Phosphorylation of Ser<sup>465</sup> and Ser<sup>467</sup> in the C Terminus of Smad2 Mediates Interaction with Smad4 and Is Required for Transforming Growth Factor-β Signaling*

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Members of the Smad family of intracellular signal transducers are essential for transforming growth factor-β (TGF-β) to exert its multifunctional effects. After activation of TGF-β receptors, Smad2 and Smad3 become phosphorylated and form heteromeric complexes with Smad4. Thereafter, these activated Smad complexes translocate to the nucleus, where they may directly transcribe transcriptional responses. Here we report that TGF-β mediates phosphorylation of Smad2 at two serine residues in the C terminus, i.e. Ser<sup>465</sup> and Ser<sup>467</sup>, which are phosphorylated in an obligate order; phosphorylation of Ser<sup>465</sup> requires that Ser<sup>467</sup> be phosphorylated. Transfection of Smad2 with mutation of Ser<sup>465</sup> and/or Ser<sup>467</sup> to alanine residues into Mv1Lu cells resulted in dominant-negative inhibition of TGF-β signaling. These Smad2 mutants were found to stably interact with an activated TGF-β receptor complex, in contrast to wild-type Smad2, which interacts only transiently. Mutation of Ser<sup>465</sup> and Ser<sup>467</sup> in Smad2 abrogated complex formation of this mutant with Smad4 and blocked the nuclear accumulation not only of Smad2, but also of Smad4. Thus, heteromeric complex formation of Smad2 with Smad4 is required for nuclear translocation of Smad4. Moreover, peptides from the C terminus of Smad2 containing phosphorylated Ser<sup>465</sup> and Ser<sup>467</sup> were found to bind Smad4 in vitro, whereas the corresponding unphosphorylated peptides were less effective. Thus, phosphorylated Ser<sup>465</sup> and Ser<sup>467</sup> in Smad2 may provide a recognition site for interaction with Smad4, and phosphorylation of these sites is a key event in Smad2 activation.

During mammalian embryogenesis and adult tissue homeostasis, transforming growth factor-β (TGF-β) performs pivotal tasks in intercellular communication (1). The cellular effects of this pleiotropic factor are exerted by ligand-induced heterologomerization of two distantly related type I and type II serine/threonine kinase receptors, TβR-I and TβR-II, respectively (2–5). The two receptors, both of which are required for signaling, act in sequence; TβR-I is a substrate for the constitutively active TβR-II kinase (6, 7). TGF-β forms part of a large family of structurally related proteins, which includes activins and bone morphogenetic proteins (BMPs) that signal in a similar fashion, each employing distinct complexes of type I and type II serine/threonine kinase receptors (2–5).

Genetic studies of TGF-β-like signaling pathways in Drosophila and Caenorhabditis elegans have led to the identification of mothers against dpp (Mad) (8) and sma (9) genes, respectively. The products of these related genes perform essential functions downstream of TGF-β-like ligands acting via serine/threonine kinase receptors in these organisms (10–12). Vertebrate homologs of Mad and sma have been termed Smad (13) or Madr (14) genes. Genetic alterations in Smad2 and Smad4/DPC4 have been found in specific tumor subsets; thus, Smad genes may function as tumor suppressor genes (15–17). Smad proteins share two regions of high similarity, termed MH1 and MH2 domains, connected with a variable proline-rich sequence (18, 19). The C-terminal part of Smad2 when fused to a heterologous DNA-binding domain, was found to have transcriptional activity (20, 21), whereas the intact Smad2 protein fused to a DNA-binding domain was latent, but transcriptional activity was unmasked after stimulation with ligand (20).

Different Smad proteins specify different responses using functional assays in Xenopus. Whereas Smad1 induces ventral mesoderm, a BMP-like response, Smad2 induces dorsal mesoderm, an activin/TGF-β-like response (22–24). Upon ligand stimulation, Smad proteins become phosphorylated on serine and threonine residues (25, 26); BMP stimulates Smad1 phosphorylation (12, 20, 31), whereas TGF-β induces Smad2 and Smad3 phosphorylation (17, 27–30).

