TβRI Phosphorylation of Smad2 on Ser^{465} and Ser^{467} Is Required for Smad2-Smad4 Complex Formation and Signaling*

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Mothers against Dpp-related or Smad proteins are essential components of serine/threonine kinase receptor signaling pathways that are regulated by phosphorylation. Recently, it was demonstrated that Smad2 interacts transiently with and is a direct substrate of the transforming growth factor-β (TGF-β) type I receptor, TβRI. Phosphorylation sites on Smad2 were localized to a carboxyl-terminal fragment containing three serine residues at positions 464, 465, and 467. In this report, we show that TβRII specifically phosphorylates Smad2 on serines 465 and 467. Serine 464 is not a site of phosphorylation, but is important for efficient phosphorylation of Smad2. Phosphorylation at both sites is required to mediate association of Smad2 with Smad4 in mammalian cells, while in yeast, Smad2 interacts directly with Smad4 and does not require phosphorylation. Mutation of either serine residue 465 or 467 prevents dissociation of Smad2 from activated TβRI and blocks TGF-β-dependent signaling and Smad2 transcriptional activity. These results indicate that receptor-dependent phosphorylation of Smad2 on serines 465 and 467 is required in mammalian cells to permit association with Smad4 and to propagate TGF-β signals.

Members of the TGF-β superfamily elicit their biological effects by binding to heteromeric complexes of transmembrane Ser/Thr kinase receptors known as type I and type II (reviewed in Refs. 1 and 2). Initiation of the signaling cascade has been most extensively studied for TGF-β and its type I and type II receptors, TβRI and TβRII. In the absence of ligand both TβRI and TβRII exist as independent homomers on the cell surface (3, 4). Formation of a heteromeric receptor complex occurs when TGF-β binds to TβRII which then leads to the recruitment of TβRI. TβRII, a constitutively active kinase, can then transphosphorylate TβRI in a conserved region of the receptor known as the “GS domain” (5). Once phosphorylated in this glycine and serine-rich domain, TβRI is activated and can signal to Smad2, also known as MAD2, a downstream substrate of the type I kinase (6).

Smad2 is a member of the Mothers Against Dpp (MAD)-related family of proteins that have been identified in organisms as diverse as Drosophila (MAD; Refs. 7–9), Caenorhabditis elegans (sma; Ref. 10), Xenopus, and man (Smad; reviewed in Refs. 1 and 11–13). Smad proteins lack any known structural motifs, but all members possess two highly conserved amino and carboxyl-terminal domains (MH1 and MH2 domains, respectively) that are separated by a nonconserved linker region. Biochemical and biological studies have clearly demonstrated that Smad proteins are essential components of Ser/Thr kinase receptor signaling pathways and that different Smad proteins can convey distinct biological signals. For example, Smad2 and Smad3 act in TGF-β/activin signaling pathways (14–18), and Smad1 appears to specifically transduce BMP signals (18–21). Smad4 (also known as DPC4), originally identified as a tumor suppressor in pancreatic carcinomas (22), appears to function in multiple pathways and can form heteromeric complexes with either Smad1, Smad2, or Smad3 in response to activation of signaling (23–26). Interestingly unlike other MAD-related family members, the recently identified Smad7 protein appears to block TGF-β signaling (27).

Several studies have indicated that Smad proteins mediate signaling through a nuclear function. Both Smad1 and Smad2 appear to accumulate in the nucleus upon activation of the appropriate signaling pathways, and several Smad proteins have been demonstrated to function as transcriptional activators in heterologous assay systems (15, 19, 20, 28). Furthermore, in Xenopus, Smad2 can associate with FAST1, a winged-helix transcription factor, to form a regulatory complex that is involved in the activin-dependent transcriptional induction of the homeobox gene, Mix.2 (16).

