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Functional interaction between Smad, CREB binding protein, and p68 RNA helicase☆

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Abstract

The transforming growth factors β control a diversity of biological processes including cellular proliferation, differentiation, apoptosis, and extracellular matrix production, and are critical effectors of embryonic patterning and development, including that of the orofacial region. TGFβ superfamily members signal through specific cell surface receptors that phosphorylate the cytoplasmic Smad proteins, resulting in their translocation to the nucleus and interaction with promoters of TGFβ-responsive genes. Subsequent alterations in transcription are cell type-specific and dependent on recruitment to the Smad/transcription factor complex of coactivators, such as CBP and p300, or corepressors, such as c-ski and SnoN. Since the affinity of Smads for DNA is generally low, additional accessory proteins that facilitate Smad/DNA binding are required, and are often cell- and tissue-specific. In order to identify novel Smad 3 binding proteins in developing orofacial tissue, a yeast two hybrid assay was employed in which the MH2 domain of Smad 3 was used to screen an expression library derived from mouse embryonic orofacial tissue. The RNA helicase, p68, was identified as a unique Smad binding protein, and the specificity of the interaction was confirmed through various in vitro and in vivo assays. Co-expression of Smad 3 and a CBP-Gal4 DNA binding domain fusion protein in a Gal4-luciferase reporter assay resulted in increased TGFβ-stimulated reporter gene transcription. Moreover, co-expression of p68 RNA helicase along with Smad 3 and CBP-Gal4 resulted in synergistic activation of Gal4-luciferase reporter expression. Collectively, these data indicate that the RNA helicase, p68, can directly interact with Smad 3 resulting in formation of a transcriptionally active ternary complex containing Smad 3, p68, and CBP. This offers a means of enhancing TGFβ-mediated cellular responses in developing orofacial tissue.

Keywords: Smad; TGFβ; Two-hybrid; Transcription; p68; Helicase; CBP

The TGFβ family of polypeptide growth factors regulates critical developmental processes including cellular proliferation, differentiation, apoptosis, migration, and extracellular matrix production (for review, see [1]). TGFβ signaling is initiated by binding to the type II TGFβ receptor (TβRII) which subsequently heterodimerizes with a type I receptor (TβRI). The receptor complex then phosphorylates a subset of the intracellular Smad proteins, of which there are three known classes: those that are phosphorylated by cell surface receptors (R-Smads, including types 1, 2, 3, 5, and 8/9), common Smads that form heterodimers with R-Smads (types 4 and 10), and inhibitory Smads that attenuate TGFβ signaling by interfering with receptor phosphorylation (types 6 and 7). Phosphorylation of receptor-regulated Smads facilitates complex formation

☆ *Abbreviations:* TGFβ, transforming growth factor β; p68, p68 RNA helicase; CBP, Creb binding protein; PCR, polymerase chain reaction; X-α-gal, 5-bromo-4-chloro-3-indolyl-α D-galactopyranoside; GST, glutathione *S*-transferase; RIPA, radioimmunoprecipitation assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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with Smad 4, translocation to the nucleus, and binding of the complex to the promoters of target genes. Depending on the cell type and the recruitment of either co-repressors (e.g., c-Ski or SnoN) or co-activators (e.g., CBP/p300), transcription is inhibited or activated, respectively. The critical role of CBP/p300 in Smad-mediated TGFβ-dependent transcriptional activation has been established. The Smad/co-activator complex forms a platform for the recruitment of a plethora of accessory proteins involved in transcription, such as histone acetyltransferases and RNA polymerase II, as well as numerous transcription factors [2].

TGFβs exhibit a wide range of cell-specific effects and function through activation or inhibition of transcription of a broad range of genes (e.g., see [3]). Smads 2 and 3 are TGFβ-regulated transcription factors, where Smad 3 recognizes a minimal nucleotide sequence of GTCT (Smad binding element, SBE [4]). The specificity, however, with which Smad 3 binds to this tetranucleotide sequence is low and additional proteins are required to form efficient transcriptional complexes that assemble onto the SBE and activate/inhibit transcription [5,6]. To gain a better understanding of the mechanisms underlying the diverse molecular and cellular effects of Smad-mediated TGFβ signaling, unique Smad binding proteins with the potential to function as transcriptional cofactors were sought using a yeast two-hybrid screen with the MH2 (transactivation) domain of Smad 3 as bait. Several novel proteins identified from this screen were reported previously [7–9]. The present report describes the interaction between Smads and p68 RNA helicase (p68), an ATP-dependent RNA and DNA helicase with significant homology to eIF-4A and diverse cellular and developmental activities [10]. Several reports have demonstrated that p68 is a cofactor for CBP-mediated transcriptional activity [11,12]. Detailed herein is evidence that p68 also can interact directly with Smads and lead to the formation of a transcriptionally active ternary complex containing Smad 3, p68, and CBP.