Smad4 is a common component of TGF-β, activin, and BMP signaling (32–34). Smad4 phosphorylation has thus far been reported only after activin stimulation of transfected cells (32). After stimulation with TGF-β or activin, Smad4 interacts with Smad2 or Smad3, and upon BMP challenge a heteromeric complex of Smad4 and Smad1 has been observed (32). Upon ligand stimulation, Smad complexes translocate from the cytoplasm to the nucleus (12, 20, 23, 28–31), where they, in combination with DNA-binding proteins, may regulate gene transcription (36).

Recently, phosphorylation of serine residues located in the C terminus of Smad2 was shown to be important for TGF-β signaling (28, 31). Here we identify Ser<sup>465</sup> and Ser<sup>467</sup> as two major ligand-dependent in vivo phosphorylation sites in Smad2 and show that they are involved in the association with Smad4; mutation of these phosphorylation sites abrogates TGF-β-induced signaling.

MATERIALS AND METHODS

Cell Lines—COS-1 cells and mink lung epithelial (Mv1Lu) cells were obtained from American Type Culture Collection. Cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% FBS, glutathione S-transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Fmoc, N-(9-fluorenyl)methoxycarbonyl; FBS, fetal bovine serum; BSA, bovine serum albumin.

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§ The abbreviations used are: TGF-β, transforming growth factor-β; TβR-I and TβR-II, TGF-β receptor type I and II, respectively; BMP, bone morphogenetic protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Fmoc, N-(9-fluorenyl)methoxycarbonyl; FBS, fetal bovine serum; BSA, bovine serum albumin.
fetal bovine serum (FBS), 100 units/ml of penicillin, and 50 µg/ml of streptomycin. For stable transfection, Mv1Lu cells were transfected with wild-type Smad2 or Smad2 mutants, subcloned in pcDNA3 vector using the calcium phosphate precipitate method as described (37). Cells were selected and cultured in the presence of 100 µg/ml of G-418 (Life Technologies). Constructs and Cell Transfection—Expression plasmids for TβRI, TβR-II, Smad4, and Smad2 were previously described (29, 38). Smad2 mutants were made by a polymerase chain reaction-directed approach and subcloned in pcDNA3 vector. Mutations and sequences of the exchanged restriction fragments in Smad2 cDNA were confirmed by DNA sequencing. Transient transfections were performed using a DEAE-dextran protocol, as described (39).

**RESULTS**

**Mapping of in Vivo Phosphorylation Sites in Smad2**—TGF-β receptor activation leads to phosphorylation of Smad2 on serine and threonine residues (17, 28, 29). To localize the phosphorylated residues in Smad2, we performed two-dimensional tryptic phosphopeptide mapping. Nontransfected as well as Smad2- and Smad2-transfected Mv1Lu cells were labeled with 32P-orthophosphate and subjected to SDS-PAGE and autoradiography, as described previously (42). To determine the expression level of Smad2 constructs, aliquots of cell lysates were analyzed by SDS-PAGE and autoradiography, as described previously (42). To determine the expression level of Smad2 constructs, aliquots of cell lysates were analyzed by SDS-PAGE and Western blotting with SED-antisera.

**Transcriptional Response Assay**—Mv1Lu cells were transiently transfected with p3TP-Lux (43) in the absence or presence of Smad2 expression plasmids using the DEAE-dextran method. After transfection, cells were incubated for 24 h in Dulbecco's modified Eagle's medium with 10% FBS and then incubated with 0.1% FBS for 5 h, after which TGF-β (10 ng/ml) was added. Luciferase activity in the cell lysates was measured after 22–24 h using the luciferase assay system (Promega Biotech Inc.), according to the manufacturer's protocol using an LKB Luminometer (LKB Bromma).

**Sequential Immunoprecipitation of Smad2 and Smad4—COS-1 cells** were transiently transfected with TβRI, TβR-II, Smad4 tagged at the N terminus with a Flag epitope, and wild-type Smad2 or Smad2/Smad4 tagged at the C terminus using the DEAE-dextran method. After 24 h, medium containing 1% Triton (PBS-T) with 0.1% BSA overnight at 4°C. After washing five times in PBS-T with 0.1% BSA and twice in PBS-T, the samples were subjected to SDS-PAGE. Proteins were electrotransferred to nitrocellulose membrane and immunoblotted with GST antisera (gift from Gino Rausala) and developed using an enhanced chemiluminescence detection system (Amersham).
in Mv1Lu cells were identical, albeit signal intensity was higher on maps of overexpressed Smad2. Overexpression of Smad2 with TßR-I and TßR-II in COS-1 cells and incubation with ligand also led to stimulation of Smad2 phosphorylation (Fig. 1A). Tryptic phosphopeptide maps of Smad2 phosphorylated \textit{in vivo} in these cells revealed a nearly identical pattern as compared with phosphorylated Smad2 from Mv1Lu cells (Fig. 1, E–G). Phosphoamino acid analysis of spots 1–18 revealed that all were phosphorylated on serine residues except for spots 11 and 18 that contained both phosphoserine and phosphothreonine residues (data not shown). None of the spots contained phosphotyrosine.