Smad proteins are regulated by rapid ligand-dependent phosphorylation in response to specific signaling pathways (6, 14, 19, 28–30). Thus, Smad1 phosphorylation is induced by BMPs through the highly related type I receptors ALK3 or ALK6 and not by TGF-β (19). In an analogous manner, Smad2 is phosphorylated in response to TGF-β or activin and not to BMPs (14). Phosphorylation of Smad2 and Smad1 occurs on the carboxyl terminus within the last three serine residues and is directly mediated by the TGF-β type I receptor, TβRII (6), or by the BMP type I receptor, ALK6 (24), respectively. Phosphorylation of Smad proteins is critical for their biological activity. For example, mutations in Smad2, identified in colorectal carcinomas, that block Smad2 phosphorylation yield functionally inactive proteins (14). Furthermore, mutation of the serine residues that encompass the phosphorylation site in Smad1 or Smad2 yields proteins that cannot accumulate in the nucleus.

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The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis.
upon activation of signaling pathways. In addition, these mutants can act as dominant-negatives in TGF-β signaling (6) and can prevent their transcriptional activity (24).

Phosphorylation of Smad proteins by Ser/Thr kinase receptors provides a rapid and specific mechanism for transducing TGF-β superfamily signals. However, precisely which residues are phosphorylated, the mechanism of phosphorylation, and the role that individual phosphorylation events play in Smad function are unknown. In this report, we demonstrate that Smad2 is phosphorylated by the TGF-β type I receptor kinase on Ser465 and Ser467. Phosphorylation can occur independently on either site, but phosphorylation of both serines is required for dissociation of Smad2 from the activated receptor complex. We also show that Ser465 is not phosphorylated by the receptor kinase, but that this serine is required for efficient phosphorylation of Smad2 on serines 465 and 467. Furthermore, we demonstrate that in mammalian cells, association of Smad4 and Smad2 requires phosphorylation of Smad2 at both sites. In functional assays, we show that Smad2 harboring point mutations of serine to alanine residues at either 465 or 467 acts as a dominant-negative in TGF-β signaling and is inactive as a transcriptional activator. Thus Smad2 phosphorylation at Ser465 and Ser467 is essential for Smad2-Smad4 complex formation and for TGF-β signaling.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—The construction of pCMV5B-FlagSmad2 and pGEX4T1-Smad2 has been described previously (14). To generate mammalian and bacterial expression constructs of mutant versions of Smad2 (S464A, S465A, S467A, S464/465A, S464/467A, S465/467A), mutations were introduced using polymerase chain reaction-based strategy and the products were subcloned into pCMV5B-FlagSmad2 and pGEX4T1-Smad2, respectively. Mouse Smad4, cloned as described previously (6), was tagged with the influenza hemagglutinin (HA) epitope at the carboxyl terminus by polymerase chain reaction. All constructs were confirmed by sequencing.

The pG5E1b-Lux construct, containing the luciferase reporter gene downstream of the GAL4-DNA binding element and the E1b TATA box, was generated by subcloning an XhoI/EcoRI insert of pG5E1b-CAT (a kind gift from A. Bonni (Division of Neuroscience, Children’s Hospital, Harvard Medical School), into XhoI/XhoI fragments of pCMV5B-GAL4-Smad2, consisting of Smad2 fused to the DNA binding and dimerizing domains of GAL4 (amino acids 1–147), was constructed by subcloning the GAL4-Smad2 insert from pG5B9-Smad2 into pCMV5B using HindIII/XhoI sites. To generate GAL4-Smad2 constructs harboring serine to alanine mutations, SalI/XhoI fragments from the mutant versions of Smad2 in pCMV5B were subcloned into SalI/XhoI-digested pCMV5B-GAL4-Smad2. pCMV5B-GAL4-WT was generated from pCMV5B-GAL4-Smad2 vector by digestion with EcoRI/XhoI.

Cell Lines and Transfections—Mv1Lu and HepG2 cells were maintained in minimal essential medium containing 10% fetal calf serum and nonessential amino acids, and COS-1 cells were grown in high glucose-Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. For transient transfections, HepG2 cells were seeded at 10–25% confluence in 24-well plates and transfected overnight with 0.5 μg DNA/well using the calcium phosphate-DNA precipitation method as described previously (19). For transfections in COS-1, cells were seeded at 50–70% confluence and were transfected with DEAE-dextran as described previously (31).

Phosphate Labeling, Immunoprecipitations, and Immunoblotting—For [32P]Phosphate labeling, transiently transfected COS-1 cells were washed and preincubated with phosphate-free medium containing 0.2% dialyzed fetal calf serum. The cells were then incubated with media containing 1 μCi/ml [32P]phosphate for 2 h at 37 °C as described previously (5). Cells were then lysed and subjected to immunoprecipitation.

