The TGF-β family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase

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Bone morphogenetic proteins (BMPs) are members of the TGF-β family that regulate cell proliferation, apoptosis, and differentiation, and participate in the development of most tissues and organs in vertebrates. Smad proteins function downstream of TGF-β receptor serine/threonine kinases and undergo serine phosphorylation in response to receptor activation. Smad1 is regulated in this fashion by BMP receptors, and Smad2 and Smad3 by TGF-β and activin receptors. Here, we report that BMP receptors phosphorylate and activate Smad1 directly. Phosphorylation of Smad1 in vivo involves serines in the carboxy-terminal motif SSXS. These residues are phosphorylated directly by a BMP type I receptor in vitro. Mutation of these carboxy-terminal serines prevents several Smad1 activation events, namely, Smad1 association with the related protein DPC4, accumulation in the nucleus, and gain of transcriptional activity. Similar carboxy-terminal serines in Smad2 are required for its phosphorylation and association with DPC4 in response to TGF-β, indicating the generality of this process of Smad activation. As a direct physiological substrate of BMP receptors, Smad1 provides a link between receptor serine/threonine kinases and the nucleus.

[*Key Words:* TGF-β; BMP; receptor; Smad1; DPC4; phosphorylation]

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The transforming growth factor-β (TGF-β) family of growth factors regulates a wide variety of cellular processes including differentiation, proliferation, adhesion, and migration (Roberts and Sporn 1990; Attisano et al. 1994; Kingsley 1994; Hogan 1996). Members of this family play essential roles during embryogenesis in mammals, amphibians, and insects, as well as in bone development, wound healing, hematopoiesis, and immune and inflammatory responses in adults. Several disorders in humans including fibrosis, immune disorders, and cancer are linked to alterations in the activity of these factors (Border and Ruoslahti 1992; Massagué 1992; Fynan and Reiss 1993; Markowitz and Roberts 1996). Therefore, how TGF-β and related factors mediate their biological effects is of great interest.

Recent progress has elucidated how several members of this family, such as the TGF-βs, the activins, and the bone morphogenetic proteins (BMPs), initiate signaling from the cell surface. These factors bind to and bring together two distinct but related serine–threonine kinase receptors, the type I and II receptors (Attisano et al. 1994; Miyazono et al. 1994). Upon ligand binding the type II receptor, acting as a primary receptor, phosphorylates the type I receptor and activates this kinase. In turn, the activated type I receptor propagates the signal to downstream substrates (Wrana et al. 1994; for review, see Massagué and Weis-Garcia 1996). The downstream function of the type I receptors is demonstrated by the ability of constitutively active mutants to signal in the absence of the ligand and the type II receptor (Wieser et al. 1995).

Progress in identifying signaling events downstream of the type I receptors has been made recently with the discovery of a novel family of proteins, the Smad family, as TGF-β signal transducers. The first known member of this family, Mad, was identified by genetic analysis of a *Drosophila* BMP-like signaling pathway, the dpp pathway (Sekelsky et al. 1995). *Mad* (*Mothers against decapentaplegic*)-related genes, called *Sma*2, *Sma*3, and *Sma*4, were identified by genetic analysis of a related pathway in *Caenorhabditis elegans*, the Daf4 pathway (Savage et al. 1996). Vertebrate homologs, called Smads (for Sma and Mad homolog) were identified shortly thereafter (Baker and Harland 1996; Eppert et al. 1996; Graff et al. 1996; Hahn et al. 1996; Hoodless et al. 1996; Lechleider et al. 1996; Liu et al. 1996; Riggins et al. 1996; Savage et al. 1996; Thomsen 1996; Yingling et al. 1996; Zhang et al. 1996).

Important insights have been gained on the role of Smads in TGF-β family signaling. In response to receptor activation Smads become phosphorylated (Eppert et al. 1996; Hoodless et al. 1996; Lagna et al. 1996; Lechleider

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et al. 1996; Yingling et al. 1996) and accumulate in the nucleus (Baker and Harland 1996; Hoodless et al. 1996; Liu et al. 1996). A direct role in transcriptional control has been inferred from the presence of transcriptional activity in various Smads (Liu et al. 1996) and the association of Smad2 with the DNA-binding protein FAST1 (Chen et al. 1996). FAST1 belongs to the winged-helix family of DNA binding proteins and its association with Smad2 mediates activin-induced activation of the *Mix.2* gene in *Xenopus* (Chen et al. 1996). Structurally, Smad proteins consist of highly conserved amino-terminal and carboxy-terminal domains separated by a linker region of variable length and sequence (Massagué 1996). The carboxy-terminal domain is considered an effector domain because it displays transcriptional activity, as shown with Smad1 and DPC4 (Liu et al. 1996), and is biologically active, as shown with Smad2 in *Xenopus* embryos (Baker and Harland 1996). These activities are repressed by the amino-terminal domain (Baker and Harland 1996; Liu et al. 1996).

On the basis of structural and functional criteria, the Smad family can be divided into two groups. One includes Smad1, Smad2, Smad3, and Smad5; Sma-2 and Sma-3; and Mad, which are highly homologous and mediate the effects of specific TGF-β family members. Smad1 (and presumably its close isoform Smad5) mediates signaling by BMP2 and BMP4, but not by TGF-β or activin (Graff et al. 1996; Hoodless et al. 1996; Liu et al. 1996; Thomsen 1996), whereas the converse is true for Smad2 (Graff et al. 1996; Baker and Harland 1996; Eppert et al. 1996; Lagna et al. 1996) and its close isoform Smad3 (Zhang et al. 1996). The second group includes Smad4/DPC4 from vertebrates and its homolog in *C. elegans* Sma4. DPC4 is a shared mediator of TGF-β family responses as it associates with pathway-restricted Smads in response to the corresponding agonists, and its inactivation disables TGF-β, activin, and BMP signaling in mammalian cells or *Xenopus* embryos (Lagna et al. 1996). *DPC4* was isolated originally as a tumor-suppressor gene on chromosome 18q21 that is deleted or mutated in a large proportion of human pancreatic carcinomas (Hahn et al. 1996) and less frequently in other cancers (Barrett et al. 1996; Kim et al. 1996; Nagatake et al. 1996; Schutte et al. 1996). *Smad2* is also located in 18q21, and is mutated in a significant proportion of colon and head and neck carcinomas (Eppert et al. 1996; Riggins et al. 1996; Uchida et al. 1996).

Although the involvement of Smad proteins in TGF-β family signaling is firmly established, the positioning of Smads within these signaling pathways, the events leading from receptor activation to Smad phosphorylation, the identity of the kinases involved, and the role of phosphorylation in Smad activation have remained unknown. Here, we report that Smad1 is phosphorylated directly by the BMP type I receptor kinase at carboxy-terminal serine residues. These phosphorylation sites are shown to be required for Smad1 association with DPC4, nuclear accumulation, and transcriptional activity. In agreement with Macias-Silva et al. (1996), who recently reported phosphorylation of similar sites in Smad2 by a TGF-β receptor immune complex, we present evidence that these sites are also required for Smad2 activation in response to TGF-β. Therefore, Smads are physiological substrates of TGF-β family type I receptor kinases and provide a direct link from receptors to the nucleus.

