad3. **C,** top panel, 1/2 μg each of GST-Smad2, GST-Smad3, GST-Smad4 and GST-Smad3A was incubated with 2 μg of constitutively active TGF-β receptor type 1 (TβRI_T204D) kinase and 0.5 μg of Mps1 at room temperature for 15 min in the presence of [γ-32P]ATP. Both kinases exhibit substantial autophosphorylation. Bottom panels, amounts of recombinant proteins used in the kinase reaction as determined by SDS-PAGE and Coomassie Blue staining. **D,** Smad4-dependent activation of Mps1 kinase activity. Equal amounts of GST, GST-Smad2, GST-Smad3, and GST-Smad4 either alone or in combination (the total amount of input was kept constant at 0.1 μg/μl by varying the amount of GST) as indicated were incubated with 0.02 μg/μl Mps1 along with 0.25 μg/μl MBP at room temperature for 15 min in the presence of [γ-32P]ATP. The reactions were terminated by boiling in SDS sample buffer and then analyzed by SDS-PAGE. The relative amount and quality of recombinant proteins used in this experiment were determined by the Coomassie Blue staining. (10× the amount of Mps1 was loaded in order to visualize the bands.)
**Mps1 Phosphorylates Smad2 and Smad3 in Vitro**—The presence of Mps1 kinase in the Smad signaling complex prompted us to investigate whether Smads are potential substrates of this kinase. To address this question, we produced and purified recombinant Smad2, Smad3, and Smad4 in *Escherichia coli* and recombinant Mps1 or the constitutively active TGF-β type I receptor kinase (TβRI_T204D) from insect cells (Fig. 3A). Purified Smad2, Smad3, and Smad4 were incubated with recombinant Mps1 or the constitutively active TGF-β type I receptor kinase (TβRI_T204D) purified from insect cells under kinase reaction conditions. Smad2 and Smad3 but not Smad4 were phosphorylated both by Mps1 and TβRI_T204D with similar efficiencies (Fig. 3, B and C). No phosphorylation of Smad2 or Smad3 was observed with the kinase dead Mps1 (Mps1_KD, Fig. 3B) (24). Phosphorylation of Smad3 by Mps1 was reduced when the conserved SSXS motif at C terminus of Smad3 was changed to AAXA, suggesting that these residues were targeted for phosphorylation in *vivo* (Fig. 3C). Furthermore, Mps1 can phosphorylate both the GST- and His₆-tagged versions of Smad2 and Smad3 in *vivo*, suggesting this reaction is independent of the protein tag employed (data not shown). Hence, Smad2 and Smad3 but not Smad4 are substrates of Mps1 *in vitro*.

**Smad4 Regulates Mps1 Kinase Activity**—Because Smad4 associates with Mps1, we tested whether Smad4 can directly regulate Mps1 kinase activity in *vivo*. Recombinant Mps1 was incubated with MBP, a substrate that is commonly used to measure its activity (32, 34), in the presence or absence of Smad2, Smad3 or Smad4. As shown in Fig. 3D, addition of Smad2 and Smad3, individually or combined, had little effect on MBP phosphorylation, although low levels of Smad2 and Smad3 phosphorylation were observed. Upon Smad4 addition, Mps1 kinase activity toward MBP was greatly increased (Fig. 3D). Moreover, the Mps1 kinase activities toward Smad2 and Smad3 were also increased. However, neither Smad2 nor Smad3 alone stimulated the kinase activity, indicating that Smad4 but not Smad2 or Smad3 is a strong activator of Mps1 *in vitro*. Furthermore, the stimulatory activity of Smad4 depended on its ability to bind Mps1 but not on the tag (GST or His₆) used to purify the Smads (data not shown). This result suggests that Smad4 stimulates Mps1 activity in *vivo*.

**Mps1 Phosphorylates Smad2 and Smad3 in Vivo**—To determine whether Mps1 can also phosphorylate the conserved SSXS motifs in Smad2 and Smad3 in cell cultures *in vivo*, mammalian expression vectors expressing either TβRI_T204D and Mps1 were cotransfected with Smad2 or Smad3 expression vectors in 293T cells (Fig. 4, A and B). Forty-eight hours after transfection, cell lysates were prepared and subjected to immunoblot analysis. Phosphorylated Smad2 and Smad3 were readily detected in lysates prepared from cells cotransfected with Mps1 with either Smad2 (Fig. 4A) or Smad3 (Fig. 4B) using antibodies that specifically recognize the forms of Smad2 (pSmad2) and Smad3 (pSmad3) phosphorylated at the SSXS motif (35, 36). Thus, both Smad2 and Smad3 can be phosphorylated by Mps1 at the C-terminal SSXS motif *in vivo*.