Materials and methods

Yeast two-hybrid expression library and bait construct. The cDNA expression library and bait construction previously have been described in detail [7,8]. Briefly, a cDNA expression library fused to the activation domain of Gal4 was prepared by Research Genetics (Huntsville, AL) using RNA isolated from embryonic maxillofacial tissue dissected from mid-gestation mouse embryos (ICR, Harlan, Indianapolis, IN) and cloned into pGADT7 (Clontech, Palo Alto, CA). The Smad 3 MH2 domain was amplified by PCR from full-length Smad 3 [13] and cloned into pGBKT7 (Clontech) to create the Gal4 DNA binding domain-Smad 3 MH2 fusion protein. The final construct was sequenced to confirm integrity.

Yeast two-hybrid assay. The Matchmaker Yeast Two-Hybrid System 3 (Clontech) was used to screen for Smad 3 binding proteins expressed in mouse embryonic maxillofacial tissue. pGBKT7-Smad 3 MH2 was transformed into *Saccharomyces cerevisiae* (strain AH109) along with the pGADT7 expression library. Transformants were plated on yeast medium in the absence of histidine, leucine, tryptophan, and adenine, and in the presence of 2.5 mM 3-amino-1,2,4-triazole and 20 μg/ml X-α-gal according to instructions provided by the manufacturer. Approximately 5×10^5 yeast transformants were screened, blue colonies bearing the $His^+/Leu^+/Trp^+/Ade^+$ phenotype were cultured , and plasmid DNA was isolated. To confirm positive clones, purified plasmid was re-introduced into *S. cerevisiae* (AH109) along with $pGBKT_7$, $pGBKT_7$-lamin C (negative control), or pGBKT7-Smad 3 MH2. cDNA clones that maintained the $His^+/Leu^+/Trp^+/Ade^+/X\text{-}\alpha\text{-}gal^+$ phenotype in the presence of Smad 3 MH2 were sequenced and identified by nucleotide comparison to sequences within GenBank using BLAST software (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD).

Glutathione S-transferase pull-down assay. To assay for in vitro interaction between Smads and p68, GST pull-down assays with purified GST-Smad fusion proteins and in vitro translated, [^{35}S]methionine-labeled p68 were performed. The preparation and purification of GST-fusion protein constructs for full-length Smads 1, 2, 3, 4, and 7, and their corresponding MH2 domains have been described previously [7]. In vitro translated, [^{35}S]methionine-labeled p68 was prepared with the TNT T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI) and 5 μl was mixed with 1 μg bacterially expressed, purified GST-Smad bound to glutathione–Sepharose (Amersham Biosciences, Piscataway, NJ). The mixture was incubated for 1 h at 4 °C in GST pull-down buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM dithiothreitol, 10% glycerol, 1 mM EDTA, 2.5 mM $MgCl_2$, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) with gentle rotation. The Sepharose:protein complex was washed three times with GST pull-down buffer and bound material was eluted by the addition of 2× Laemmli sample loading buffer [14], followed by boiling for 5 min and separation on an 8–16% polyacrylamide gel under reducing/denaturing conditions. Gels were fixed for 30 min with 50% methanol:10% acetic acid and exposed to Kodak X-Omat AR film for 2 days at −80 °C. Following autoradiography, dried gels were stained with Coomassie blue to compare the loading efficiency for each sample.

Cloning of full-length p68. The cDNA for full-length p68 was cloned using a PCR-based approach with mouse p68-specific primers and first-strand cDNA synthesized from embryonic orofacial tissue RNA. The PCR primers were synthesized based on the published cDNA sequence of mouse p68 (GenBank Accession No. NM_007840) and were designed to amplify amino acids 2–614 (forward, 5′-aag gta cca tcg agt tat tct agt gac cga g-3′; reverse, 5′-gc gat atc tta ttg aga ata ccc tgt tgg-3′). The forward and reverse primers were designed to contain unique *Kpn*I and *Eco*RV restriction sites at the 5′- and 3′-ends of the amplified cDNA, respectively. The subsequent PCR product was digested with *Kpn*I and *Eco*RV, and cloned into the *Kpn*I–*Eco*RV site of the pcDNA3.1-His/Xpress expression vector (Invitrogen, Carlsbad, CA) creating a modified p68 that contained a His/Xpress tag (HHHHHHMASMTGGEEMGRDLYDDDDK) at the amino terminus. The PCR program consisted of 35 cycles of: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. A single product of the expected size of 1800 base pairs was obtained and cloned into pcDNA3.1-His/Xpress. The cloned p68 cDNA was sequenced in full and matched the published sequence of mouse p68 with the exception of two changes: thymidine at positions 336 and 477, both of which lead to His → Gln substitutions at amino acids 112 and 159, respectively. Although different from the published mouse sequence, these residues correspond to the published human p68 sequence, which contains Gln at both positions (GenBank Accession No. NM_004396). Synthesis of full-length p68 from pcDNA-Xpress-p68 was demonstrated by in vitro translation (data not shown).