To identify the positions of phosphorylated residues in the phosphopeptides, the release of radioactivity upon Edman degradation of peptides extracted from the two-dimensional chromatography plate, was determined. The peptide corresponding to spot 15, which contained only phosphoserine, yielded $^{32}$P radioactivity released in the third and fifth cycles, with some trailing in the following cycles, which is characteristic for this method. This indicates that Ser$^{465}$ and Ser$^{467}$ in the C terminus of Smad2 were phosphorylated, since the corresponding tryptic peptide is the only one in Smad2 with serine residues in positions 3 and 5 (Fig. 2, A and B). The C-terminal tryptic peptide also contained another phosphorylatable amino acid residue, \textit{i.e.} Ser$^{464}$. However, radiochemical sequencing and phosphoamino acid analysis did not reveal phosphoserine at position 2 in phosphopeptides with the migration position expected if Ser$^{464}$ would have been phosphorylated alone or in combination with Ser$^{465}$ and Ser$^{467}$. Thus, Ser$^{464}$ in Smad2 appears not to be phosphorylated in response to TGF-ß-stimulation.

To confirm that Ser$^{465}$ and Ser$^{467}$ are phosphorylation sites in Smad2, these residues and the neighboring Ser$^{464}$ were mutated to alanine residues singly and in combinations. Analysis of two-dimensional tryptic phosphopeptide maps of wild-type and mutant Smad2 from $^{32}$P-labeled, TGF-ß-stimulated COS-1 cells revealed that spot 15, corresponding to a peptide with Ser$^{465}$ and Ser$^{467}$ phosphorylated, was not seen in the phosphopeptide map of the Smad2/S465A,S467A mutant (Fig. 2C). As expected, the phosphopeptide map of the Smad2/S464A mutant contained spot 15; the map of the Smad2/S464A mutant was nearly identical to that of wild-type Smad2, although the intensity of phosphorylation was somewhat lower. Moreover, the phosphopeptide map of the triple Smad2/S464A,S465A,S467A mutant also lacked spot 15 and was identical to that of the Smad2/S465A,S467A mutant. Thus, Ser$^{465}$ and Ser$^{467}$ are \textit{in vivo} phosphorylation sites in Smad2.
Phosphorylation of Ser\textsuperscript{465} Requires That Ser\textsuperscript{467} Be Phosphorylated—Since Ser\textsuperscript{465} and Ser\textsuperscript{467} are located in close proximity of each other, we examined the possibility that they are phosphorylated sequentially. We characterized the T\textsuperscript{b}R-I-mediated phosphorylation in transfected COS-1 cells of Smad2 mutants in which Ser\textsuperscript{465} or Ser\textsuperscript{467} were converted to alanine or aspartic acid residues. Two-dimensional tryptic phosphopeptide mapping of Smad2/S465A, Smad2/S465D, Smad2/S467A, and Smad2/S467D mutants after co-expression with TGF-\textit{b} receptors and stimulation with TGF-\textit{b} showed a loss of spot 15 in all cases. Notably, a new spot appeared in the maps of the Smad2/S465A, Smad2/S465D, and Smad2/S467D mutants but not in the map of the Smad2/S467A mutant (Fig. 3A). The new peptide had a shorter migration distance in electrophoresis at pH 1.9 compared with that of spot 15, which is in agreement with a lower degree of phosphorylation of the novel phosphopeptide and suggested that it was phosphorylated at one rather than two residues. When we performed radiochemical sequencing of the novel spot from the map of the Smad2/S465A mutant, radioactivity eluted at the fifth cycle, which is consistent with the expected phosphorylation of the C-terminal tryptic peptide at Ser\textsuperscript{467} after mutation of Ser\textsuperscript{465} (Fig. 3B). The fact that the new spot was not seen in the map of the Smad2/S467A mutant suggests that phosphorylation of Ser\textsuperscript{465} requires phosphorylation of Ser\textsuperscript{467}. The finding that the map of the Smad2/S467D mutant showed the novel spot suggested that introduction of a negative charge at position 467 could rescue the phosphorylation of Ser\textsuperscript{465}. Radiochemical sequencing of the spot revealed that the peak of radioactivity eluted at the third cycle (Fig. 3). Phosphoamino acid analysis revealed the presence of only phosphoserine in these new phosphopeptides of Smad2/S465A and Smad2/S467D mutants (data not shown). This result indicates that Ser\textsuperscript{465} was phosphorylated in the C-terminal peptide of the Smad2/S467D mutant and suggests that the requirement for phosphorylation at Ser\textsuperscript{467} was bypassed by the introduction of a negative charge at this residue.