Results

Smad1 carboxy-terminal domain phosphorylation in response to BMP receptor activation

To study receptor kinase activity we used R-1B/L17 mink lung epithelial cells, which can be made responsive to TGF-β, activin, or BMP by transfection of the appropriate receptors (Cárcamo et al. 1994; Liu et al. 1995). Smad2 phosphorylation in these cells is stimulated by TGF-β and activin through the highly related type I receptors TβR-I and ActR-IB, respectively (Eppert et al. 1996; Lagna et al. 1996), whereas Smad1 phosphorylation is induced by BMP2 or BMP4 through the BMP type I receptors BMPR-IA or BMPR-IB (Hoodless et al. 1996; Yingling et al. 1996). Smad1 transfected alone into these cells contained basal phosphorylation, as determined by precipitation from [^{32}P]phosphate-labeled cells through a Flag epitope tag (Fig. 1A, lane 2). The phosphorylation level of transfected Smad1 was strongly increased by both BMP2 and BMP4 acting through the type II receptor BMPR-II or the shared BMP/activin type II receptor ActR-IIB, together with the type I receptors BMPR-IA or BMPR-IB (Fig. 1A, lanes 5,6; and data not shown). The shared BMP/activin type II receptor ActR-IIB stimulated Smad1 phosphorylation in combination with BMP2/BMP4 and BMPR-I, but not in combination with activin and the type I receptor ActR-I (Fig. 1A, lanes 4–6). Activin and ActR-I in concert with another activin type II receptor, ActR-II, mediated a small increase in Smad1 phosphorylation (Fig. 1A, lane 3). However, this small increase observed when overexpressing both Smad1 and the activin receptors might not be physiologically significant. Indeed, an endogenous protein precipitated with Smad1 antibodies was phosphorylated in response to BMP receptor activation but not in response to activin or TGF-β receptor activation (Fig. 1B). In conclusion, Smad1 phosphorylation was specified by the activation of BMPR-I.

Our initial attempts to identify the sites of Smad1 phosphorylation were based on proteolytic digestion of ^{32}P-labeled Smad1 from BMP-stimulated R-1B/L17 cells with various enzymes and subsequent analysis of the resulting phosphopeptides by one- and two-dimensional separations, and peptide microsequencing. This revealed that the serine-rich linker region of Smad1 is phosphorylated (data not shown). However, mutation of potential phosphorylation sites within this region did not abolish BMP-induced phosphorylation, although it decreased the basal phosphorylation significantly (M. Kretzschmar and J. Massagué, unpubl.). Therefore, we searched for the BMP-dependent phosphorylation sites in other domains of Smad1. Because certain mutations in the carboxyl-terminal domains of Smad1 and Smad2 can inhibit ago-

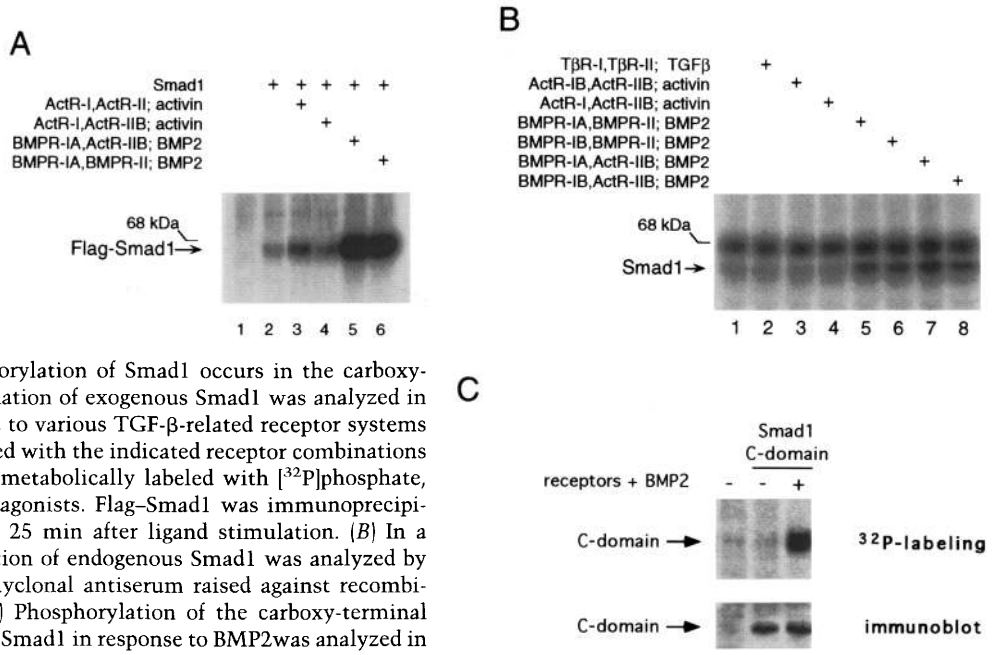

Figure 1. BMP-induced phosphorylation of Smad1 occurs in the carboxy-terminal domain. (*A*) Phosphorylation of exogenous Smad1 was analyzed in mink R-1B/L17 cells in response to various TGF-β-related receptor systems and ligands. Cells were transfected with the indicated receptor combinations and Flag epitope-tagged Smad1, metabolically labeled with [^{32}P]phosphate, and treated with the respective agonists. Flag–Smad1 was immunoprecipitated from cell lysates prepared 25 min after ligand stimulation. (*B*) In a similar experiment phosphorylation of endogenous Smad1 was analyzed by immunoprecipitation with a polyclonal antiserum raised against recombinant human Smad1 protein. (*C*) Phosphorylation of the carboxy-terminal domain (amino acids 268–465) of Smad1 in response to BMP2was analyzed in a similar experiment. Cells were transfected with the Smad1 construct and a combination of BMP type I and II receptors as indicated. Expression levels of Flag–Smad1 (amino acids 268–465) were controlled by immunoblotting with anti-Flag antibody.

nist-induced phosphorylation (Eppert et al. 1996; Hoodless et al. 1996), we analyzed a construct encoding only the Smad1 carboxy-terminal domain. When transfected into R-1B/L17 cells, this construct was devoid of basal phosphorylation but became phosphorylated in response to BMP (Fig. 1C), suggesting that the BMP-dependent phosphorylation sites are located in the carboxy-terminal domain.

Requirement of Smad carboxy-terminal serines for agonist-dependent phosphorylation

One of the mutations that inhibits BMP-induced phosphorylation of Smad1 affects a glycine in the GWG sequence near the carboxyl-terminus (Hoodless et al. 1996). Therefore, we decided to investigate whether serine residues located near the carboxyl-terminus of Smad1 might be sites for BMP-dependent phosphorylation. Alanine mutation of each of the serines (S415 and S431) flanking the GWG sequence (Fig. 2A) had only a minor effect on BMP-induced Smad1 phosphorylation (data not shown). However, alanine mutation of the three serines in the carboxy-terminal SSVS sequence (Fig. 2A), either simultaneously or individually, abolished BMP-induced phosphorylation of Smad1 (Fig. 2B). BMP-induced phosphorylation was retained when individual serines were replaced with threonine (Fig. 2B). The carboxy-terminal sequence SSXS is conserved in Smad2 (Baker and Harland 1996; Graff et al. 1996), which is a TGF-β and activin-responsive Smad, but not in DPC4 (Hahn et al. 1996) (Fig. 2A). As in Smad1, alanine mutation of the SSXS serines in Smad2 blocked phosphorylation by the corresponding agonist, TGF-β (Fig. 2C).