Cell culture, transfections, and luciferase reporter assay. Mink lung epithelial (Mv1Lu) cells were obtained from ATCC (Manassas, VA) and maintained in Opti-MEM (GIBCO, Gaithersburg, MD) supplemented with 5% heat-inactivated fetal bovine serum (Sigma, St. Louis,

MO) at 37 °C in 5% CO_2/95% air. For reporter assays, Mv1Lu cells were seeded the day before transfection at 1.5×10^5 cells per 35 mm tissue culture dish and were 50–70% confluent the following day, when transfections were performed. Cell cultures were transfected with plasmid DNAs (as below) using Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's recommendations. For reporter assays, 0.6 μg pGAL4$_2$-tk80-luc [15] and 0.05 μg pRL-CMV (Promega) were added to each cell culture. The control plasmid pRL-CMV was co-transfected in order to normalize for transfection efficiencies within individual experiments. In order to test for a functional interaction between p68, CBP, and Smad 3 proteins, the cell cultures were transfected with the two reporter plasmids and the following plasmids either singly or in combination: 1.0 μg pcDNA3-His/Xpress-p68; 0.1 μg pCMV-myc-Smad 3, and 0.2 μg pGal4-CBP$^{1891–2441}$ which encodes a fusion protein between the DNA binding domain of Gal4 and the C-terminus of CBP [16]. Sixteen hours post-transfection, cells were washed with fresh culture medium and stimulated with TGFβ_1 (1 ng/ml, R&D Systems, Minneapolis, MN) for 24 h. Cells were harvested, lysates were prepared, and luciferase activity was determined with the Dual Luciferase Reporter Assay System (Promega) according to the method detailed by the manufacturer. Reporter luciferase (firefly luciferase) activity was assayed and expressed relative to control luciferase (*Renilla* luciferase) activity for each sample. Each condition was assayed in triplicate and the experiment was performed two times with comparable results.

Co-immunoprecipitation of p68 and Smad 3. For immunoprecipitation studies, 1 μg each of pcDNA3-His/Xpress-p68 and pCMV-myc-Smad 3 was transfected into Mv1Lu cells as described above for the reporter assay, except that cells were grown in 60 mm tissue culture dishes. Cells were harvested 48 h post-transfection in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Lysates were prepared by brief sonication and clarified by centrifugation at 10,000*g* for 30 min. p68 was immunoprecipitated by incubation of the lysate with 2 μg/ml anti-Xpress monoclonal antibody (Invitrogen) for 1 h at 4 °C, followed by the addition of 15 μl protein A/G-agarose (50% slurry, Santa Cruz Biotechnology, Santa Cruz, CA) and subsequent overnight incubation at 4 °C with gentle rotation. Protein-bound agarose beads were washed three times with RIPA buffer and bound protein eluted by addition of 2× SDS–PAGE sample buffer [14] and heating to 90 °C for 5 min. The eluted material was analyzed by Western blotting with antibodies specific for the myc amino terminal tag of Smad 3 (BD Biosciences Clontech, Palo Alto, CA). Western blotting was performed as described previously [17]. Blots were reprobed with anti-Xpress antibodies to demonstrate immunoprecipitation of His/Xpress-p68.