Mutation of Ser\textsuperscript{464}, Ser\textsuperscript{465}, and/or Ser\textsuperscript{467} in Smad2 Interferes with TGF-\textit{b}-mediated Signaling—To determine the importance of Ser\textsuperscript{464}, Ser\textsuperscript{465}, and Ser\textsuperscript{467} for TGF-\textit{b} signaling, we measured the ability of Smad2 mutants, in which these residues were replaced with alanine or aspartic acid residues singly or in combinations, to block the TGF-\textit{b}-mediated transcriptional response using a p3TP-Lux reporter plasmid (Fig. 4). Consistent with previous observations (33), we found no differ-
ence in signaling in the absence or presence of transfected wild-type Smad2, indicating that level of endogenous Smad2 is sufficient for full response. We found that expression of any Smad2 with Ser\(^{465}\) or Ser\(^{467}\) replaced with alanine residue(s) led to a decrease of the luciferase signal. The introduction of aspartic acid residues to mimic the negative charge of the phosphate group did not rescue the stimulation of luciferase expression. Notably, Smad2/S464A also acted as a dominant negative inhibitor. However, in experiments with a lower expression level, the inhibitory effect of this mutant on TGF-\(\beta\) signaling was less pronounced than those of Smad2/S465A and Smad2/S467A mutants.\(^2\) Thus, Ser\(^{464}\) and in particular the phosphorylatable residues Ser\(^{465}\) and Ser\(^{467}\) in Smad2 are required for TGF-\(\beta\) signaling.

**Association of Wild-type Smad2 and Smad2 Mutants with T\(\beta\)R-I**—The interaction of Smad2 with the TGF-\(\beta\) receptor complex was investigated by co-expression of wild-type Smad2 and Smad2 mutants with TGF-\(\beta\) receptors in COS-1 cells (Fig. 5). Smad2-receptor interaction was determined by the ability of an antiserum against Smad2 to co-immunoprecipitate the receptors, cross-linked with \(125\)I-TGF-\(\beta\). We found that single as well as double mutants of Ser\(^{465}\) and Ser\(^{467}\) interacted with wild-type T\(\beta\)R-I or a kinase-inactive T\(\beta\)R-I mutant in complex with T\(\beta\)R-II. Interestingly, although Ser\(^{464}\) is not an \textit{in vivo} phosphorylation site, Smad2/S464A interacted with T\(\beta\)R-I as efficiently as the Smad2/S465A and Smad2/S467A mutants (Fig. 5). Affinity-labeled T\(\beta\)R-II was abundantly immunoprecipitated with Smad2 antiserum, which is probably caused by the higher expression of T\(\beta\)R-II versus T\(\beta\)R-I that was achieved after transfection. In accordance with previous results (28), we found that wild-type Smad2 interacted with T\(\beta\)R-I, provided that T\(\beta\)R-I was kinase-inactive and phosphorylated by T\(\beta\)R-II kinase; in contrast, a Smad2 mutant with the three C-terminal serine residues altered to alanine residues was able to bind with high affinity also to activated wild-type T\(\beta\)R-I. These data are in concordance with the dominant negative effects of Smad2 mutated at Ser\(^{464}\), Ser\(^{465}\) and/or Ser\(^{467}\) on TGF-\(\beta\)-mediated stimulation of gene expression (Fig. 4) and suggest that the mechanism may involve competition of the Smad2 mutants with endogenous Smad2 for binding to T\(\beta\)R-I.