For anti-Flag immunoprecipitations, cells lysates were subjected to immunoprecipitation with anti-Flag M2 antibody (Eastman Kodak Co.) followed by adsorption to protein G-Sepharose (Pharmacia Biotech Inc.) as described previously (19). For TGF-β receptor complex or anti-HA immunoprecipitations, cell lysates were subjected to immunoprecipitation with anti-TβRII antibody C16 (Santa Cruz Biotechnology) or anti-HA (12CA5) antibody, respectively, followed by adsorption to protein A-Sepharose (Pharmacia). Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography. For determination of Flag/Smad2 or Smad4/HA protein levels, cell lysates were separated by SDS-PAGE and assayed by immunoblotting as described previously (19) or alternatively were subjected to anti-Flag or anti-HA immunoprecipitation and immunoblotting, as indicated.

In Vitro Kinase Assay—Receptor complexes were immunoprecipitated from Mv1Lu cells and incubated with wild type or mutant Smad2 proteins as a substrate in the kinase assay, as described previously (6). Smad2 proteins were expressed in bacteria as a glutathione S-transferase fusion protein and purified as described (32). Protein phosphorylation was analyzed by SDS-PAGE and autoradiography.

Transcriptional Response Assay—For p3TP-Lux reporter and GAL4-Smad2 transcriptional assays, HepG2 cells were transiently transfected with the reporter plasmids p3TP-Lux and pG5E1b-Lux, respectively, pCMVβ-gal, and the indicated Smad2 or GAL4-Smad2 constructs. Cells were treated overnight with or without TGF-β, and luciferase activity was measured in cell lysates using the luciferase assay system (Promega) in a Berthold Lumat LB 9501 luminometer. To determine transfection efficiency, β-galactosidase activity assay was performed as described (33). The luciferase assay results were normalized for β-galactosidase activity.

RESULTS

Mutation of the COOH-terminal Serine Residues of Smad2 Blocks Phosphorylation in Vivo—Phosphorylation of Smad2 by the TGF-β receptor complex is essential for its function as an intracellular transducer of TGF-β signals and is mediated by the type I receptor kinase. Our previous studies mapped the ligand-dependent phosphorylation site to a five-amino acid tryptic peptide at the COOH-terminal tail of Smad2. This peptide contains three serine residues, and mutation of all three residues to alanine abolishes phosphorylation, thus confirming this region as the site of TGF-β-dependent phosphorylation (6).

Recently, the same region was also identified on Smad1 as the site of BMP-dependent phosphorylation (24).

To determine more precisely which of the three serine residues located within this tryptic peptide are phosphorylated, we generated a series of single, double, and triple mutants of Smad2 that contain all combinations of serine to alanine mutations in the COOH-terminal peptide (Fig. 1). The phosphorylation of these mutants by the TGF-β type I receptor was then evaluated in intact cells. COS-1 cells were transiently transfected with wild type or mutant Smad2 in combination with either wild type or constitutively active TβRI. This constitutively active receptor, which was constructed by substitution of a threonine in the GS domain to an aspartic acid, signals TGF-β responses in the absence of ligand or type II receptor (34). Cells were labeled with [32P]phosphate and receptor-mediated phosphorylation examined by immunoprecipitation of Flag epitope-tagged Smad2 using α-Flag antibodies. As described previously, the phosphorylation of wild type Smad2 in the absence or presence of wild type TβRI was low (Fig. 2A).
at wild type Smad2 yielded a distinct phosphopeptide that migrated toward the anode (Fig. 2B) and corresponded to the COOH-terminal tryptic peptide, as described previously (6). Interestingly, tryptic phosphopeptide maps of the Smad2(S464A) mutant displayed the same pattern as for wild type Smad2, although the relative level of phosphorylation at the COOH-terminal peptide was considerably lower. In contrast, when tryptic maps of the other single mutants, Smad2(S465A) and (S467A), were examined, the characteristic COOH-terminal tryptic phosphopeptide was never observed (Fig. 2B). Thus, while mutation of any serine in the COOH terminus of Smad2 affects its net phosphorylation in intact cells, only mutations at serines 465 or 467 failed to yield a phosphorylated COOH-terminal tryptic peptide. These observations suggest that the last two COOH-terminal serine residues on Smad2 (Serine residues 465 and 467) may be the target sites for phosphorylation by TβRI. Furthermore, since the pattern of phosphorylation of Smad2(S464A) is similar to wild type, but is present at reduced levels, our observations suggest that serine 464 is not a phosphorylation site, but is required for efficient phosphorylation of Smad2 in intact cells.