Mps1 kinase activity is cell cycle regulated and is maximal during mitosis. To determine the specificity of Smad2 and Smad3 phosphorylation, we overexpressed other protein kinases known to be active in mitosis, Bub1, BubR1, and TOPK, in 293T cells by transient transfection with Smad2 and tested their ability to phosphorylate Smad2 *in vivo* (Fig. 4C). Only Mps1 and TβRI_T204D appear to specifically phosphorylate the SSXS motif *in vivo*, suggesting that the phosphorylation of Smad2 by these two kinases is highly specific.

**Mps1 Is Involved in Nocodazole-induced Smad2 Phosphorylation in Vivo**—We sought to find physiologically relevant events that could trigger Mps1-mediated Smad2 phosphorylation. Previous studies have implicated Mps1 as an essential kinase for normal mitotic progression and mitotic checkpoint signaling in yeast, zebra fish, and vertebrate cells (32–34, 37, 38). Mps1 kinase activity is elevated 30-fold upon treatment with nocodazole, a microtubule-depolymerizing drug, which causes mitotic arrest because of activation of the mitotic checkpoint signaling response (32). Interestingly, nocodazole has

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**FIGURE 4. Mps1 phosphorylates Smad2 and Smad3 at the SSXS motif in vivo.** A, Mps1 and TβRI_T204D phosphorylate Smad2 in vivo. Smad2 was cotransfected with either Mps1 or TβRI_T204D or both into 293T cells. Cell lysates were prepared 48-h post-transfection and subjected to immunoblotting analysis with an anti-phospho-Smad2 antibody. B, Mps1 and TβRI_T204D phosphorylate Smad3 in vivo. The experiment is identical to A except Flag-Smad3 was used instead of Flag-Smad2. C, Mps1, but not other mitotic checkpoint kinases, phosphorylates Smad2 in vivo. 293T cells were transfected with empty vector (for kinase expression), Bub1, BubR1, Mps1 PBK/TOPK, or TβRI_T204D each in combination with Flag-Smad2. Phospho-Smad2 was determined by immunoblotting analysis. An IP-kinase assay was used to determine the kinase activity toward MBP.
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been observed to stimulate phosphorylation of Smad2 in mink lung cells at the SSXS motif and activate Smad transcriptional response (22, 39). The protein kinase(s) responsible for this phosphorylation has not been identified, although it has been speculated that the TGF-β type I receptor may be involved in this process (22) after Smad dissociates from the microtubules.

To further investigate nocodazole-induced endogenous Smad2 phosphorylation and whether this phosphorylation depends on TGF-β type I receptor, we treated wild type (CCL64) and type I receptor-defective mink lung cells (R1B) (40) with either TGF-β or nocodazole for various times (Fig. 5A). Upon treatment with TGF-β, Smad2 is phosphorylated at the SSXS motif in wild-type but not R1B cells (Fig. 5A, compare lanes 2 versus 7). In contrast, nocodazole treatment of wild-type and mutant cells resulted in comparable Smad2 phosphorylation (Fig. 5A), even in the presence of SB431542, an ALK4/5/7 small molecule inhibitor (Fig. 5B). These data suggest that nocodazole-induced Smad2 phosphorylation is independent of TGF-β type I receptor kinase.

To determine whether activated Mps1 kinase contributes to endogenous Smad2 phosphorylation, we used HeLa cells because they are amendable to RNAi analysis. HeLa cells synchronized by double thymidine treatment were released into growth media in the presence of nocodazole for the indicated times. Consistent with previous observations, nocodazole induced both Smad2 phosphorylation at the SSXS motif and Mps1 kinase expression in a time-dependent manner (Fig. 5C). Nocodazole-induced Smad2 phosphorylation peaks at around 13 h of the treatment and appears to correlate with Mps1 expression levels (Fig. 5C) (32). If Mps1 contributes to Smad2 phosphorylation in vivo, we would expect that knockdown of Mps1 expression would reduce Smad2 phosphorylation in response to nocodazole treatment. HeLa cells transfected with control, Mps1, and Bub1 siRNA duplexes were treated with nocodazole for the indicated times. As shown in Fig. 5, D and E, a siRNA duplex targeting Mps1 led to a significant reduction in nocodazole-induced Smad2 phosphorylation whereas neither control nor Bub1 siRNA knockdown had any effect. The incomplete elimination of Smad2 phosphorylation could be caused by our inability to completely deplete Mps1 from cells by RNAi or an unidentified redundant kinase also involved in nocodazole-induced Smad2 phosphorylation. Nevertheless, knockdown of Mps1 expression consistently lowered the levels of pSmad2 but had little effect on total Smad2 levels. Thus, we conclude that Mps1 is
involved in nocodazole-induced Smad2 phosphorylation, and endogenous Smad2 appears to be a substrate for Mps1 in vivo.