Smad1 phosphorylation by BMP type I receptor immune complexes

To investigate whether Smad1 is phosphorylated by a BMP receptor-associated kinase, we assayed in vitro BMP receptors immunoprecipitated from transfected COS cells. Kinase assays using highly purified, bacterially expressed Smad1 as a substrate showed that Smad1 is phosphorylated by immunoprecipitated BMPR-IB (Fig. 3A). In contrast, an equivalent amount of ActR-I, which does not mediate BMP-induced Smad1 phosphorylation in vivo (see Figs.1A,B), did not phosphorylate Smad1 over background in vitro (Fig. 3A).

We also tested the constructs BMPR-IB(QD) and ActR-I(QD) that contain an activating mutation affecting the penultimate amino acid before the kinase domain. Aspartic acid replacement of the glutamine or threonine invariably present at this position in all type I receptors confers constitutive signaling activity in vivo (Wieser et al. 1995). In the cell, BMPR-IB(QD) stimulates Smad1 phosphorylation in a ligand-independent manner (Hoodless et al. 1996). In our Smad1 kinase assay, BMPR-IB(QD) was more active than the wild-type BMPR-IB, whereas ActR-I(QD) was still inactive (Fig. 3A). The Smad1 kinase activity associated with BMPR-IB was observed at substrate concentrations in the nanomolar range (Fig. 3B), suggesting a high affinity for this substrate. BMPR-IB did not phosphorylate histone H1 or myelin basic protein (data not shown).

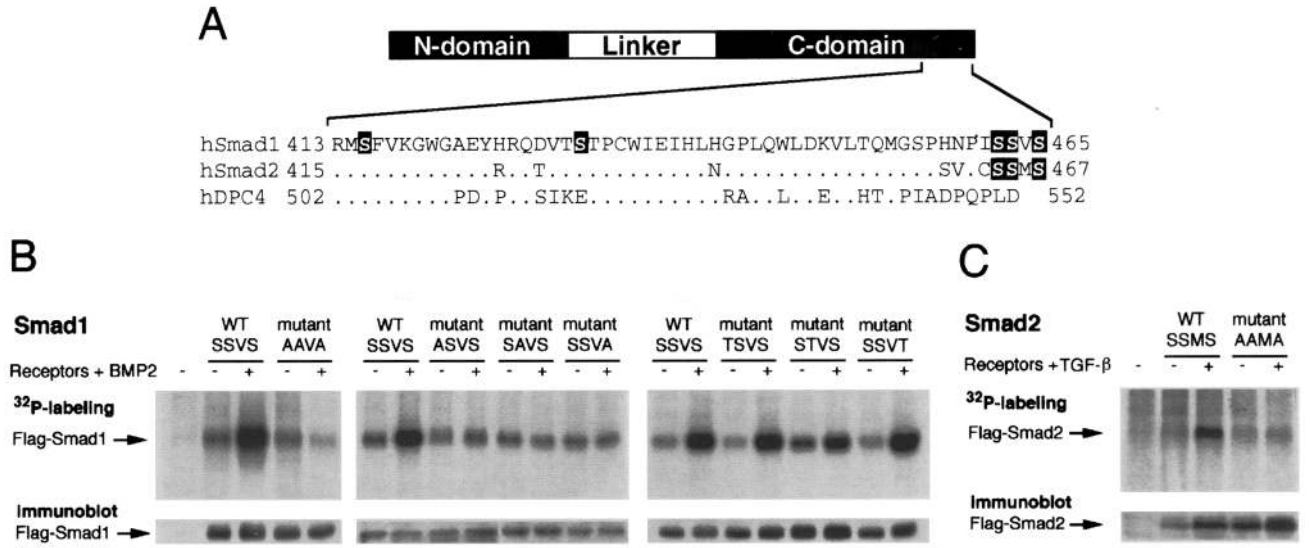

Figure 2. Mutations of the carboxy-terminal serines prevents agonist-induced phosphorylation of Smad1 or Smad2. (*A*) Sequence comparison of the carboxyl termini of Smad1, Smad2, and DPC4. The highlighted residues were mutated as indicated in the text. (*B*) Flag-tagged Smad1 with the indicated serine to alanine or serine to threonine mutations at its carboxyl terminus was analyzed for BMP-induced phosphorylation. The mutations affected serines 462, 463, and 465 of Smad1. R-1B/L17 cells were transfected with the various Flag-tagged Smad1 constructs and the receptor combination (BMPR-IA/BMPR-II) as indicated. Expression levels of Smad1 constructs were controlled by anti-Flag immunoblotting. (*C*) In a similar experiment, Flag-tagged Smad2 with serine to alanine mutations at its carboxyl terminus was analyzed for TGF-β-induced phosphorylation. The mutations affected serines 464, 465, and 467 of Smad2. The receptor combination used was TβRI and TβRII.

To determine whether the in vivo and in vitro phosphorylations of Smad1 may occur at the same sites, we compared tryptic phosphopeptide maps obtained from these two sources. In vivo phosphorylated Smad1 yielded one major phosphopeptide and a minor one (Fig. 3C, in vivo spots 1 and 2, respectively). The major phosphopeptide was also obtained from in vitro phosphorylated Smad1 (Fig. 3C, in vitro), as confirmed by mixing the two samples (Fig. 3C, mix). The in vitro phosphorylated Smad1 yielded a high proportion of the second phosphopeptide. Given the less anionic character of this peptide and the presence of multiple serines at the carboxyl-terminus of Smad1, this phosphopeptide might correspond to an incompletely phosphorylated form of peptide 1.

Direct phosphorylation of Smad1 by the BMP type I receptor kinase

Receptor preparations obtained by immunoprecipitation from cells could contain other kinases in addition to the receptor itself. Therefore, it was important to determine whether the receptor itself has the ability to phosphorylate Smad1. To this end we expressed the BMPR-IB cytoplasmic domain as a glutathione *S*-transferase (GST) fusion protein in bacteria, and purified this product to near-homogeneity (Fig. 4A). In vitro assays showed Smad1 phosphorylation by the recombinant BMPR-IB kinase at nanomolar concentrations of the substrate (Fig. 4B). A control GST fusion (GST–farnesyl-transferase-α subunit) prepared in the same way as the recombinant receptor, was inactive in this assay (Fig. 4A,B). Similar to the results with immunoprecipitated full-length receptors, a GST–BMPR-IB cytoplasmic domain containing the Q to D mutation (Fig. 4A) was approximately fivefold more active than the wild-type GST–BMPR-IB as a Smad1 kinase (Fig. 4B). Therefore, BMPR-IB has intrinsic Smad1 kinase activity that is proportional to the level of in vivo signaling activity of this receptor.

