CONTRIBUTION OF ARGININE 462 AND CYSTEINE 463 IN THE C TERMINUS OF SMAD2 FOR SPECIFICITY

Ihor Yakymovych, Carl-Henrik Heldin, and Serhiy Souchelnytskyi

From the Ludwig Institute for Cancer Research, Box 595, SE-751 24 Uppsala, Sweden

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Abstract

Transforming growth factor-β (TGFβ) is a potent regulator of cell proliferation, differentiation, motility, and apoptosis. TGFβ binds to and activates serine/threonine kinase receptors that phosphorylate Smad2 and Smad3 intracellular signal transducers at two C-terminal serine residues. Here we show that substitutions of Arg-462 and Cys-463 residues, which are in proximity of the C-terminal serine residues, inhibited TGFβ type I receptor-dependent phosphorylation of the C-terminal Smad2 peptides and full-length GST-Smad2 proteins in vitro. In vivo, mutation of Arg-462 and Cys-463 inhibited TGFβ-stimulated phosphorylation of the C-terminal serine residues in Smad2. Moreover, Smad2 with mutated Arg-462 and Cys-463 was less efficient in activation of the Smad2-responsive activin-responsive element-containing luciferase reporter ARE-luc, as compared with the wild-type protein. Thus, Arg-462 and Cys-463, which are in proximity of the C-terminal serine residues, contribute to recognition and phosphorylation of the C terminus of Smad2 by type I TGFβ receptor.

EXPERIMENTAL PROCEDURES

Materials, Reagents, Constructs—The constructs of constitutively active GST-TβR-I, GST-BMPR-II, GST-Smad2, and FLAG-Smad2 were described earlier (20–22). Mutants of GST-Smad2 and FLAG-Smad2 were generated by site-directed mutagenesis using a QuikChange kit (Stratagene). Mutations were confirmed by sequencing of the constructs, and expression of proteins was evaluated by SDS-PAGE and by immunoblotting assay. Mv1Lu, NIH3T3, and COS1 cells were obtained from ATCC (LGC, Teddington), and Smad2−/− mouse embryonic fibroblasts (MEFs) were obtained from Anita Roberts and Ester Fierk. All cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum.

In Vitro Phosphorylation Assay—For phosphorylation assay, peptides were diluted in the reaction mixture (20 mM HEPES (pH 7.4), 10 mM MgCl₂, 2 mM MnCl₂, 1 mM dithiothreitol, 5 μM ATP plus 0.5 μCi of [γ-³²P]ATP (Redivue; Amersham Biosciences)). The peptides used in this study were synthesized using the Fmoc (N-(9-fluorenlymethoxycarbonyl) chemistry as described (20). Three lysine residues were added to the N terminus of each peptide to allow peptide binding to the P81 phosphocellulose paper or to the plastic. Phosphorylation reaction was initiated by addition of constitutively active GST-TβR-I adsorbed on glutathione-Sepharose beads. After 20 min of incubation at 22°C, reaction mixtures were centrifuged, and the supernatants were spotted onto Whatman P81 phosphocellulose paper. Filters were washed in three changes of 0.75% phosphoric acid, rinsed in acetone, dried, and

faces between the L45 loop and the GS domain of type I receptors and the L3 loop, the adjacent basic surface, and the α-H1 helix in the MH2 domain of receptor-regulated Smads as determinants of specificity in recognition of Smads by receptor kinases (8–13).

Phosphoryl groups at Ser-465 and Ser-467 of the C terminus of Smad2 were found to mediate the interaction with Smad4 (7). Crystallography studies showed that upon phosphorylation of Ser-465 and Ser-467, the C terminus of Smad2 acquires certain structural features, allowing interaction with the MH2 domain of another Smad molecule (14–16). Phosphorylation of the two C-terminal serine residues is also important for relief of the inhibitory intramolecular interaction between the MH1 and the MH2 domains, leading to activation of Smads (6, 7, 17). Therefore, phosphorylation of the C terminus of receptor-activated Smads is crucial for initiation of TGFβ signaling.