**Nocodazole Treatment Induces Nuclear Accumulation of Smad4**—A key step in TGF-β signaling is the nuclear translocation of Smad2/3 and Smad4. To determine whether nocodazole exposure also regulates nuclear translocation of Smad2 or Smad4, we created SW480 and Mink lung epithelial cell lines stably expressing GFP-Smad4 and YFP-Smad2 respectively. Although we were able to stably express YFP-Smad2 in mink lung cells, stable expression of GFP-Smad4 in mink lung cells was not possible, perhaps because of the presence of endogenous Smad4 and an intact growth inhibitory response to Smad4. However, GFP-Smad4 can be stably expressed in SW480 cells in which endogenous Smad4 is deleted. As expected, GFP-Smad4 and YFP-Smad2 predominantly localize in the cytosol in the basal state (Fig. 6, A and B), which agrees with previous observations that the nuclear export rates of Smad2 and Smad4 exceed their import rates in the absence of ligand (14, 41). When exposed to TGF-β for 2 h, GFP-Smad4 and YFP-Smad2 localized to the nucleus (Fig. 6AB). Both GFP-Smad4 and YFP-Smad2 return to the cytosol upon prolonged TGF-β treatment (10 h) as observed (14, 42). Compared with TGF-β, nocodazole treatment induces more persistent nuclear retention of GFP-Smad4 (Fig. 6A) with nuclear accumulation of GFP-Smad4 still evident at 10 h after the nocodazole addition. At the 10-h time point, close to 25% of SW480 cells were arrested in mitosis. In mitotic cells, YFP-Smad2 is diffused throughout the cells as opposed to preferentially binding to chromosomes observed with Smad4 (Fig. 6B). Therefore, compared with TGF-β, nocodazole treatment induces similar but persistent nuclear accumulation of Smad4, and nuclear accumulation of Smad2 is slow and less robust.

**Mps1 Depletion Attenuates Nocodazole-induced TGF-β Transcriptional Activity**—Previous studies suggest that nocodazole treatment can trigger transcriptional activation of TGF-β responsive promoters which correlates with the elevated levels of Smad2 phosphorylation in mink lung cells (22). It is not clear whether induction of TGF-β-responsive promoters by nocodazole is Smad4-dependent. To test the requirement of Smad4 in this response, we compared induction of p3TP-lux promoter in response to TGF-β, nocodazole or both in SW480 and SW480M4 cells. As shown in Fig. 6C, there is very little induction of 3TP promoter in the Smad4-null SW480 cells in the presence of either nocodazole or TGF-β. However, in SW480M4 cells which Smad4 is re-expressed, TGF-β treatment leads to at least a 3-fold induction of luciferase activity, which is consistent with previous observations (43). Nocodazole can also independently activate this promoter although the induction is slightly low with nocodazole alone (Fig. 6C). When SW480M4 cells were treated with both TGF-β and nocodazole, the induction was higher than with either treatment alone, suggesting that the effects may be additive. Differences between Smad4-null and Smad4-revertant SW480 cells in transcriptional responses to nocodazole suggest that nocodazole-induced TGF-β transcriptional response is Smad4-dependent. Immunoblotting analysis of cell lysates with each treatment indicates that both TGF-β and nocodazole can activate Smad2 phosphorylation at the SSXS motif, which appears to be Smad4-independent. However, we did observe that nocodazole-induced Smad2 phosphorylation is always more efficient in Smad4-positive cells than Smad4-null cells (Fig. 6D), which implies that Smad4 may promote nocodazole-induced Smad2 phosphorylation.

