Two Short Segments of Smad3 Are Important for Specific Interaction of Smad3 with c-Ski and SnoN

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c-Ski and SnoN are transcriptional co-repressors that inhibit transforming growth factor-β signaling through interaction with Smad proteins. Among receptor-regulated Smads, c-Ski and SnoN bind more strongly to Smad2 and Smad3 than to Smad1. Here, we show that c-Ski and SnoN bind to the “SE” sequence in the C-terminal MH2 domain of Smad3, which is exposed on the N-terminal upper side of the toroidal structure of the MH2 oligomer. The “QPSMT” sequence, located in the vicinity of SE, supports the interaction with c-Ski and SnoN. Sequences similar to SE and QPSMT are found in Smad2, but not in Smad1. The N-terminal MH1 domain and linker region of Smad3 protrude from the N-terminal upper side of the MH2 oligomer. Smurf2 induces ubiquitin-dependent degradation of SnoN, since it appears to be located close to Smad2 through binding to the linker region of Smad2. In contrast, transcription factors Mixer and FoxH3 (FAST1) bind to the bottom side of the Smad3 MH2 toroid; therefore, c-Ski does not affect the interaction of Smads with these transcription factors. Our findings thus demonstrate the stoichiometry of how multiple molecules can associate with the Smad oligomers and how the Smad-interacting proteins functionally interact with each other.

Cytokines of the transforming growth factor-β (TGF-β)1 superfamily bind to two different serine/threonine kinase receptors, type I and type II, and transmit intracellular signals through Smad proteins (1). Receptor-regulated Smads (R-Smads) are directly phosphorylated by type I receptors, and form complexes with common-partner Smad (Co-Smad). The R-Smad-Co-Smad complexes translocate into the nucleus, where they regulate transcription of target genes through interaction with various transcription factors and transcriptional co-activators or co-repressors. Of the eight different mammalian Smad proteins, Smad1, Smad5, and Smad8 are R-Smads activated by bone morphogenetic proteins (BMPs) and anti-Müllerian hormone, whereas Smad2 and Smad3 are R-Smads activated by TGF-βs, activins, and nodal. Smad4 is the only Co-Smad in mammals.

Transcription of target genes by TGF-β is up-regulated by binding of Smads to transcriptional co-activators, including p300 and CBP, which induce acetylation of histones (2–4). In contrast, transcriptional co-repressors, including c-Ski and its related protein SnoN, physically interact with Smads and repress TGF-β superfamily signaling through recruitment of histone deacetylases (5–9). Ski was originally identified as the oncoprotein present in the avian Sloan-Kettering retroviruses (10). Although c-Ski regulates transcriptional activities of some other proteins (11, 12), Smads may be one of the most important partners of c-Ski in transcriptional regulation.

R-Smads and Co-Smads are composed of the N-terminal MH1 domains, linker regions, and the C-terminal MH2 domains. Among the various regions of Smads, the MH2 domains play important roles in binding to type I receptors, formation of Smad oligomers, and interaction with DNA-binding proteins (13). Upon ligand stimulation, R-Smads and Co-Smads may form heterotrimers or heterodimers through their MH2 domains, although their exact oligomeric structures have not been fully determined (14–16). Smads interact with various transcription factors and transcriptional co-activators/co-repressors through the MH2 domains. Among them, Mixer and FoxH3 (originally termed FAST1) have been shown to bind to α-helix 2 (H2) of the MH2 domains of Smad2/3 (17–19). However, sites of binding to other proteins in Smads have not been determined in detail. Interestingly, SnoN was shown to be degraded by a HECT type E3 ubiquitin ligase Smurf2, which binds to the linker region of Smad2 (20). SnoN does not directly bind to Smurf2, but it may be located in the vicinity of Smurf2 when it binds to Smad2. It is thus important to determine through which regions c-Ski/SnoN bind to Smad2 and Smad3.