For a number of kinases, specificity of phosphorylation is determined by sequences adjacent to the site of phosphorylation (18). Consensus sequences have been established for more than 50 kinases, suggesting that substrate recognition is mediated by a complementation between amino acid residues in a substrate and in a kinase substrate-binding region in or immediate to the catalytic cleft (19). Previously we showed that the C-terminal Smad2 peptide specifically inhibited TβR-I kinase, but not other receptor kinases (20). This suggests that the substrate recognition site of TβR-I kinase has preferences for the C-terminal peptide of Smad2. Here we have shown that Arg-462 and Cys-463 in the C terminus of Smad2 are important for specific recognition and phosphorylation of the C-terminal serine residues of Smad2 by TβR-I.
counted in a scintillation counter. Every experimental condition was performed in triplicate, and experiments were repeated three to four times. In some experiments, phosphorylated peptides were separated by a Tricine-polyacrylamide gel electrophoresis (23). Gels were dried and exposed by phosphorimaging. Phosphorylation of GST-Smad2 proteins in vitro was performed as described earlier (20). After SDS-PAGE, the proteins were transferred onto the nitrocellulose membrane, and phosphorylation of the C terminus was analyzed by two-dimensional phosphopeptide mapping.

**Detection of Smad2 C-terminal Phosphorylation**—Immunoblotting with pS2 antibodies that specifically recognize phosphorylated C-terminal Ser-465 and Ser-467 in Smad2 was performed as described earlier (20).

** Luciferase Reporter Assays**—The cells were transiently transfected using LipofectAMINE (Invitrogen) according to the manufacturer’s recommendations or using a 25-kDa branched polyethyleneimine (Sigma) as described (24). Activin-responsive element (ARE)-luc reporter assays were performed as previously described (21). In all reporter assays, the β-galactosidase reporter plasmid pCMV-LacZ was used for normalization of efficiency of transfection.

**RESULTS**

**TβR-I Phosphorylates Specifically the C-terminal Peptide of Smad2**—Amino acid residues adjacent to sites of phosphorylation are for several kinases crucial for substrate recognition, e.g. Pro is required at the +1 position for phosphorylation by Erk (extracellular signal-regulated kinase) 1/2 (19). To explore whether phosphorylation of Smads by TβR-I kinase is dependent on surrounding amino acids, we performed in vitro kinase assays with peptides corresponding to the C-terminal tails of Smad2 and Smad5 as substrates (Fig. 1A). The use of peptides allowed us to exclude an influence of the N-terminal sequence, e.g. of the MH2 domain. Crystallography studies could not identify a defined structure for the C terminus of Smad, suggesting that the C terminus is not an intrinsic part of the MH2 domain (17).

We found that TβR-I kinase phosphorylated the C-terminal...
Smad2 peptide (Sm2S), but not the Smad2 peptide with the five C-terminal serine residues substituted to alanines (Sm2A). Neither was Smad5 C-terminal peptide with mutated serines (Sm5A) phosphorylated by TβR-I, and wild-type Smad5 peptide (Sm5S) showed very weak signal (Fig. 1B). A similar specificity of TβR-I-dependent phosphorylation was observed with the same peptides coupled to plastic surfaces (data not shown). Furthermore, phosphorylation of the Smad2 peptide was specific for TβR-I kinase because other serine/threonine kinases receptors, e.g. bone morphogenetic protein receptor type II (BMPR-II), bone morphogenetic receptor type I (BMPR-IB), and type II TGFβ receptor did not phosphorylate the peptide (Fig. 1C). Preservation of the specificity of TβR-I kinase toward a substrate was also shown previously (20).

In vitro phosphorylation assay with various concentrations of the Sm2S and Sm5S peptides (31, 62, 125, 250, 500, and 1000 μM) showed that the Sm2S peptide was strongly phosphorylated at a concentration of 31 μM with a maximal level achieved at 500 μM (Fig. 1D). Weak, but detectable, phosphorylation of Smad5 peptide was observed only at 1000-μM concentration of the peptide. This suggests that under non-physiological conditions the C-terminal tail of BMP Smad5 can be phosphorylated by TβR-I.

To explore which residue(s) in the C-terminal peptide of Smad2 defines specificity of Smad2 recognition by the TβR-I kinase, we generated peptides with substitutions of Ser-460, Arg-462, and Cys-463 to Ala, Ile and Ala, respectively (Fig. 1A). We found that the strongest decrease of phosphorylation was observed when both Arg-462 and Cys-463 were mutated (Sm2R462I,C463A peptide; Fig. 1E). Single mutations of Arg-462 and Cys-463 decreased phosphorylation of peptides as well. We did not see changes in peptide phosphorylation when Ser-460 or when Ser-458, Ser-460, and Ser-464 were mutated (Fig. 1E). Thus, Arg-462 and Cys-463 are of importance for phosphorylation of the C-terminal peptide of Smad2 by the TβR-I kinase. Mutation of Arg-462 and Cys-463 Prevents Phosphorylation of the C-terminal Serine Residues in GST-Smad2 by TβR-I—