BMP type I receptor phosphorylation of the carboxy-terminal SSXS sequence

To demonstrate that Smad1 is a physiological substrate of the BMP–type I receptor, we then sought evidence that the receptor can phosphorylate the sites involved in BMP-induced Smad1 phosphorylation in vivo. Both the full-length BMPR-IB and the GST–BMPR-IB cytoplasmic domain were able to phosphorylate the recombinant carboxy-terminal domain of Smad1 in vitro (Fig. 5A,B). Furthermore, a Smad1 carboxy-terminal domain containing the SSVS to AAVA mutation was a poor substrate for the full-length BMPR-IB receptor or the GST–BMPR-IB kinase (Fig. 5A,B). To determine whether these serines are actual phosphorylation sites, we generated a recombinant Smad1 carboxy-terminal domain containing a SSVS to TTVT mutation. The TTVT mutant was nearly as good a substrate of BMPR-IB as the wild-type carboxy-terminal domain (Fig. 5A,B). Furthermore, phosphoamino acid analysis of the products phosphorylated by GST–BMPR-IB showed the presence of phosphoserine but not phosphothreonine in the wild-type Smad1 carboxy-terminal domain, whereas both phosphothreonine

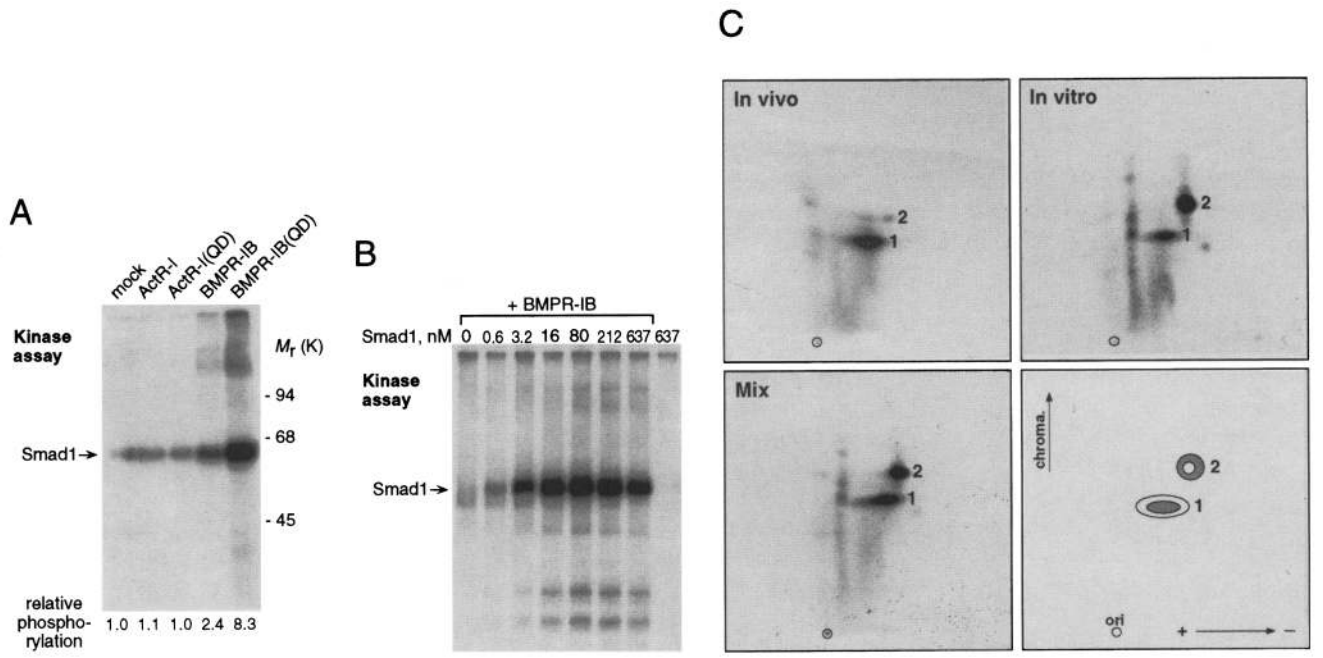

Figure 3. Smad1 is phosphorylated by a BMP receptor complex in vitro. (*A*) Purified recombinant Smad1 was subjected to in vitro kinase assays using the indicated receptors. Hemagglutinin (HA)-tagged receptors were prepared by immunoprecipitation from transfected COS-1 cells, and amounts were normalized by anti-HA immunoblotting of cell lysates (not shown). At the end of the reaction, Smad1 was recovered by affinity chromatography and analyzed by SDS-PAGE and autoradiography. (*B*) In a similar assay the efficiency of phosphorylation by the BMPR-IB receptor was tested by titration of the substrate Smad1. (*C*) Two-dimensional phosphopeptide mapping demonstrates overlapping phosphorylation patterns of Smad1 phosphorylated by BMPR-IB in vitro or in response to BMP2 in vivo. In vitro or in vivo phosphorylated Smad1 proteins were digested with trypsin and the resulting phosphopeptides were analyzed separately or as a mix by two-dimensional separation. The mixed sample contained similar cpm of in vivo and in vitro phosphorylated material. The spots marked with numbers are found in both samples. In the scheme the shaded areas represent in vitro phosphorylated peptides, whereas the open areas represent in vivo phosphorylated peptides. The location of sample application (○, marked ori) and the direction of electrophoresis and chromatography are indicated.

and phosphoserine were present in the TTVT mutant (Fig. 5B). The same result was obtained when full-length BMPR-IB immunoprecipitated from COS cells was used as the kinase (data not shown). These results demonstrate that BMPR-IB phosphorylates Smad1 at the carboxy-terminal serines, which are essential for phosphorylation in vivo. The biological relevance of the additional serine phosphorylation remains to be determined.

Smad activation prevented by phosphorylation site mutations

To determine the functional significance of carboxy-terminal phosphorylation of Smad1 or Smad2, we tested our mutants in various functional assays. An early biochemical event occurring in response to BMP2/BMP4 or TGF-β is the association of Smad1 or Smad2, respectively, with the shared partner DPC4 (Lagna et al. 1996). When analyzed in transfected COS cells, this event was abolished by alanine mutations of all three carboxy-terminal serines in Smad1 or Smad2 (Fig. 6A). The BMP-induced association of Smad1 with DPC4 was also abolished by mutation of each carboxy-terminal serine individually (Fig. 6A), thus correlating with the inhibitory effect of such mutations on BMP-dependent Smad1 phosphorylation (refer to Fig. 2B). Smad homo-oligomerization (Lagna et al. 1996) was not affected by these mutations (Fig. 6A), suggesting that the overall tertiary structure was preserved in these mutant Smads.

Smad proteins are thought to mediate their biological effects by acting as transcriptional regulators. Smad1 fused to the DNA-binding domain of Gal4 has BMP2/BMP4-inducible transcriptional activity (Liu et al. 1996). This response was inhibited significantly when the carboxy-terminal SSVS to AAVA mutations were introduced into a Gal4–Smad1 fusion construct (Fig. 6B). Another consequence of BMP2/BMP4 stimulation is the accumulation of Smad1 in the nucleus, which is a prerequisite for its role in transcriptional regulation. The carboxy-terminal SSVS to AAVA mutation prevented the BMP2/BMP4-induced nuclear accumulation of transfected Smad1, as determined by immunofluorescence microscopy. In accordance with previous observations (Hoodless et al. 1996; Liu et al. 1996) wild-type Smad1 was distributed throughout the cell in the absence of BMP2/BMP4 (Fig. 7, top) and became mostly nuclear in 43%–56% of the cells upon BMP addition (Fig.