Next, we determined whether Mps1 is involved in nocodazole-induced Smad-dependent transcription. SW480M4 cells were first transfected with the control or Mps1 siRNA followed by p3TP-lux. Cells were treated with Me2SO, nocodazole, TGF-β, or both nocodazole and TGF-β for 24 hours after transfection. As expected, TGF-β and nocodazole alone or in combination activate TGF-β-responsive transcription with the control siRNA (Fig. 6E). Depletion of Mps1 in SW480M4 cells did not affect the basal levels of 3TP promoter activity (Fig. 6E), however, nocodazole-induced transcriptional activation is reduced by at least 50%, barely rising above basal levels. There is also a slight reduction in TGF-β-induced transcriptional activation of the 3TP promoter, the reason for which is not clear at this point. Mps1 expression is clearly reduced in SW480M4 cells upon RNAi (Fig. 6F) and so are the levels of phosphorylated Smad2. Taken together, our data suggest that partial depletion of Mps1 causes a reduction in phosphorylation of Smad2 at the SSXS motif and attenuates nocodazole-induced TGF-β transcriptional activity. Thus we conclude that Mps1 is involved in a non-canonical Smad signaling pathway in response to nocodazole treatment.

**DISCUSSION**

To identify novel partners of the Smad signaling complex in colon cancer cells, we performed tandem affinity purification of Smad4 and discovered that Mps1/TTK, a protein kinase implicated in normal mitotic progression and mitotic checkpoint signaling, associates with Smad4. We demonstrated that Mps1 is involved in nocodazole-induced phosphorylation of Smad2 and Smad3 at the SSXS motif that is normally targeted by the activated TGF-β type I receptor. Our data implicate Mps1 in a non-canonical activation of Smad signaling independent of TGF-β ligand.

The canonical function of Smad in mediating TGF-β signaling is well established. However, several studies show that Smad signaling can be activated by stimuli other than the ligands in the TGF-β superfamily. For example, it has been shown that HGF and EGF can induce Smad2 phosphorylation at the SSMS motif in mink lung cells and induction of phosphorylation is independent of TGF-β type I receptor (21). Both HGF and EGF also activate TGF-β-responsive 3TP promoter activity, and this activation requires functional Smad4 (21). It was proposed that Smad2 may act as a common effector to integrate TGF-β- and
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A

SW480 Cells

Control 2 h TGF-β 2 h Noco 10 h TGF-β 10 h Noco

GFP-Smad4

DAPI

Merge

Mink Lung cells

Control 2 h TGF-β 2 h Noco 10 h TGF-β 10 h Noco

YFP-Smad2

DAPI

Merge

B

C

D

E

F
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HGF-induced signals and modulate cross-talk between receptor serine/threonine kinase and receptor tyrosine kinase signaling pathways (21). Disruption of microtubule networks by nocodazole or colchicine also activates TGF-β transcriptional activity by induction of Smad2 phosphorylation, although it was not clear whether activation of Smad signaling and transcriptional activity require TGF-β type I receptor or Smad4 (22, 39). Here we showed that nocodazole-induced Smad signaling is independent of the TGF-β type I receptor but requires Smad4, similar to HGF or EGF. The kinase(s) responsible for the specific phosphorylation of Smad2 had not been identified in either situation. Our results suggest that Mps1 is likely responsible for Smad2 phosphorylation during nocodazole treatment, because knockdown of Mps1 expression reduced nocodazole-induced Smad2 phosphorylation in vivo. Because Mps1 RNAi treatment only partially depletes endogenous Mps1, it is not clear whether the residual amount of kinase is still sufficient to phosphorylate Smad2. Therefore, we cannot rule out that additional kinases are also involved in mediating Smad2 phosphorylation. Definitive answers will likely come from future Mps1 knock-out studies. Nevertheless, Smad2 phosphorylation by Mps1 appears to be very specific because other mitotic and checkpoint kinases such as Bub1, BubR1, and TOPK do not phosphorylate this site in cells. Phosphorylation of Smad2 induced by nocodazole occurs independently of the TGF-β type I receptor. Mps1 is now the only known kinase other than the TGF-β type I receptor that can phosphorylate Smad2 at the SSXS motif. It would be interesting to test whether Mps1 is also involved in HGF-induced Smad2 phosphorylation.

Even though both canonical and non-canonical signals can activate Smad2 phosphorylation, the amplitude and duration of Smad2 phosphorylation appear to be different. TGF-β-induced Smad2 phosphorylation is strong and sustained whereas HGF-induced Smad2 phosphorylation is much lower and transient (21). Induction of Smad2 phosphorylation by nocodazole is sustained but the amplitude is lower than TGF-β. Another major difference between these two treatments is the rate of appearance of phospho-Smad2. It takes a few hours to detect pSmad2 upon nocodazole treatment as opposed to 30 min after the addition of TGF-β. The delayed phosphorylation kinetics could be a result of delayed activation of the protein kinase responsible for this phosphorylation.