We further explored the importance of the amino acid residues in the region from Ser-460 to Cys-463 for the specific phospho-
Fig. 3. Mutations of Arg-462 and Cys-463 lead to inhibition of efficient phosphorylation of Smad2 in vivo. FLAG-Smad2 constructs were transfected in COS1 (A), Smad2−/− mouse embryonic fibroblasts (MEFs; B and C), Mv1Lu (D), and in NIH3T3 cells (E) as indicated. After transfection, cells were treated with TGFβ/1 (5 ng/ml) for 1 h (MEFs) or 30 min (Mv1Lu, NIH3T3), and FLAG-Smad2 was precipitated with anti-FLAG antibodies. Phosphorylation of the C-terminal serine residues was monitored by immunoblotting with pS2 antibodies, and expression of FLAG-Smad2 constructs was monitored by immunoblotting with anti-FLAG antibodies as indicated. Arrows show migration positions of FLAG-Smad2 proteins. WT, wild-type; RC/PI, FLAG-Smad2R462P,C463I; RC/A, FLAG-Smad2R462A,C463I; and SS/AA, FLAG-Smad2S465A,S467A mutants. Upon high expression of FLAG-Smad2 constructs in COS1 cells, stability of the proteins is not affected (A). In other cell lines, FLAG-Smad2 constructs were expressed at lower levels (B, D, E). Experiments shown are representative of four (B, C) and two (D, E) performed. WCE, whole cell extracts; IP, immunoprecipitation; IB, immunoblotting; Ctr, control cells transfected with empty vector.
Fig. 4. Delayed phosphorylation of Smad2 with mutated Arg-462 and Cys-463 residues. Wild-type (WT) and R462F,C463I mutant (RC/PI) of FLAG-Smad2 were transfected in Smad2−/−MEFs. Transfected cells were treated with TGFβ1 (5 ng/ml) for the indicated times, and proteins were immuno-precipitated with anti-FLAG antibodies. A, phosphorylation of the C-terminal serine was evaluated by immunoblotting with pS2 antibodies (upper panel). Expression of proteins was monitored by immunoblotting with anti-FLAG antibodies (lower panel). Migration positions of FLAG-Smad2 proteins are shown by arrows. B, quantification of FLAG-Smad2 phosphorylation, normalized to protein expression, is shown. Figure is representative of three experiments performed.

We observed a phosphopeptide with migration corresponding to the wild-type peptide (Fig. 2C) in phosphopeptide maps of double mutants GST-Smad2S460A,V461N and GST-Smad2V461N,R462P (Fig. 2, D and E). The observed slight deviations in migrations of the peptide is because of the substitutions made and in the case of GST-Smad2V461N,R462P mutant because of abrogation of trypsin cleavage C-terminal to Arg-462. A double mutation of Arg-462 and Cys-463 abrogated GST-Smad2 phosphorylation, as we could not observe any phosphorylated C-terminal Ser-465 and Ser-467 (Fig. 3). As a negative control, we used FLAG-Smad2 with the two C-terminal serines substituted by alanine residues (FLAG-Smad2SA). We found that none of the mutations affected the stability of proteins, whether proteins were expressed at low or at high levels (Fig. 3).

We observed that substitution of both Arg-462 and Cys-463 to either Pro-462 and Ile-463 (FLAG-Smad2R462P,C463I; RC/PI mutant) or to Ile-462 and Ala-463 (FLAG-Smad2R462I,C463A; RC/IA mutant) inhibited TGFβ1-dependent phosphorylation at the C-terminal serine residues (Fig. 3, B–E). In Mv1Lu epithelial cells and in NIH3T3 mouse fibroblasts we did not observe any detectable C-terminal phosphorylation of RC/PI or RC/IA mutants of FLAG-Smad2; neither was there any phosphorylation of the FLAG-Smad2S465A,S467A (SS/AA) mutant as expected (Fig. 3, D and E). We could observe some phosphorylation of the FLAG-Smad2 RC/PI and RC/IA mutants upon expression in Smad2−/− mouse embryonic fibroblasts. However, quantification of the phosphorylation showed significant reduction in efficiency of phosphorylation (Fig. 3, B and C). Moreover, analysis of time-dependence of Smad2 phosphorylation upon TGFβ1 treatment showed that the RC/PI mutant was phosphorylated at a slower rate than the wild-type FLAG-Smad2 (Fig. 4). The rate of Smad phosphorylation may be affected by various interacting proteins and could therefore be cell type-de-
specificity of Smad2 phosphorylation by TβR-I