Among mammalian Smad proteins, Smad2, Smad3, and Smad4 interact with c-Ski, but Smad1 or Smad5 does so only weakly (6). In the present study, we have shown that the SE and QPSMT parts in the MH2 domain of Smad3 interact with c-Ski and SnoN. SE and QPSMT are located on the N-terminal upper side of the toroidal structure of the MH2 oligomer, toward which the MH1 domain and the linker region face. Since Smurf2 binds to the linker region of Smad2, it may be located in the vicinity of SnoN. Amino acid sequences similar to SE and QPSMT are found in Smad2, but not in Smad1 or Smad4. Thus,
these sequences may be responsible for specific interaction of Smad2/3 with c-Ski and SnoN.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The constructions of HA-tagged constitutively active forms of human BMP type IB and TGF-β type I receptors (ALK-6(QD) and ALK-5(TD), respectively) were previously described (21). The construction of FLAG-Smad chimeras were described (6, 22). Smad1 and Smad3 chimeras and mutants were prepared by a PCR-based approach (23). To generate the chimeras, restriction enzyme sites were introduced, if not present, into the MH2 domains of Smad3 and Smad1 by PCR without changing amino acid residues. Phosphorylation of the Smad chimeras and mutants used in the present study was efficiently induced by constitutively active ALK-5 or ALK-6 (data not shown). c-Ski and its deletion constructs were described previously (6). The cDNAs for Mixer (17), SnoN (11), and Smurf2 (24) were provided by Dr. C. S. Hill, Dr. T. Nomura, and Dr. X.-H. Feng, respectively. All of the PCR products were sequenced.

DNA Transfection, Immunoprecipitation, and Immunoblotting—COS7 cells and 293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics and were transiently transfected using FuGENE6 (Roche Diagnostics). Amounts of transfected DNAs were adjusted to 2.0 μg for each sample. Immunoprecipitation and immunoblotting were performed as described (21). For inhibition of proteasomal degradation, cells were incubated with 2.5 μM of lactacystin (Calbiochem) for 24 h.

Molecular Modeling of the Smad3 MH2 Oligomer—Sequences of MH2 domains of human Smad3 and Smad2 were aligned using ClustalW (25), and comparative modeling of the Smad3 MH2 domain monomer was performed by program MODELLER (26) using coordinates of phosphorylated form of the Smad2 MH2 domain (Protein Data Bank code 1khx) as a template. The modeled monomer was tripled and superposed onto subunits A, B, and C of the crystal structure of the oligomeric Smad1 MH2 domain (Protein Data Bank code 1khu) to make root mean square deviation of the Cα atom positions minimum.

RESULTS AND DISCUSSION

Role of a Region of the Smad3 MH2 in Interaction with c-Ski—Smad2 and Smad3 interact with c-Ski, but Smad1 does so only weakly (Ref. 6 and see below). The MH2 domain of Smad3 has been shown to be the major binding site to c-Ski (6, 7). To determine the binding site of the MH2 domain of Smad3 to c-Ski in more detail, we divided the MH2 domain of Smad3
into four parts (termed 2a through 2d regions in the present study) and generated chimeric Smad1/Smad3 molecules (Fig. 1, A and B). The 2d region contains the L3 loop, which is important for the interaction of Smads with type I receptors (27). The 2c region includes H2, which is important for interaction with Mixer (17, 18) and FoxH3 (19).

We characterized Smad1 chimeras containing the MH2 domains, parts of which were sequentially replaced by corresponding regions of Smad3 from the C terminus (Fig. 1, A). Immunoprecipitation of the Smad1 chimeras followed by immunoblotting of c-Ski revealed that the chimeras containing the 2b region of Smad3, i.e. 1-1-3 and 1-1-1333, interacted with c-Ski, and that this interaction was increased more in the presence than in the absence of constitutively active TGF-β1 type I receptor, ALK-5 (Fig. 1, A). In contrast, the chimeras containing the 2b region of Smad1 failed to efficiently interact with c-Ski. Consistent results were obtained using Smad3 chimeras in which only one of the four regions of the MH2 domain was replaced by the corresponding region of Smad3 also revealed that the 2b region of Smad3 is most important for the interaction with c-Ski (data not shown; see Fig. 1E and supplementary Fig. S1).