Another difference between canonical and non-canonical activation of Smad signaling lies in Smad4 translocation. While TGF-β induces rapid transient nuclear accumulation of Smad4, the effect of nocodazole on Smad4 nuclear accumulation is as rapid but more persistent. Interestingly, unlike TGF-β, nocodazole-induced Smad4 nuclear translocation is not coupled with concomitantly elevated Smad2 nuclear accumulation. This observation is consistent with the finding that Smad2 phosphorylation in response to nocodazole treatment is delayed (Fig. 5). Given Smad4 can activate Mps1 kinase activity in vitro and phosphorylation of Smad2 is reduced in the absence of Smad4 in vivo, we speculate that association of Smad4 with Mps1 may precede phosphorylation of Smad2 by Mps1. However, we do not know the exact subcellular location and cell cycle stage at which the Smad4-Mps1 interaction occurs. A definitive answer to this question requires development of FRET-based technology to visualize dynamic protein-protein interaction in vivo as biochemical analysis of protein complex formation cannot exclude the possibility of protein association in lysates upon cell disruption.

Mps1 kinase activity is strongly elevated after a few hours treatment with nocodazole (44). Mps1 kinase activity is tightly regulated during cell cycle progression with the lowest activity in interphase cells and highest in mitotic cells (33, 44). Nocodazole activates mitotic checkpoint signaling and causes cell cycle arrest in mitosis. Recent studies also implicate the importance of Mps1 in DNA damage response during G2 phase of the cell cycle (45, 46). The striking parallel between Mps1 kinase activation and Smad2 phosphorylation suggests that nocodazole-induced Smad2 activation may occur in late G2 or early mitosis. Thus, our results highlight a potential new aspect of Smad regulation, i.e. cell cycle-specific activation of Smad signaling.

What might be the function of Smad2/3 phosphorylation by Mps1? Phosphorylation of Smad2/3 at their C-terminal serines by the TGF-β type I receptor promotes their complex formation with Smad4 and is required for activation of target genes (1). Thus, one possible function for Mps1-induced Smad2/3 phosphorylation is that phosphorylated R-Smads form a complex with Smad4 and regulate the transcription of certain genes critical for mitotic checkpoint responses. Conceptually, this is analogous to the TGF-β signaling response except that it represents cell autonomous activation of Smads by checkpoint signaling pathways. Future studies are needed to demonstrate that Mps1 can indeed promote complex formation between R-Smads and Co-Smad and to identify Smad target genes that may play a role in cell cycle progression and mitotic checkpoint signaling.