Discussion

Here we have provided evidence that Arg-462 and Cys-463 in the C terminus of Smad2 are important for recognition and phosphorylation of the serine residues 465 and 467 by TβR-I. Our findings expand the current view on determinants of specificity in TGFβ signaling. Previously, it has been shown that the physical interaction between TβR-I and Smad2 is mediated by the L45 loop and GS region of the receptor and by the L3 loop and the adjacent basic surface of Smad protein (8–13). For Smad1, a member of the BMP Smad family, the importance of the α-H1 helix in the MH2 domain for interaction with ALK1 and ALK2 receptors has also been shown, in addition to the L3 loop (11). The role of the C-terminal sequences of receptor-regulated Smads in determination of signaling specificity has not been addressed thoroughly. The fact that mutation of the phosphorylatable serine residues increases the interaction with TβR-I suggests that the C terminus, or at least C-terminal phosphorylation, affects the interaction between receptor and Smad protein (4–7).

The reported inhibition of TβR-I by the C-terminal peptide specific for Smad2 and the inability of the Smad5-specific C-terminal peptide to inhibit the TβR-I kinase suggest that the kinase may be able to selectively recognize the substrate even in the absence of the MH2 domain (20). Several kinases are dependent on specific sequences adjacent to the site of phosphorylation (19). Consensus sequences for at least 50 kinases have been identified (18), and residues at +1 or −3 and −4 have been found to be of particular importance for recognition of substrates by kinases (19). An early attempt to identify a
consensus phosphorylation sequence for TβR-I using a peptide library showed that the presence of basic charges at the −4 and −3 positions would be preferable for phosphorylation by TβR-I (26). The presence of arginine residue at the −5 and −3 positions, compared with phosphorylatable serines in the C terminus of Smad2, is in agreement with this suggestion.

Here we have shown that TβR-I kinase can specifically recognize the Smad2 C-terminal sequence and that Arg-462 and Cys-463 are crucial determinants for the specificity. The importance of Arg-462 and Cys-463 was observed in vitro for peptides and in the context of the whole Smad2 molecule as well as in vivo for expressed Smad2 proteins (Figs. 1–4). Some difference in the phosphorylation efficiency of peptides, compared with full-length proteins, may be because of the upstream sequences in proteins, e.g. the MH1 and MH2 domains. However, the similarity of results obtained with peptides that do not have any of the MH2 domain structural features, and with full-length proteins, supports the notion that Arg-462 and Cys-463 are important determinants of recognition and phosphorylation of the Smad2 C terminus.

Positioning of the C-terminal peptide in the substrate-binding region of TβR-I showed that Arg-462 and Cys-463 of Smad2 may interact with residues in the activation segment of the kinase (Fig. 6) (27). The activation segments of TGFβ- and BMP-specific receptors differ, with strong similarities of the sequences within the groups of receptor. Thus, the presence of aspartic acid residues in the activation segments of TβR-I and ActR-IB, e.g. Asp-359 and Asp-363, may provide negatively charged surfaces that interact with positively charged C-terminal residues of Smad2 (Fig. 6). Significant similarity in the activation segments has also been observed for ALK7, which is known to phosphorylate Smad2 and Smad3. The activation segments of BMPR-IA and BMPR-IB receptor kinases have sequences that differ from the TGFβ receptors. Another group, defined on the basis of the sequences of activation segments, consists of ActR-I A and ALK1 receptors. Thus, it is tempting to suggest that the activation segments of receptor kinases provide a surface for specific recognition of the C-terminal peptide of Smad proteins. However, for the conclusive answer, the structure of the TβR-I kinase with bound substrate has to be determined.

Our findings add a new aspect to our understanding of the determination of specificity in TGFβ signaling. Whereas the interfaces between the L3 loop, basic surface, and α-H1 structures of Smad proteins and the L45 loop and G8 domain of the type I receptors are involved in docking of Smads to receptors, specific recognition of the C-terminal phosphorylation sites adds to the specificity of substrate recognition. Importance of adjacent amino acid residues for specific recognition and phosphorylation of substrates is well established for many kinases. The data presented here extend this notion to the type I serine/threonine kinases receptors and their substrates.

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