**SE Is Responsible for Differential Binding of Smad1 and Smad3 to c-Ski**—Comparison of the amino acid sequences of the 2b region of Smad1 and Smad3 (Fig. 1, B) showed that two parts in this region are more varied than other parts between Smad1 and Smad3. The "SE" sequence of Smad3 and its corresponding part of Smad1 (NKN) are almost opposite to each other in its electrostatic properties. The "AAVEL" sequence of Smad3 and its corresponding part of Smad1 (STIEN) are different from each other in its hydrophobic properties. To identify locations of these two parts in the three-dimensional structure of monomer and trimer forms of Smads, we explored the crystal structure of the Smad1 MH2 domain (15) and homology-modeled Smad3 structure based on the crystal structure of the Smad2 MH2 domain (16). Interestingly, SE and AAVEL are distantly located, facing the N-terminal upper and bottom sides of the toroidal structure of MH2 domain trimer, respectively (Fig. 1, C and D).
Smad Segments for Interaction with c-Ski/SnoN

We therefore generated Smad1 and Smad3 mutants in which the SE or AAVEL parts were replaced by the corresponding parts of the other Smads. As shown in Fig. 1E, Smad1(AA), the “STIEN” to AAVEL mutant of Smad1, did not interact with c-Ski. In contrast, Smad1(SE), the “NNK” to SE mutant of Smad1, interacted with c-Ski as efficiently as the Smad chimera 1-1-1311. Our results thus suggest that only two amino acid residues of Smad3 is crucial for interaction of Smad3 with c-Ski. However, Smad3(NK), the SE to NKN mutant of Smad3, still bound to c-Ski (data not shown), suggesting that other parts of Smad3 may also participate in the interaction with c-Ski.

**QPSMT Is Located Close to SE**—We next inspected sequence alignment and three-dimensional structures of the Smad1 and Smad3 MH2 domains. We identified the QPSMT sequence, which is present in the 2α region in the Smad3 MH2 domain and is highly varied from the corresponding part of Smad1 (Fig. 2A, upper panel). QPSMT and SE are located apart from each other in the primary structure, but positioned in the close vicinity on the N-terminal upper surface of the MH2 trimer toroid (Fig. 2A, lower panels).

We constructed mutants of Smad1 and Smad3 in which the QPSMT part was replaced by the corresponding part of the other Smads. As shown in Fig. 2B, Smad1(QP), the STSVL to QPSMT mutant of Smad1 weakly bound to c-Ski. Interestingly, Smad1(Q/S) with mutations in both the QPSMT and SE parts in Smad1 bound to c-Ski more strongly than Smad1(SE) did. Similarly, Smad3(NK) and Smad3(ST), which have the NKN sequence in SE and the “STSVL” sequence in QPSMT, respectively, efficiently bound to c-Ski, but Smad3(S/N) with double mutations in QPSMT and SE in Smad3, bound to c-Ski much less efficiently than wild-type Smad3 (data not shown). These findings suggest that QPSMT does not strongly bind to c-Ski by itself, but plays a role in supporting SE in interacting with c-Ski.

We also studied the interaction of SnoN, a structurally related protein to c-Ski, with the Smad chimeras (data not shown) and mutants (Fig. 2C). Similar to c-Ski, SnoN weakly interacted with Smad1(SE), and more strongly with Smad1(Q/S). Thus, SE and QPSMT are the major sites of specific interaction of Smad3 with c-Ski and SnoN.