**Mutation of Smad2 at Ser\(^{465}\) and Ser\(^{467}\) Abrogates TGF-\(\beta\)-dependent Nuclear Translocation of Smad4**—Complex formation among Smad2, Smad3, and Smad4 and nuclear translocation of these Smad proteins are thought to be important steps in TGF-\(\beta\) signaling. Using sequential immunoprecipitation with antisera specific for Smad2 or Smad4, we found that the mutation of Ser\(^{465}\) and Ser\(^{467}\) in Smad2 led to abrogation of complex formation of Smad2 with Smad4 (data not shown), suggesting an involvement of Ser\(^{465}\) and Ser\(^{467}\) in the interaction between Smad2 and Smad4. Upon stimulation of cells with

\(^2\) S. Souchelnytskyi, unpublished data.
TGF-β, Smad2 and Smad4 translocate to the nuclei (12, 20, 23, 28, 29, 33). To investigate whether phosphorylation of Ser\(^{465}\) and Ser\(^{467}\) in Smad2 is important for TGF-β-dependent nuclear translocation of the Smad proteins, we used Mv1Lu cells stably transfected with wild-type Smad2 or its mutants. Intracellular localization of Smad proteins was determined by immunofluorescence, using Smad4 (HPP) or Smad2 (DQQ) antisera. Interestingly, single or double mutations of Ser\(^{465}\) and/or Ser\(^{467}\) led to abrogation of nuclear translocation not only of Smad2 but also of Smad4 (Fig. 6 and data not shown). In wild-type Smad2-transfected cells we observed, as expected, nuclear translocation of Smad2 and Smad4 in response to TGF-β stimulation. This finding indicates that nuclear translocation of Smad4 upon TGF-β stimulation is dependent on phosphorylation of the C terminus of Smad2.

**GST-Smad4 Binds to Phosphorylated Peptides Derived from the C Terminus of Smad2**—To investigate whether phosphorylated Ser\(^{465}\) and Ser\(^{467}\) of Smad2 are directly involved in heteromeric interaction with Smad4, we made phosphorylated and nonphosphorylated peptides corresponding to the C terminus of Smad2 and tested their ability to bind GST-Smad4 fusion proteins. Peptides were coupled to a solid support and incubated with GST-Smad4; after washing, the amount of bound GST-Smad4 was analyzed by SDS-PAGE and immunoblotting with a GST antiserum. We found that Smad4 bound to a doubly phosphorylated peptide containing the four C-terminal amino acid residues of Smad2 (SSpMSP, where Sp represents phosphorylated serine), but not to the corresponding nonphosphorylated counterpart (SSMS) (Fig. 7A). GST protein showed no binding to phosphorylated peptides coupled to beads, and GST-Smad4 showed no binding to beads without coupled peptides (data not shown). We also found that the phosphorylated form of a longer peptide containing the 14 C-terminal amino acid residues of Smad2 (TQMGSPSVRCSSpMSP) bound GST-Smad4 even more efficiently than the SSMSp peptide. The phosphorylated long peptide also bound GST-Smad4 more efficiently than the nonphosphorylated counterpart; however, binding was observed also to the long nonphosphorylated peptide. The interaction was shown to be specific, since it was blocked by an excess of short or long phosphorylated peptides but not by an excess of an irrelevant phosphopeptide, phosphoserine, or a peptide derived from a proline-rich linker region of Smad2 (Fig. 7B). In addition, no interaction was seen between GST-Smad4 and a Smad2-related peptide with the two C-terminal serine residues replaced with alanine residues (Fig. 7C).

**DISCUSSION**

Genetic and biochemical studies have shown that Smad family members are key components in the intracellular signaling pathway of TGF-β superfamily members (18, 19). Upon TGF-β stimulation, Smad2 and Smad3 are directly phosphorylated by the TβR-I kinase and form heteromeric complexes with Smad4 that are translocated from the cytoplasm to the nucleus. In the present study we have determined two TGF-β-induced in vivo phosphorylation sites in the C terminus of Smad2, i.e. Ser\(^{465}\) and Ser\(^{467}\), and demonstrated that they are phosphorylated sequentially and are important in TGF-β-induced signal transduction. Mutation of Ser\(^{465}\) and Ser\(^{467}\) blocked nuclear translocation of both Smad2 and Smad4, and, using peptides derived from the C terminus of Smad2, we were able to demonstrate an...
involvement of phosphorylated Ser465 and Ser467 in interaction with Smad4.