FIGURE 6. Nocodazole treatment activates Smad signaling by targeting Mps1. A, nocodazole induces nuclear translocation of Smad4. GFP-Smad4 was stably expressed in Smad4-deficient SW480 cells. Cells were treated with either TGF-β (100 ng/ml) or nocodazole (100 ng/ml) for 2 or 10 h, respectively. Cells were fixed with paraformaldehyde and stained with DAPI prior to imaging. B, mink lung epithelial cells stably expressing YFP-Smad2 were treated with either TGF-β (100 ng/ml) or nocodazole (100 ng/ml) for 2 or 10 h, respectively and imaged as described in A. At least 100 cells were scored for each treatment, and representative images are shown. C, microtubule disruption enhances TGF-β transcriptional responses and Smad2 phosphorylation. SW480 or SW480M4 cells were transiently transfected with a TGF-β responsive reporter gene (p3TP-Lux). Twenty-four hours after transfection, cells were treated with or without nocodazole (100 ng/ml) or TGF-β (100 ng/ml) or in combination for 18 h, harvested in cell lysis buffer (Promega) and processed for luciferase activity measurements. D, same lysates used for measuring luciferase activity were also immunoblotted with anti-Mps1, anti-Smad2, and anti-phospho-Smad2 antibodies. Equal amounts of lysates used in this experiment were determined by normalizing for protein concentration measurements. As observed previously with HeLa cells (32, 33), nocodazole treatment increased the steady state levels of Mps1 in both SW480 and SW480M4 cells. E, knockdown expression of Mps1 by siRNA negates nocodazole-induced Smad2 phosphorylation and elevated TGF-β transcriptional responses. SW480 and SW480M4 cells were transfected with a nonspecific siRNA duplex or a siRNA duplex targeted to Mps1 using the Gensilencer reagent. 24 h after transfection, cells were split and seeded into 12-well plates and transfected with p3TP-Lux reporter gene using FuGENE 6. Sixteen hours later, cells were treated with vehicle, TGF-β, nocodazole, or in combination for an additional 18 h prior to harvest. A portion of the lysates was used to measure the luciferase activity, while the remainder was used to measure protein concentrations or immunoblot with anti-Mps1, anti-Smad2, or anti-phospho-Smad2 antibodies in F.
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REFERENCES
1. Massague, J. (1998) Annu. Rev. Biochem. 67, 753–791
2. Kingsley, D. M. (1994) Genes Dev. 8, 133–146
3. Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997) J. Biol. Chem. 272, 27678–27685
4. Souchelnytskyi, S., Tamaki, K., Engstrom, U., Wernstedt, C., ten Dijke, P., and Heldin, C. H. (1997) J. Biol. Chem. 272, 28107–28115
5. Liu, X., Sun, Y., Constantinescu, S. N., Karam, E., Weinberg, R. A., and Lodish, H. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10669–10674
6. Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L., and Wrana, J. L. (1998) Mol. Cell Biol. 18, 10671–10679
7. Laiho, M., Weis, M. B., and Massague, J. (1990) Mol. Biol. Cell 1, 272, 28107–28115
8. Lin, X., Duan, X., Liang, Y. Y., Su, Y., Wrighton, K. H., Long, J., Hu, M., Davis, C. M., Wang, J., Brunicardi, F. C., Shi, Y., Chen, Y. G., Meng, A., and Feng, X. H. (2006) Cell 125, 915–928
9. Inman, G. J., Nicolas, F. J., and Hill, C. S. (2002) Mol. Cell 10, 283–294
10. Kretzschmar, M., Doody, J., Timokhina, I., and Massague, J. (1999) Genes Dev. 13, 804–816
11. Matsuura, I., Denissova, N. G., Wang, G., He, D., Long, J., and Liu, F. (2004) Nature 430, 226–231
12. Wicks, S. J., Lui, S., Abdel-Wahab, N., Mason, R. M., and Chantry, A. (2000) Mol. Cell Biol. 20, 8103–8111
13. Kretzschmar, M., Doody, J., and Massague, J. (1997) Nature 389, 618–622
14. Kifir, S., Ehrlich, M., Goldshmidt, A., Liu, X., Kloor, Y., and Henis, Y. I. (2005) Mol. Cell Biol. 25, 8239–8250
15. Graff, J. M., Bansal, A., and Melton, D. A. (1996) Cell 85, 479–487
16. de Caestecker, M. P., Parks, W. T., Frank, C. J., Castagnino, P., Bottaro, D. P., Roberts, A. B., and Lechleider, R. J. (1998) Genes Dev. 12, 1587–1592
17. Dong, C., Li, Z., Alvarez, R., Jr., Feng, X. H., and Goldschmidt-Clermont, P. J. (2000) Mol. Cell 5, 27–34
18. Zhang, Y., Feng, X., We, R., and Derynck, R. (1996) Nature 383, 168–172
19. Fisk, H. A., and Winey, M. (2001) Cell 106, 95–104
20. Navaux, R. K., Costanzi, E., Haas, M., and Verma, I. M. (1996) J. Virol. 70, 5701–5705
21. Knuesel, M., Wan, Y., Xiao, Z., Holinger, E., Lowe, N., Wang, W., and Liu, X. (2003) Mol. Cell Proteomics 2, 1225–1233
22. Schütte, M., Hruban, R. H., Hedrick, L., Cho, K. R., Nadasdy, G. M., Weinstein, C. L., Bova, G. S., Isaacs, W. B., Cairns, P., Nawroz, H., Sidransky, D., Casero, R. A., Jr., Meltzer, P. S., Hahn, S. A., and Kern, S. E. (1996) Cancer Res. 56, 2527–2530
23. Zhao, Y., Feng, X. H. (2006) Cancer Res. 66, 12527–12530
24. Shen, H.,_listen to him, Y., Yeh, H. Y., Tyan, S. W., Sun, T. P., Shen, C. Y., and Shieh, S. Y. (2005) J. Biol. Chem. 280, 7748–7757