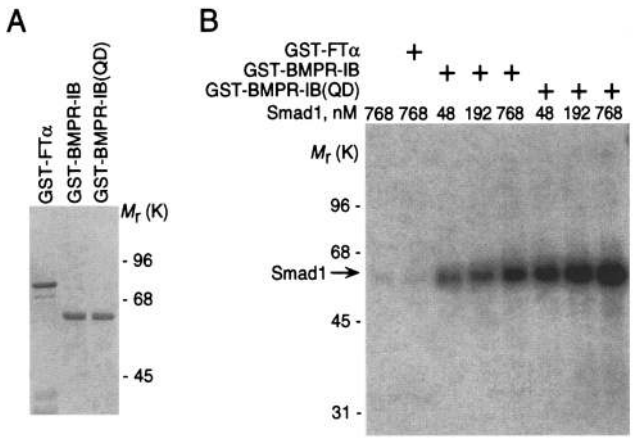

Figure 4. Smad1 is a direct substrate of the BMP type I receptor in vitro. (*A*) GST fusions with the cytoplasmic domain of the BMPR-IB wild-type or QD mutant, or with farnesyl-transferase α-subunit as a control, were overexpressed in bacteria, purified, resolved by SDS-PAGE, and visualized by Coomassie Blue staining. (*B*) GST fusion proteins were tested for kinase activity with the indicated concentrations of Smad1 as the substrate.

7, middle). In contrast, BMP did not induce nuclear accumulation of Smad1 containing the SSVS to AAVA mutation (Fig. 7, bottom).

Therefore, loss of agonist-induced Smad phosphorylation correlates with failure of a Smad to associate with DPC4, accumulate in the nucleus, and activate transcription. We conclude that direct receptor phosphorylation of the carboxy-terminal serines of Smad1 and Smad2 is essential for functional activation of these proteins.

Discussion

The recent identification of Mad in *Drosophila* (Sekelsky et al. 1995) and Smad1 in vertebrates (Graff et al. 1996; Hoodless et al. 1996; Liu et al. 1996; Thomsen 1996) has provided the first major component of the pathway leading from BMP receptors to the processes they control (Hogan 1996). This raises the question of how Smad1 is activated by the BMP receptor signal. Smad1 phosphorylation in response to BMP2 and BMP4 has been documented (Hoodless et al. 1996), but the identity of the kinase and the functional relevance of this phosphorylation have remained unknown. In the present studies, we provide evidence that BMP receptors can phosphorylate Smad1 directly. Furthermore, we show that phosphorylation of the relevant sites is essential for Smad1 association with DPC4, its accumulation in the nucleus, and its activation as a transcriptional regulator. Additional evidence suggests that this mechanism of activation is conserved in Smads that respond to other TGF-β family members. Therefore, a signaling pathway can be traced with Smads as a central link between receptor serine/threonine kinases and the nucleus.

Smad1 as a direct substrate of the BMP type I receptor

Substrates for the type I receptors have been sought since the discovery that these kinases function downstream of type II receptors and are responsible for transducing TGF-β family signals (Miyazono et al. 1994; Massagué and Weis-Garcia 1996). A number of proteins have been identified that bind to the cytoplasmic domain of the TGF-β type I receptor in yeast two hybrid screens, but none of these proteins has been shown to mediate TGF-β signaling (Wang et al. 1994, 1996a,b; Kawabata et al. 1995; Okadome et al. 1996; Ventura et al. 1996; Chen et al. 1997). In contrast, genetic and biochemical evidence demonstrates that Smad proteins act as mediators of every TGF-β family response examined to date, and are phosphorylated in response to these agonists (Derynck and Zhang 1996; Massagué 1996; Lagna et al. 1996). To investigate the process of Smad1 phosphorylation, we first mapped the BMP-dependent phosphorylation sites in Smad1. Initially we found that the carboxy-terminal domain is the target of this modification. Subsequent

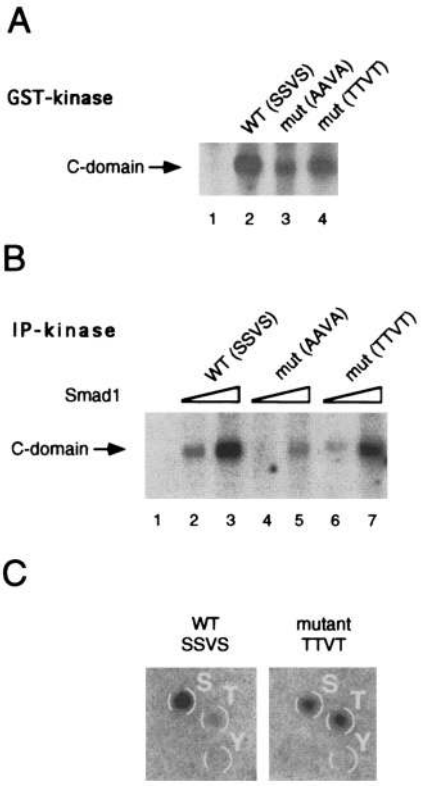

Figure 5. The BMP type I receptor phosphorylates Smad1 at the carboxy-terminal serines. (*A*) Purified recombinant Smad1 carboxy-terminal domains, either wild-type or containing the indicated mutations, were used as substrates in kinase reactions with GST–BMPR-IB(Q203D). Equivalent amounts of wild-type or mutant proteins were used as substrates, as normalized by titration on Coomassie Blue-stained gels (not shown). (*B*) Similar kinase reactions were carried out using full-length BMPR-IB(Q203D) immunoprecipitated from overexpressing COS-1 cells and a fivefold increment in the substrate concentrations. (*C*) Phosphoamino acid analysis of the wild-type and the SSVS to TTVT mutant Smad1 carboxy-terminal domains after the above kinase reactions with GST–BMPR-IB(Q203D).