The TGF-β Receptor Complex Phosphorylates Smad2 on Serine Residues 465 and 467—The low level of phosphorylation observed in intact cells for the Smad2 mutants made it difficult to conclusively demonstrate that serines 465 and 467 were the sites phosphorylated by the receptor. Thus, to show that these are the phosphorylation sites and to gain insights into the mechanism of phosphorylation at these adjacent sites, we employed an in vitro kinase assay utilizing isolated TGF-β receptor complexes and recombinant Smad2 proteins (6). Endogenous TGF-β receptors were isolated from Mv1Lu cells incubated in the presence or absence of TGF-β, using antibodies directed against TβRII. Immunoprecipitates were then subjected to an in vitro kinase assay using bacterially expressed wild type or mutant GST-Smad2 as a substrate. When receptors were isolated in the absence of TGF-β addition, only low levels of Smad2 phosphorylation were observed (Fig. 3A). However, pretreatment of the cells with TGF-β resulted in an increase in the phosphorylation of both wild type and mutant forms of Smad2 (Fig. 3A). To characterize the sites of phosphorylation, the phosphorylated proteins were then analyzed by tryptic phosphopeptide mapping. Both wild type Smad2 and Smad2(S464A) displayed similar phosphopeptide maps, which consisted of one major phosphopeptide corresponding to the phosphopeptide obtained in vivo and an additional peptide previously identified as a phosphoisomer of the COOH-terminal peptide (Fig. 3B; Refs. 6 and 35). These observations are consistent with the data obtained in intact cells and confirm that serine 464 is unlikely to be a phosphorylation site.

In contrast to Smad2(S464A), the maps derived from the single mutants S465A and S467A lacked the phosphopeptide observed in vivo (Fig. 3B). However, both displayed a phosphopeptide that corresponded to the phosphoisomer identified in the wild type maps. Furthermore, this phosphoisomer was also clearly detected in maps derived from either Smad2 (S464/465A) or (S464/467A). In the case of Smad2(S465/467A), neither of the two phosphopeptides were observed (Fig. 3B). Interestingly, although all the phosphoisomers analyzed here have the same charge and migrated similar distances in the electrophoretic dimensions, changes in hydrophobicity obtained upon substitution of serine for alanine in the mutants yielded slight differences in the migration in the second dimension. Together, these mapping data demonstrate that mutation of either serine 465 or 467 prevents the appearance of the more highly phosphorylated tryptic peptide normally observed in intact cells. These results suggest strongly that these two ser-
Phosphorylation Mutants of Smad2 Stably Associate with Smad4. To investigate this, COS-1 or HepG2 cells were transiently transfected with wild type or mutant Flag epitope-tagged Smad2 along with wild type or constitutively active TβRI. Cell extracts from these transfectants were then subjected to anti-Flag immuno- blotting to show more clearly the state of phosphorylation of wild type and mutant Smad2. Similar levels of expression of Flag-Smad2 and TβRI proteins were confirmed by immunoblotting of total cell lysates using anti-Flag-M2 and anti-HA antibodies, respectively (bottom panel).

Phosphorylation of both serine 465 and 467 is required for dissociation from the TGF-β receptor complex. COS-1 cells were transfected with empty vector (−) alone or combinations of wild type (WT) or activated (A) TβRI together with wild type (WT) or mutant versions of Smad2 harboring single or triple serine to alanine mutations as indicated. Cells were labeled with [32P]phosphate and FlagSmad2 and TβRI/HA were purified by immunoprecipitation with anti-Flag M2 or anti-HA antibodies, respectively, and were analyzed by SDS-PAGE and autoradiography (32PO4). The migration of coimmunoprecipitating, activated TβRI is indicated (top panel). A 3-fold lower exposure of the autoradiograph shown in the upper panel is included (middle panel) to show more clearly the state of phosphorylation of wild type and mutant Smad2. Similar levels of expression of Flag-Smad2 and TβRI proteins were confirmed by immunoprecipitation of total cell lysates using anti-Flag-M2 and anti-HA antibodies, respectively.
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mation levels of Smad4, aliquots of total lysates were immunoprecip-