Radiochemical sequencing data and phosphoamino acid analysis of phosphorylated peptides of Smad2 led to the identification of Ser465 and Ser467 as \textit{in vivo} phosphorylation sites. It has recently been reported that the C-terminal part of Smad2 contains TGF-β-dependent serine phosphorylation sites (28, 30), but it has not been demonstrated which of these three serine residues are phosphorylated \textit{in vivo}. We identified Ser465 and Ser467 as phosphorylation sites, while Ser464 was not phosphorylated upon receptor activation (Fig. 2). However, intactness of Ser464 was found to be important for efficient Smad2 phosphorylation and dissociation from TβR-I (Figs. 2, 4, and 5). Analogously, Ser462 in the related 462SSVS C-terminal motif of Smad1 was recently shown to be important for BMP-dependent phosphorylation of Smad1 at C-terminal serines (31). Two-dimensional maps of Smad2/S465S, S467A and Smad2/S464A, S465A, S467A mutants showed not only an expected loss of the C-terminal phosphopeptide (spot 15) but also a disappearance of most of the other acidic phosphopeptides. The reason for the heterogeneity in the peptide maps remains to be elucidated. Whereas posttranslational modification of the C-terminal peptide has not been excluded, treatment with O- or N-glycosidase did not alter the migration of spot 15 or other spots. Moreover, radiochemical sequencing of the acidic phosphopeptides showed elution of radioactivity in cycles different from the third and fifth cycle, suggesting phosphorylation of residues in other tryptic peptides. It appears possible, therefore, that phosphorylation of Ser465 and Ser467 is required for subsequent phosphorylation at other sites by TβR-I kinase or cytoplasmic kinases.

An abundantly phosphorylated peptide (spot 18) was observed (Fig. 1); this peptide was brought down with antiserum raised against a peptide from a sequence in a large tryptic peptide that covers almost completely the proline-rich linker region (SED peptide) or a peptide phosphorylated on two tyrosine residues (RH peptide) or by an excess of phosphoserine (pSer). C, peptide with alanine substitution of two C-terminal serines, related to Ser465 and Ser467 (S2Ap), does not bind GST-Smad4. LNp and LPp are nonphosphorylated and phosphorylated peptides, respectively, coupled to beads. The migration position of GST-Smad4 is shown.

![Fig. 7. Phosphorylated peptides derived from the C terminus of Smad2 bind to Smad4.](image-url)

**A** Short C-terminal (phospho) peptide

| Protein | GST | GST-Smad4 |
|---------|-----|----------|
| peptide beads | SNp | SNp | SPp |
| GST-Smad4 | 31 | 66 | 97 |

**B** Interaction of GST-Smad4 with phosphorylated long peptide is not affected by an excess amount (200 μM) of irrelevant nonphosphorylated peptide derived from Smad2 proline-rich linker region (SED peptide) or a peptide phosphorylated on two tyrosine residues (RH peptide) or by an excess of phosphoserine (pSer).

**C** Peptide with alanine substitution of two C-terminal serines, related to Ser465 and Ser467 (S2Ap), does not bind GST-Smad4.
their importance for regulation of Smad2 activity are currently being analyzed.

We found an obligate order in the phosphorylation of Ser465 and Ser467 in Smad2 (Fig. 3). The TβR-I kinase apparently initially recognizes only Ser467, which upon phosphorylation then provides a new recognition sequence, allowing phosphorylation of Ser465. The requirement of Ser467 phosphorylation could be bypassed by replacement of Ser467 with an aspartic acid residue. Thus, the appearance of a negative charge at the position of Ser467 is sufficient for recognition of Ser465 as a phosphate acceptor. The need for sequential phosphorylation of Smad2 may possibly be to increase the specificity of phosphorylation, thereby limiting phosphorylation at these sites to TβR-I kinase only, and thus providing a safeguard against inappropriate activation by other kinases. In addition, it may create a certain threshold level for Smad2 activation, in which activation occurs only after both serine residues have become phosphorylated.