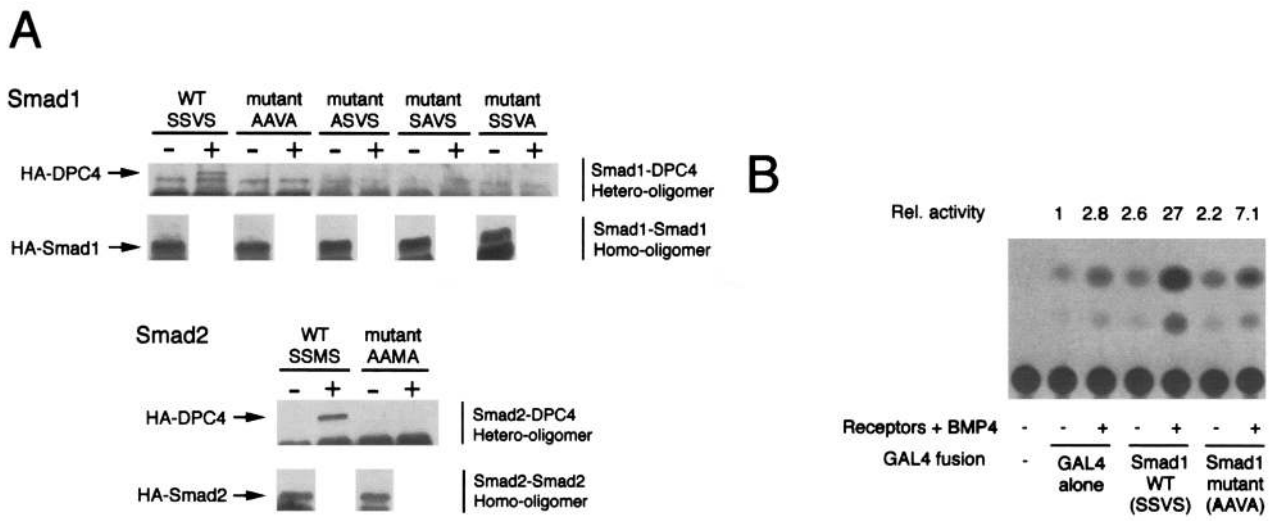

Figure 6. Mutations of the carboxy-terminal serines abolish agonist-induced Smad activation. (*A*) Ability of wild-type and mutant Smad1 and Smad2 to associate with DPC4 and with themselves. Flag–Smad1 constructs were cotransfected with HA-tagged DPC4, and with BMPR-IA(QD) as indicated. Similarly, Flag–Smad2 constructs were cotransfected with HA–DPC4 and with TβRI(T204D) as indicated. Transfections including the receptors were also treated with BMP4 or TGF-β1. Anti-Flag immunoprecipitates were subjected to anti-HA immunoblotting. Smad homo-oligomerization was determined in a similar fashion using cells cotransfected with Flag–Smad and HA–Smad constructs. (*B*) The Gal4 DNA-binding domain (amino acids 1–147) alone or fusion constructs of this domain with wild-type Smad1 or mutated Smad1 (SSVS to AAVA) were tested for BMP-dependent transcriptional activation of a Gal4-responsive chloramphenicol acetyltransferase (CAT) reporter construct. R-1B/L17 cells were transfected with reporter, Smad constructs and receptors as indicated. CAT assays were analyzed by thin layer chromatography and autoradiography, and quantitated by scintillation counting (*top*). BMP had a small effect on the background activity of GAL4 in this particular experiment.

mutational analysis and results of in vivo and in vitro phosphorylation experiments identified the serines of the carboxy-terminal SSVS motif as the BMP2/BMP4-dependent phosphorylation sites. Interestingly, substitution of any of these serines with alanine completely eliminates BMP-induced phosphorylation, whereas substitution with threonine does not. On the basis of the crystal structure of the homologous carboxy-terminal domain of DPC4 (Y. Shi, A. Hata, R. Lo, J. Massagué, and N. Pavletich, in prep.), it can be inferred that the last few carboxy-terminal amino acids of Smad1, like the corresponding region in DPC4, fold outside the conserved core structure of the carboxy-terminal domain, and thus are unlikely to affect the overall structure of the protein. It is possible that recognition of the SSVS motif by the catalytic center of the BMPR-IB kinase requires all three hydroxyl groups. Eliminating any of these polar groups might alter sufficiently the biophysical properties of the carboxy-terminal tail to prevent an efficient catalytic interaction.

Our attempts to determine which and how many of the three carboxy-terminal serines are phosphorylated in response to BMP2/BMP4 have so far failed to yield conclusive results. Phosphoamino acid analysis of the single serine to threonine mutant Smad1 proteins phosphorylated in response to BMP2/BMP4 in vivo have revealed only a small or no increase in the amount of phosphothreonine (M. Kretzschmar, J. Doody, and J. Massagué, unpubl.). This suggests that a threonine replacing any of the three serines may not itself be phosphorylated efficiently, but, in contrast to the single serine to alanine mutations, it will not interfere with phosphorylation of the remaining two serine residues. A preference of the receptor kinase for serines is consistent with the partial loss of Smad1 phosphorylation in vitro when threonines replace the carboxy-terminal serines.

Our conclusion that Smad1 is a physiological substrate of the BMP type I receptor BMPR-IB is based, in part, on the observations that full-length BMPR-IB immunoprecipitated from overexpressing COS cells is able to phosphorylate Smad1 at nanomolar concentrations. Similar preparations of ActR-I, a type I receptor that does not mediate Smad1 phosphorylation in vivo, do not phosphorylate Smad1 in vitro. Significantly, the Smad1 kinase activity of immunoprecipitated BMPR-IB can be reproduced with highly purified preparations of bacterially expressed BMPR-IB cytoplasmic domain. This provides strong evidence that BMPR-IB has intrinsic Smad1 kinase activity. It also suggests that the Smad1 kinase activity that coprecipitates with the full-length BMPR-IB is attributable to this kinase rather than to a different kinase present in the immune complex.

Additional evidence indicates that the BMPR-IB-dependent Smad1 phosphorylation in vivo and the BMPR-IB-mediated phosphorylation in vitro occur at the same sites. This is demonstrated by the overlap of the phosphopeptide maps obtained from Smad1 phosphorylated under these two conditions and, more specifically, by the effect of mutations in the carboxy-terminal SSVS sequence. Alanine mutation of all three serines strongly

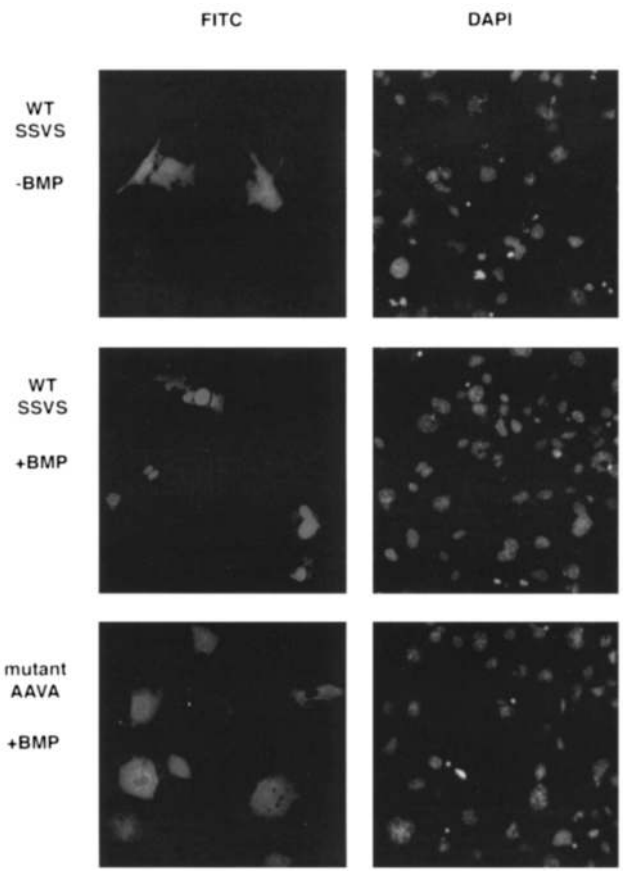

Figure 7. Mutations of the carboxy-terminal serines prevent BMP2/BMP4-induced nuclear accumulation of Smad1. COS-1 cells were transfected transiently with Flag-tagged wild-type Smad1 or mutant Smad1 (SSVS to AAVA). BMP2 (5 nM) was added for 30 min before immunostaining with M2 anti-Flag monoclonal antibodies and FITC-conjugated secondary antibodies. The same slides were counterstained with DAPI to visualize nuclei. Three independent experiments were carried out with essentially identical results.