data demonstrate that Smad2 interacts with Smad4 in a TGF-

FIG. 5. Smad4 Interacts with wild type Smad2 but not with phosphorylation site mutants in mammalian cells. COS-1 cells (A) or HepG2 cells (B) were transiently transfected with empty vector alone (-) or Smad4, together with either wild type (WT) or activated (A) TpRl and wild type (WT) or mutant Smad2. Cell lysates were subjected to immunoprecipitation with anti-Flag M2 antibody and then immunoblotting using anti-HA antibody. To confirm equivalent levels of wild type or mutant Smad2 protein expression, the same membranes were rebotted with anti-Flag M2 antibody (α-Flag blot). To determine expression levels of Smad4, aliquots of total lysates were immunoprecipitated using anti-HA antibody and then immunoblotted using anti-HA antibody (α-HA blot).

munoprecipitation and associated Smad4 detected by immunoblotting using anti-HA antibody. Equivalent levels of protein expression for Smad2 and Smad4 were confirmed by immunoblotting. When either HepG2 or COS-1 cells were transfected with wild type Smad2 and Smad4, either alone or together with wild type TpRl, little or no interaction between these two proteins could be detected (Fig. 5). However, in the presence of the activated receptor, Smad4 could clearly be detected coprecipitating with Smad2, similar to previous observations (23) (Fig. 5). In contrast, when the single or triple serine to alanine mutants of Smad2 were tested, interactions between the mutant proteins and Smad4 were abolished (Fig. 5). During the course of these experiments we occasionally detected weak interactions between Smad2(S464A) and Smad4, which were barely detectable and difficult to reproduce. This is likely due to the low levels of phosphorylation observed for this mutant, which would subsequently yield levels of interaction with Smad4 which fall below the detection limit in this assay. These data demonstrate that Smad2 interacts with Smad4 in a TGF-

Specific Phosphorylation of Smad2 COOH Terminus Tail Is Required for TGF-β Signaling—To examine the role of phospho-

Smad2 and Smad4 were expressed as fusion proteins with either the GAL4 activation domain or the GAL4 DNA-binding domain. Yeast cells coexpressing various combinations of these fusion proteins and the appropriate control vectors were generated and the presence of protein interactions was determined by measuring the activation of a GAL4-responsive promoter that drives expression of a lucZ reporter gene. Smad2 or Smad4 alone were not able to induce β-galactosidase when expressed as fusions with either the GAL4 activation domain or the GAL4 DNA-binding domain, and consistent with previous findings (36, 37), strong activation of the reporter construct was ob-

Smad2 Phosphorylation and Interaction with Smad4

and Smad4 was also confirmed in vitro by our observation that Smad4 produced in a transcription/translation system interacted with recombinant Smad2 expressed in bacteria (data not shown). Together these data suggest that Smad2 and Smad4 interact directly and that phosphorylation is not absolutely required to mediate their association. However, since phosphory-

Smad2 and Smad4 in intact cells, these results may reflect the presence of inhibitory components in mammalian cells that prevent constitutive associ-

pected patterns and that TGF-β-dependent phosphorylation of Smad2 is required for this association.

To determine whether Smad2 and Smad4 interact directly, we tested their association using a yeast two-hybrid system. Smad2 and Smad4 were expressed as fusion proteins with either the GAL4 activation domain or the GAL4 DNA-binding domain. Yeast cells coexpressing various combinations of these fusion proteins and the appropriate control vectors were generated and the presence of protein interactions was determined by measuring the activation of a GAL4-responsive promoter that drives expression of a lucZ reporter gene. Smad2 or Smad4 alone were not able to induce β-galactosidase when expressed as fusions with either the GAL4 activation domain or the GAL4 DNA-binding domain, and consistent with previous findings (36, 37), strong activation of the reporter construct was ob-