It has recently been shown that mutation of all three C-terminal serine residues in Smad2 leads to abrogation of TGF-β signaling (28). We found that mutation of any of these residues, even Ser464, which is not a phosphorylation site, inhibited TGF-β-stimulated gene expression (Fig. 4). This effect of the different Smad2 mutants could be explained in part by their strong interaction with wild-type TβR-I, which may prevent interaction with and phosphorylation of wild-type Smad2, thereby blocking signal transduction (Fig. 5). Interestingly, a Smad2 mutant with Ser467 replaced by Asp, in which phosphorylation of Ser465 occurs, also had a dominant negative effect. Thus, the appearance of a negative charge at the position of Ser467 allows phosphorylation of Ser465 by TβR-I, but this is not sufficient for the efficient dissociation of phosphorylated Smad2 from the receptor kinase. Likewise, a Smad2 mutant in which both Ser465 and Ser467 were replaced with aspartic acid residues showed a strong interaction with wild-type TβR-I and a dominant negative effect on TGF-β signaling (Fig. 5). Our observations suggest a need for simultaneous phosphorylation of Ser465 and Ser467 for efficient dissociation of activated Smad2 from the receptor kinase. In MvLu cells, the Smad2/S464A mutant was shown to act in a dominant negative fashion, albeit somewhat less efficiently than the Smad2/S465A and Smad2/S467A mutants. A possible explanation for these findings is that Ser464 mutation to alanine interferes, in part, with phosphorylation at Ser465 and Ser467. The detailed molecular mechanisms of the sequential phosphorylation of Smad2 and its subsequent dissociation from the kinase require further investigation.

We found that mutation of the in vivo phosphorylation sites Ser465 and Ser467 led to abrogation of TGF-β-dependent complex formation of Smad2/S465A,S467A and Smad4. This finding is in agreement with the recently reported inhibition of complex formation between Smad4 and Smad2 with all three C-terminal serines mutated to alanine residues (31). Moreover, we found that the TGF-β-dependent nuclear translocation of Smad2 was inhibited in cells stably transfected with the Smad2 mutants in which Ser465 and/or Ser467 were replaced with alanine residues (Fig. 6; data not shown). This finding indicates a role of Smad2 in the intracellular redistribution of Smad4. These observations, together with data of Zhang et al. (33) showing that ligand-independent nuclear translocation of Smad1, -2, and -3 in transfected COS cells is dependent on Smad4, suggest that a complex of Smad4 and other Smad proteins is formed in cytoplasm, and only complexed Smad proteins translocate from cytoplasm to nucleus.

Phosphorylated peptides derived from the C terminus of Smad2 bind with higher affinity to a GST-Smad4 fusion protein than nonphosphorylated peptides. Phosphorylation of Ser465 and Ser467 in Smad2 may thus contribute directly to heteromeric complex formation with Smad4. The finding that a phosphorylated peptide containing 14 C-terminal residues bound Smad4 more efficiently than a phosphorylated peptide with four C-terminal residues suggests that the immediate upstream region may contribute to the interaction. However, it remains to be elucidated whether this is due to a specific interaction mediated by amino acid residues in the upstream region or whether the longer peptide has a spacer function that causes a more favorable presentation of the phosphorylated C-terminal serines. It will also be of interest to determine whether the C terminus of Smad2 is involved in an intramolecular interaction or possibly in an intracellular interaction with another Smad2, which may be perturbed upon phosphorylation of Ser465 and Ser467. Recently, Hata et al. (45) demonstrated an interaction between the C- and N-terminal domains of Smad2 and, moreover, that the interaction between Smad2 and Smad4 is inhibited by the N-terminal domains.

Recent studies using the yeast two-hybrid interaction screen indicated that the C-terminal domains of Smad2 and Smad3 are important for the heteromeric interactions with Smad4 (45, 46). In addition, mutant forms of Smad2 or Smad3 with short deletions in their C termini were found not to interact with Smad4 in this assay (45, 46). Our observation that C-terminal peptides of Smad2 bind GST-Smad4 is consistent with these data. Our future studies will be aimed at elucidating which region in Smad2 interacts with the phosphorylated C terminus of Smad3 and at the identification of other phosphorylation sites in Smad2 and their functional significance.

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