inhibits Smad1 phosphorylation by purified BMPR-IB in vitro or by BMPR-IB stimulation in vivo. Mutation of these serines to threonines allows Smad1 phosphorylation by BMPR-IB in vitro. Furthermore, this phosphorylated product contains phosphothreonine, whereas the phosphorylated wild-type Smad1 contains only phosphoserine. Our in vitro experiments also reveal that BMPR-IB can phosphorylate the Smad1 C domain at a serine outside the carboxy-terminal SSVS sequence. The location of this site and its physiological relevance remain to be determined, but it leaves open the possibility of an additional phosphorylation event that is secondary to phosphorylation of the SSXS motif in vivo.

We have also investigated the Smad1 kinase activity of recombinant BMPR-IB containing a glutamine to aspartic acid substitution at the penultimate position before the kinase domain. Aspartic acid substitution at this position activates constitutively various type I receptors, allowing them to signal in the absence of type II receptor or the ligand (Wieser et al. 1995; Attisano et al. 1996; Hoodless et al. 1996; Wiersdorff et al. 1996). In our in vitro assays, this mutation increases significantly the Smad1 kinase activity of BMPR-IB. Therefore, the Smad1 kinase activity of the receptor is proportional to its ability to signal in vivo, providing further support for the hypothesis that Smad1 is a direct substrate of the BMP type I receptor.

Phosphorylation is essential for Smad1 activation

Previous studies have identified the carboxy-terminal domain in Smad proteins as a functional effector domain. When fused to the DNA-binding domain of Gal4, the carboxy-terminal domains of Smad1 or DPC4 can activate potently transcription of a Gal4-responsive promoter (Liu et al. 1996). The carboxy-terminal domain of Smad2 is biologically active when injected into *Xenopus* embryos (Baker and Harland 1996). The functional importance of the carboxy-terminal domain is also suggested by the fact that most of the missense mutations found in Smad2 and DPC4 in human cancer (Barrett et al. 1996; Eppert et al. 1996; Hahn et al. 1996; Kim et al. 1996; Nagatake et al. 1996; Riggins et al. 1996; Schutte et al. 1996; Uchida et al. 1996) as well as the missense mutations isolated in genetic screens of *Mad* and *Sma* genes in *Drosophila* and *C. elegans* (Selelsky et al. 1995; Savage et al. 1996) fall in the carboxy-terminal domain, and several of them affect residues near the carboxyl-terminus of these proteins. Therefore, the localization of BMP-dependent phosphorylation sites within this domain suggests that phosphorylation plays an important role in the regulation of Smad1 function.

Our analysis of the phosphorylation site mutants in available functional assays confirms this assumption. The analysis shows that BMP-induced phosphorylation is required for each of the known Smad1 activation events, namely association with DPC4, accumulation in the nucleus, and gain of transcriptional activity. How exactly phosphorylation regulates these events remains to be established, but it is conceivable that phosphorylation at the carboxy-terminal serines causes a conformational change exposing interaction sites for DPC4 and perhaps for other molecules. It is also possible that Smad1 phosphorylation might relieve the carboxy-terminal domain from the inhibitory function of the amino-terminal domain (Baker and Harland 1996; Liu et al. 1996).

Carboxy-terminal SSXS motif phosphorylation as a general event in Smad activation

Smad2, like Smad1, is a pathway-specific Smad regulated in this case by the related TGF-β and activin receptors but not by BMP receptors (Eppert et al. 1996; Lagna et al. 1996). The carboxyl-terminus of Smad2 is highly homologous to that of Smad1 and, importantly, also contains a SSXS motif at the very end. Our analysis of mutant Smad2 demonstrates that the carboxy-terminal serines are required for TGF-β-induced phosphorylation.

Furthermore, these residues are required for functional activation of Smad2 in response to TGF-β, as determined by the ability to associate with DPC4. These results are in complete agreement with the recent report of Macias-Silva et al. (1996) who mapped the TGF-β-dependent phosphorylation sites to these three serines and demonstrated their requirement for Smad2 accumulation in the nucleus. Their studies also showed that TGF-β receptor immunoprecipitated from transfected cells has Smad2 kinase activity. From our results with bacterially expressed BMPR-IB, it can be surmised that the Smad2 kinase activity present in TGF-β receptor immune complexes may be intrinsic to the TGF-β type I receptor. Collectively, these results suggest that phosphorylation of pathway-restricted Smads at the carboxy-terminal SSXS motif is catalyzed by TGF-β family type I receptor kinases and is a key trigger of Smad activation.

DPC4, a shared partner of Smad1 and Smad2, is homologous to these Smads in most of its carboxy-terminal domain but it diverges completely at the very carboxyl-terminus and lacks a serine-rich motif in this region. Consistent with that, DPC4 does not become phosphorylated in cells with TGF-β or BMP4 treatment (Lagna et al. 1996; Zhang et al. 1996), although DPC4 phosphorylation has been detected in response to activin (Lagna et al. 1996).

It is of note that the sequences preceding the SSXS motifs in Smad1 or Smad2 do not resemble the lysine-rich sequence that was suggested to be part of the optimal substrate peptide for the TGF-β receptors as determined by analysis of combinatorial peptide libraries (Luo et al. 1995).

Smads provide a direct link between the receptors and transcriptional control

The present evidence argues that serine–threonine kinase receptors signal to the nucleus through a relatively simple pathway akin to that of the Jak kinase-coupled cytokine receptors that signal through STAT proteins (Darnell 1996; Ihle 1996). Many other responses to extracellular factors, such as those mediated by tyrosine kinase receptors or G protein-coupled receptors, usually involve multiple steps from the cell surface to the nucleus (Schlessinger and Bar-Sagi 1994; Hunter 1995).

Characteristically, substrates of tyrosine kinase receptors bind tightly to docking sites present in the receptors. In contrast, a stable interaction between Smads and type I receptors either in solution, in yeast, or in mammalian cells has been difficult to detect (Hoodless et al. 1996; Zhang et al. 1996). The interaction between TGF-β family type I receptors and Smads might be limited to the phosphotransfer reaction involving the serine residues of the SSXS motif. A transient interaction with the receptor is consistent with the role of Smads as transcriptional regulators.