strong activation of the reporter construct was observed in cells expressing both Smad2 and Smad4 in either combination of fusion proteins. We observed a similar level of interaction between Smad2 and Smad4 when the mutant, Smad2(3SA), was tested. A direct interaction between Smad2 and Smad4 was observed when using yeast two-hybrid system. Smad2 and Smad4 were expressed as fusion proteins with either the GAL4 activation domain or the GAL4 DNA-binding domain. Yeast cells coexpressing various combinations of these fusion proteins and the appropriate control vectors were generated and the presence of protein interactions was determined by measuring the activation of a GAL4-responsive promoter that drives expression of a lucZ reporter gene. Smad2 or Smad4 alone were not able to induce β-galactosidase when expressed as fusions with either the GAL4 activation domain or the GAL4 DNA-binding domain, and consistent with previous findings (36, 37), strong activation of the reporter construct was observed in cells expressing both Smad2 and Smad4 in either combination of fusion proteins. We observed a similar level of interaction between Smad2 and Smad4 when the mutant, Smad2(3SA), was tested. A direct interaction between Smad2

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Smad2 Phosphorylation and Interaction with Smad4

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TGF-β, coexpression of GAL4-Smad2 (wild type) yielded a strong luciferase activity was detected in the presence of TGF-β-domain, the promoter activity was low, and no induction of luciferase activity in lysates prepared from cells treated overnight and pG5E1b-Lux. Ligand-dependent transcriptional activation were transfected with the heterologous GAL4 fusion constructs various Smad2 proteins as transcriptional activators, HepG2 cells malian expression vector. To measure the function of the var-

GAL4 DNA-binding domain and were subcloned into a mam-

E1b TATA box (Fig. 7 of a promotor containing five GAL4 DNA-binding sites and an pG5E1b-Lux, which encodes a luciferase gene under the control

sensitive and specific assay involving a heterologous GAL4 reporter gene construct (top) and the GAL4-Smad2 fusion constructs (bottom) is shown. The wild type or mutant GAL4-Smad2 constructs consisted of the DNA binding and dimerizing domains of GAL4 (amino acid 1–147) fused to wild type (WT) or mutant versions of Smad2. B, effect of phosphorylation site mutants on GAL4-Smad2 transcriptional activity. HepG2 cells were cotransfected with the reporter gene along with empty vector, GAL4 or wild type (WT), or mutant GAL4-Smad2 as indicated. Cells were incubated overnight without (open columns) or with (solid columns) 100 pM TGF-β, and luciferase activity was deter-

determined in cell lysates. Luciferase activity was normalized to β-galacto-

disase activity and was expressed as the mean ± S.D. of triplicates from a representative experiment.

Phosphorylation at Serines 465 and 467 Is Required for TGF-

β-dependent Activation of Smad2—To further characterize the effect of the individual serine mutations, we examined Smad2 function as a transcriptional activator. For this, we developed a sensitive and specific assay involving a heterologous GAL4 transcription system based on a similar approach used previously to measure Smad1 activity (20). A reporter construct, pG5E1b-Lux, which encodes a luciferase gene under the control of a promoter containing five GAL4 DNA-binding sites and an E1b TATA box (Fig. 7A) was first constructed. Various wild type and mutant forms of Smad2 were then fused to a yeast GAL4 DNA-binding domain and were subcloned into a mammalian expression vector. To measure the function of the var-

ious Smad2 proteins as transcriptional activators, HepG2 cells were transfected with the heterologous GAL4 fusion constructs and pG5E1b-Lux. Ligand-dependent transcriptional activation of the reporter gene was then determined by measuring luciferase activity in lysates prepared from cells treated overnight with or without TGF-β. In cells transfected with pG5E1b-Lux alone or together with the vector expressing only the GAL4 domain, the promoter activity was low, and no induction of luciferase activity was detected in the presence of TGF-β. How-

ever, coexpression of GAL4-Smad2 (wild type) yielded a strong TGF-β-dependent increase in the reporter gene activity as re-