**Smad Oligomers Interact with c-Ski and Mixer or FoxH3 at Different Sites**—Mixer is a transcription factor that specifically interacts with Smad2/3. Mixer interacts with Smad3 through a region around H2 in the MH2 domain (see Fig. 1B) (17, 18), which is located on the outer region of the bottom side of the Smad3 homotrimer toroid (Fig. 3A). FoxH3 also interacts with Smad3 through H2 in the MH2 domains (19). We examined whether c-Ski affects the interaction of Smad3 with Mixer. As shown in Fig. 3B, increasing amounts of c-Ski did not affect the binding of Mixer to Smad3. Similarly, c-Ski did not affect the interaction of FoxH3 with Smad2 and Smad3 (data not shown). In contrast, increasing amounts of FoxH3 competed with Mixer for association with Smad3 (data not shown). Thus, Mixer and FoxH3 interact with Smad3 on the bottom side of the MH2 oligomer toroid, and they can interact with Smad3 even in the presence of c-Ski/SnoN.

**SnoN and Smurf2 Are Located in the Vicinity through Interacting with Smad2**—SnoN is degraded by an E3 ubiquitin ligase Smurf2, which binds to the linker region of Smad2 (20). Although SnoN does not directly bind to Smurf2, it may be located in the vicinity of Smurf2 through Smad2. The amino acid sequences of the SE part of Smad2 and SnoN are identical. Moreover, the QPSMT parts are identical in Smad2 and SnoN with the exception of one amino acid exchange (QPSLT in Smad2). As shown in Fig. 4A, wild-type Smad2 efficiently bound to SnoN. In contrast, Smad2(S/N) with double mutations in QPSMT and SE in Smad2 bound to SnoN much less efficiently than the wild-type Smad2.

When ubiquitin-dependent degradation of SnoN was examined in the presence of Smurf2, wild-type Smad2 enhanced the degradation of SnoN, but Smad2(S/N) did so less efficiently than the wild-type Smad2 (Fig. 4, B and C). These findings suggest that Smurf2 may be located close to SnoN upon binding to Smad2, allowing efficient degradation of SnoN by Smurf2. In contrast, Smad1 mutants, Smad1(SE) and Smad1(Q/S), failed to induce degradation of SnoN by Smurf2 (data not shown). Since amino acid sequences of the linker regions are divergent between Smad1 and Smad2, it is possible that SnoN is not located close enough to Smurf2 when it binds to these Smad1 mutants.

**Stoichiometry of Interaction of Multiple Proteins with Smads**—The amino acid sequences of the SE and QPSMT parts of Smad2 and Smad3 are similar, whereas those of Smad1, Smad5, and Smad8 are highly divergent from those of Smad3, suggesting a reason for differential binding of c-Ski to R-Smads. Moreover, amino acid sequences of the parts corresponding to SE and QPSMT of Smad4 are highly divergent from those of Smad2/3. Qin et al. (28) reported that Ski (17–45),
containing amino acids 17–45 of c-Ski, directly interacts with Smad3. In contrast, Wu et al. (29) recently reported that a region containing amino acids 219–312 of c-Ski interacts with the L3 loop of Smad4. Thus, Smad4 binds to c-Ski through a region different from those of Smad2/3. Since Smad4 binds to c-Ski, BMP signals are inhibited by c-Ski (30) even though BMP-specific R-Smads do not strongly interact with c-Ski. In contrast, ubiquitin-dependent degradation of SnoN by Smurf2 occurs independently of Smad4, since Smurf2 does not bind to Smad4 (24).

We have shown in the present study that c-Ski and SnoN bind to the N-terminal upper side of the toroidal structure of MH2 oligomer. Although we were not able to determine in detail which region of the MH2 domain of R-Smads interacts with transcriptional co-activators p300 and CBP, c-Ski competed with p300 for interaction with Smad3 (data not shown; Ref. 6), suggesting that p300/CBP may bind to R-Smads on the N-terminal upper side of the MH2 domains. Wu et al. (29) reported that c-Ski binds to the L3 loop of Smad4, resulting in disruption of the complex formation of R-Smads and Smad4. In contrast, binding of c-Ski to Smad2/3 does not affect the oligomer formation of Smads. Understanding the mode of interaction between Smads and c-Ski/SnoN may therefore provide information important for elucidation of the functional protein interaction and for development of new strategies for regulation of the activities of TGF-β superfamily proteins.

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