The identification of Smad proteins as direct substrates of TGF-β receptor kinases allows us to trace for the first time TGF-β signaling pathways from the cell surface to their target genes. In these pathways, two receptor kinases (receptor types I and II) acting in sequence phosphorylate a pathway-restricted Smad that then associates with DPC4 and subsequently in the nucleus, interacts with a sequence-specific DNA-binding protein such as FAST1 (Chen et al. 1996). This model provides a basis to analyze the combinatorial specificity of the system that underlies the variety of biological responses elicited by TGF-β family growth factors. The pathway-restricted Smads (e.g., Smad1 in the BMP2/BMP4 pathway) may provide signaling specificity by acting as both receptor-specific substrates and selectors of sequence-specific DNA-binding partners. As a shared component, the associated DPC4/Smad4 might provide a common function such as interaction with the transcriptional machinery.

Materials and methods

Transfection, metabolic labeling, immunoprecipitation, and Western blot analysis

R-1B/L17 cells (Wrana et al. 1994) were cotransfected transiently by the DEAE–dextran method with Flag-tagged Smad1 or Smad2 (Liu et al. 1996) or combinations of various type I and II receptors (Attisano et al. 1993; Franzén et al. 1993; Cárcamo et al. 1994; ten Dijke et al. 1994; Liu et al. 1995) as indicated. All constructs were in pCMV5. Smad1 and Smad2 mutant constructs were obtained by standard in vitro mutagenesis procedures. Twenty-four-hour post-transfection cells were split into two dishes. Forty-eight to 72 hr post-transfection cells from one dish were labeled metabolically for 3 hr with [^{32}P] phosphate, treated with the indicated factors for 25 min (1 nM TGF-β1, 5 nM activin A, or 10 nM BMP2), and lysed (Wrana et al. 1994). Flag–Smad1 was precipitated with monoclonal anti-Flag antibody (M2; Kodak Scientific) and endogenous Smad1 with a polyclonal rabbit antiserum that we raised against bacterially expressed human Smad1. Proteins were resolved by SDS-PAGE and visualized by autoradiography. Cells from the second dish were lysed, subjected to SDS-PAGE, and transferred onto nitrocellulose membranes. Western blot analysis was done with anti-Flag antibody M2 and by chemiluminescence (enhanced chemiluminescence, Amersham).

Production of recombinant Smad1 and GST fusion proteins

Smad1 full length or its carboxy-terminal domain (amino acids 268–465) was subcloned into a pET expression vector (Novagen) encoding an amino-terminal hexahistidine tag. Overexpression in BL21(DE3) cells and cell lysis was carried out (Kretzschmar et al. 1992) with no EDTA in the lysis buffer. Smad1 proteins were collected on Ni–NTA agarose beads, washed with buffer containing 10 mM imidazole, and eluted with 200 mM imidazole. Farnesyl-transferase α-subunit (Chen et al. 1991) and wild-type and mutant BMPR-IB cytoplasmic domains (amino acids 150–502) (ten Dijke et al. 1994) were amplified by PCR and subcloned into the pGEX-4T-1 GST expression vector (Pharmacia). Proteins were overexpressed in DH5α cells by induction with 0.2 mM IPTG. Cells were harvested 40 min after-induction and lysed by ultrasonication. Proteins were then collected on glutathione–Sepharose beads (Pharmacia). Beads were washed extensively and proteins eluted in kinase buffer containing 10 mM glutathione.

In vitro kinase assays

Purified hexahistidine-tagged Smad1 proteins were preincubated (30 min, 4°C) with recombinant or immunoprecipitated receptors in a buffer containing 50 mM Tris-HCl (pH 7.3), 100 mM NaCl, 10 mM $MnCl_2$, 10% (vol/vol) glycerol, 5 mM dithiothreitol, and 0.05% (vol/vol) Triton X-100. Upon addition of [γ^{32}P]ATP reactions were carried out at 28°C for 20 min. Reactions were stopped by addition of a buffer containing 6 M guanidinium–HCl and Smad1 was recovered with Ni–NTA agarose. The beads were washed and bound proteins resolved by SDS-PAGE and visualized by autoradiography. Hemagglutinin (HA)-tagged full-length receptors were prepared by overexpression in transfected COS-1 cells and immunoprecipitation with anti-HA monoclonal antibody (12CA5; BAbCo) (Cárcamo et al. 1995; Weis-Garcia and Massagué 1996), and were normalized using anti-HA Western immunoblotting. BMPR-IB(QD) containing a Gln203Asp mutation and ActR-I(QD) containing a Gln207Asp mutation were generated by in vitro mutagenesis standard procedures.

Phosphopeptide mapping and phosphoamino acid analysis

In vivo or in vitro phosphorylated Smad1 proteins were separated by SDS-PAGE, transferred to nitrocellulose, digested with trypsin (Worthington), and subjected to two-dimensional phosphopeptide mapping (Boyle et al. 1991) using the HTLE-7000 system (CBS Scientific Co.). The first dimension was done in pH 1.9 buffer and the second dimension used phosphochromatography buffer. For phosphoamino acid analysis SDS-PAGE gel pieces containing phosphorylated Smad proteins were crushed and proteins were eluted in 50 mM ammonium bicarbonate (pH 7.5), 0.7 M β-mercaptoethanol, and 0.1% sodium dodecyl sulfate. Phosphoamino acid analysis was done by subjecting the proteins to acid hydrolysis and separation by two-dimensional electrophoresis (Boyle et al. 1991). Nonradioactive phosphoamino acid standards were included in the electrophoresis and detected with ninhydrin.

Smad association assay

COS-1 cells transfected transiently with the indicated HA or Flag-tagged Smad constructs were split into two plates 24 hr after transfection. Two days post-transfection cells were rinsed twice with DMEM containing 0.2% fetal calf serum and incubated in the presence or absence of 10 nM BMP4 or 100 pM TGF-β1 for 1 hr. Cells were lysed in TNE buffer [10 mM Tris (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1% NP-40] containing protease inhibitors. Cell lysates were subjected to immunoprecipitation with anti-Flag M2 antibody. Immunoprecipitates were washed, separated by SDS-PAGE, and transferred to membranes. HA-tagged DPC4 was detected using anti-HA monoclonal antibody 12CA5 and chemiluminescence.

CAT assay

Chloramphenicol acetyltransferase (CAT) assays were performed essentially as described (Liu et al. 1996) with some modifications. In brief, R-1B/L17 cells were cotransfected with 2 μg of the G1E1BCAT reporter (Lillie and Green 1989), 0.5 μg of the Gal4 derivatives, 2 μg of BMPR-IA, and 0.15 μg of BMPR-II. Twenty-four hours after transfection some cultures were treated with 2 nM BMP4. Nineteen hours later cells were collected and assayed for CAT activity.

Immunofluorescence

COS-1 cells were transfected transiently with pCMV5 vector encoding either Flag-tagged wild-type or mutant Smad1. Twenty-four-hour post-transfection cells were transferred onto chamber slides (Nunc, Inc.). Forty-eight-hour post-transfection cells were incubated with 5 nM BMP2 for 30 min and processed for immunofluorescence as described (Harlow and Lane 1988). Immunostaining was done with M2 anti-Flag monoclonal antibodies (Kodak) and FITC-conjugated secondary antibodies (Pierce). The same slides were counterstained with DAPI to visualize nuclei.

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