Ported previously (Fig. 7B; Ref. 27). In contrast, when GAL4-

Smad2 fusion proteins containing either of the single site mu-
tants at serine 465 or 467 were tested, TGF-β-dependent induction of luciferase activity was abolished. Since mutation of either of these phosphorylation sites disrupts TGF-β-dependent transcriptional activity, these data demonstrate that phos-

phorylation at both sites is essential for ligand-dependent ac-

tivation of Smad2. We also tested the transcriptional activity of Smad2(S464A) in this assay. Although the response of this mutant was significantly reduced, a low level of TGF-β-dependent induction was detected. This observation is consistent with the demonstration that this serine is not phosphorylated but is important for efficient regulation of Smad2 by the TGF-β rece-

ceptor. Furthermore, it supports the notion that a minimum threshold of Smad2 phosphorylation may be required to ini-

tiate maximal TGF-β signaling.

DISCUSSION

Smad2 plays a critical role in TGF-β signaling, functioning as a direct substrate of the TGF-β receptor to transmit signals directly from the cell surface into the nucleus. Although receptor-dependent phosphorylation of Smad2 is essential for TGF-β signaling, little is known about the mechanism of phosphory-

lation or how phosphorylation regulates Smad protein function to initiate TGF-β signaling. In this report we have investigated the role of individual serine residues within the COOH-termi-

nal tail in Smad2 function. We show that serine residue 464 is required for efficient receptor-dependent phosphorylation of Smad2 but is not itself a target of the receptor kinase. Furthe-

more, we demonstrate that the TGF-β type 1 receptor phosphor-

ylates Smad2 on residues 465 and 467 at the COOH-terminal tail of Smad2. Individual mutation of either of these phosphorylation sites blocks release of Smad2 from the TGF-β receptor. In mammalian cells, phosphorylation of Smad2 is also required to mediate association with Smad4. Furthermore, we show that mutation of Smad2 phosphorylation sites yields a protein that can act in a dominant-negative manner to block TGF-β-dependent signaling.

Serine Residue 464 Is Required for Efficient Phosphorylation of the COOH Terminus—The mutagenesis studies presented here have shown that the serine at position 464 is critical for TGF-β-dependent regulation of Smad2 phosphorylation. However, this residue is not likely to be a site of phosphorylation by TβRI, but rather is required for efficient phosphorylation at residues 465 and 467. This requirement is not due to disruption of the association of Smad2 with the receptor, since the triple mutant, Smad2(3SA), interacts efficiently with the TGF-β receptor. Instead, serine 464 may play a role in correctly posi-

tioning the COOH terminus within the catalytic cleft of the kinase and thus might be considered as part of the type I receptor kinase recognition sequence. Interestingly, there appears to be a stringent requirement for a serine at this position. For example, we have found that substitution of a threonine at position 464 also blocks efficient phosphorylation of Smad2.2 Furthermore, all of the receptor-regulated Smad proteins char-

acterized thus far possess a serine residue at an analogous position and this may be a general requirement for all type I Ser/Thr kinase receptors (13).

Consistent with these observations, Smad2(464A) had re-

duced signaling capacity relative to wild type when it was fused to a GAL4 DNA binding domain. Interestingly, the mutant also functions as a dominant-negative to block TGF-β signaling through endogenous Smad2. This mutant may act by decreasing the efficiency of phosphorylation, thereby decreasing the rate of dissociation from the receptor and thus may effectively

2 M. Macías-Silva and J. L. Wrana, unpublished data.
function as a competitive inhibitor of the type I kinase domain. This suggests that not only is phosphorylation of specific sites important for signaling, but that the magnitude of phosphorylation may be critical to attain a threshold of phosphorylated Smad2 sufficient for initiation of signaling.

**Phosphorylation on Both Serine Residues 465 and 467 Is Required for TGF-β-dependent Signaling**—Analysis of point mutants of Smad2 by tryptic phosphopeptide mapping shows that the TGF-β receptor phosphorylates Smad2 on the COOH-terminal serine residues, 465 and 467. Furthermore, phosphorylation at one residue does not appear to be dependent upon phosphorylation at the other site. This suggests that the receptor kinase does not phosphorylate these serines in a sequential manner. This is in contrast to glycogen synthase kinase, which also phosphorylates clusters of serines. This cytosolic kinase requires a phosphorylated serine residue to phosphorylate the next serine in the cluster and thus generally requires priming of the phosphorylation site by a second kinase (38, 39). It is interesting to note that other receptor-regulated Smads possess a conserved SSXS motif at their carboxyl terminus. Thus, this motif may represent an optimal core sequence for phosphorylation by type I receptor Ser/Thr kinase receptors.

Phosphorylation of COOH-terminal residues by kinases is unusual. Structural analysis of a number of Ser/Thr kinases has shown that there are extensive contact sites between the kinase and the sequence downstream of the phosphorylation target (40). Furthermore, these downstream residues often play a critical role in defining the specificity of the interaction between the kinase and the peptide substrate (41). The absence of these residues in Smad proteins suggests that other regions of the molecule may be important for mediating specific recognition and phosphorylation by the receptors. It also suggests that these carboxyl-terminal residues may not be good targets for other serine kinases in the cell and this could provide one mechanism for preventing spurious activation of TGF-β and BMP signaling pathways in vivo.

We have observed that in mammalian cells phosphorylation of both residues is required to induce dissociation of Smad2 from the type I receptor. Currently it is unclear why dissociation requires phosphorylation at both sites, but perhaps this event is essential, either directly or indirectly, to disrupt protein-protein interactions. Regardless of the mechanism, it is clear that dissociation of Smad2 from the receptor would be essential to allow this protein to signal in the nucleus. The functional analyses of the single mutants of Smad2 support and extend these conclusions. Mutation of either serine residue abolishes TGF-β-dependent activation of Smad2 transcriptional activity, and both can act as dominant-negatives to inhibit TGF-β signaling. Thus, phosphorylation of Smad2 at both serines 465 and 467 is required for the initiation of signaling. Interestingly, a MAD-related protein Smad7 was recently identified (27). Smad7 lacks COOH-terminal SSXS motifs, associates stably with the TGF-β receptor, and inhibits TGF-β signaling by preventing access and phosphorylation of Smad2 by the type I receptor kinase. Thus, by acting in a manner analogous to that of the SSXS mutants described here, this Smad represents a naturally occurring antagonist of the TGF-β receptor.

**Phosphorylation of Smad2 Is Required in Mammalian Cells for Association with Smad4**—Previous studies have shown that TGF-β induces Smad2 and Smad4 to form a heteromeric complex in mammalian cells (23), and here we demonstrate that this interaction requires receptor-dependent phosphorylation of Smad2 at the COOH terminus. Recently, it was shown that COOH-terminal serine residues in Smad1 are required for its BMP-dependent phosphorylation and association with Smad4 (24). Analysis of Smad2 and Smad4 interaction in a yeast two-hybrid system indicates that it is a strong and direct association that does not require accessory proteins (Refs. 36 and 37 and our results). However, these latter studies indicate that this interaction can occur independently of phosphorylation and suggest that phosphorylation is specifically required for formation of heteromeric complexes only in mammalian cells. Since Smad2 and Smad4 can colocalize to the cytoplasm in resting cells (data not shown), the failure of these two proteins to associate in the absence of signaling is not due to differential subcellular localization. One possible explanation is that in mammalian cells, Smad2 interaction with Smad4 may be prevented by inhibitory molecules that bind Smad2, Smad4, or both. Phosphorylation could function to disrupt these inhibitory interactions, thereby allowing complex formation and the initiation of signaling. Interestingly, the presence of limiting amounts of an inhibitor may also explain the observation that overexpression of Smad3 and Smad4 leads to their ligand-independent interaction and causes initiation of TGF-β signaling in the absence of receptor activation (36). Although still speculative, inhibitory molecules have also been postulated to play a role in modulating Smad protein activity in resting cells by retaining the protein in the cytosol in the absence of signaling (15).

In summary, our data show that ligand-dependent activation of the TGF-β type I receptor results in recruitment of Smad2 to the type I receptor. Smad2 is phosphorylated by the activated type I receptor kinase on serine residues 465 and 467. This results in release of Smad2 from the receptor and association with Smad4. Phosphorylated Smad2 then mediates the nuclear translocation of this heteromeric complex and interaction with nuclear targets, such as FAST-1, to ultimately activate expression of target genes. In the future, it will be important to define other nuclear targets for Smad proteins and to characterize the exact function played by Smad4 and other components that might modulate signaling through these pathways.

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