Results

Yeast two-hybrid assay

To identify novel Smad binding proteins expressed in developing orofacial tissue, a yeast two-hybrid screen was employed using the MH2 domain of the TGFβ-regulated Smad 3 (in pGBKT7) as bait to screen a mouse embryonic orofacial cDNA expression library prepared in pGADT7. Of approximately 5×10^5 transformants from the initial screen, approximately 100 colonies proliferated on nutritionally restrictive medium (i.e., medium lacking histidine, tryptophan, adenine, and leucine) and expressed α-galactosidase as detected by the addition of X-α-gal. Redundant clones were identified by PCR and unique clones were purified, sequenced, and compared to sequences in GenBank with BLAST. Several novel Smad binding proteins were identified from this screen and have been reported previously [7–9]. One of the Smad binding proteins identified in this screen and not previously reported is the p68 RNA helicase (p68, gene designation, *Ddx5*). The isolated p68 cDNA clone was truncated at the 5′ end and encoded amino acids 348–614. Purified pGADT7-p68 was reintroduced into yeast along with pGBKT7-Smad 3 MH2, pGBKT$_7$-lamin C or pGBKT7 vector and plated onto medium without histidine, tryptophan, adenine, or leucine. These results are summarized in Table 1. These data confirmed that p68 interacted specifically with Smad 3 and not with lamin C, a negative control protein. Additionally, p68 did not confer colony growth when co-expressed with the bait vector (pGBKT7), thus demonstrating that p68 alone could not lead to colony growth in this assay. Likewise, Smad 3 was also unable to confer colony growth when co-expressed with the prey vector (pGADT7). Thus, colony formation only occurred in the presence of both Smad 3 and p68, demonstrating the specificity of the interaction.

Table 1
Summary of yeast two-hybrid screen

Co-transformants	Yeast two-hybrid interaction
pGBKT7-Smad 3 MH2 and pGADT7	−
pGBKT7-Smad 3 MH2 and pGADT7-p68	+
pGBKT7-lamin C and pGADT7-p68	−
pGBKT7 and pGADT7-p68	−

The indicated plasmids were co-transformed into *S. cerevisiae* AH109 and plated onto yeast medium in the absence of tryptophan, leucine, adenine, and histidine, and in the presence of X-α-gal as described in Materials and methods. The formation of colonies secreting X-α-gal was taken as an indication of an interaction between the expressed proteins and is noted by (+). No interaction between expressed proteins is noted with (−).

GST pull-down assay

To confirm the interaction between p68 and Smad 3, and to test for interaction with additional Smad proteins, a GST pull-down assay was conducted using various GST-Smad fusion proteins which were mixed with in vitro translated, [^{35}S]methionine-labeled p68. Both full-length Smads and the MH2 domains of each Smad 3 were tested individually for interaction with p68 (Fig. 1). p68 interacted with all Smads tested. The strongest interaction was noted between p68 and the MH2 domains of Smad 3, 4, and 7, while the interaction between p68 and their full-length counterparts was notably weaker (Fig. 1). Similar binding of Smads to p68 was observed between full-length Smads 1 and 2, and their respective isolated MH2 domains. A weak band, similar in intensity to that seen with GST alone, was observed with a mutant of Smad 3 missing the MH2 domain

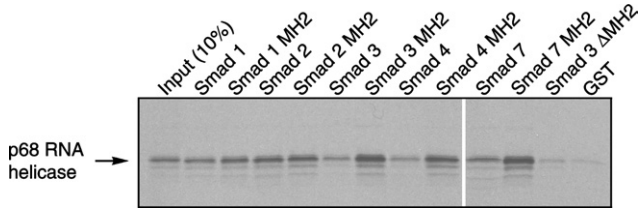

Fig. 1. GST pull-down assay demonstrating interaction between Smad and p68 RNA helicase. [^{35}S]Methionine-labeled, in vitro translated p68 was mixed with 1 μg bacterially expressed, purified GST-Smad as indicated above each lane. Protein complexes were isolated with glutathione–Sepharose and analyzed by SDS–PAGE and autoradiography. The lane labeled as "input" is 10% of the in vitro translation reaction added to each sample. The autoradiograms presented are representative of two independent experiments. Results confirm binding of p68 to Smad 3 MH2 and identify binding of p68 to additional Smads.

(Smad 3 ΔMH2). This most likely represents non-specific association of p68 with the Sepharose beads. Failure of p68 to bind Smad 3 ΔMH2 demonstrates that the binding site for p68 lies within the Smad MH2 domain.

Co-immunoprecipitation of p68 and Smad 3

To determine if full-length p68 and Smad interact in vivo, mouse p68 cDNA was amplified from first-strand cDNA prepared with embryonic orofacial RNA via PCR with p68-specific primers. The p68 cDNA was cloned into the expression vector, pcDNA-His/Xpress, resulting in a His/Xpress-tagged version of p68. To test for in vivo interaction between p68 and Smad, His/Xpress-p68, and myc-Smad 3 MH2 were co-transfected into Mv1Lu cells and co-immunoprecipitation assays were performed. Whole-cell lysates from transfected cells were incubated with anti-Xpress antibodies and the resulting immunoprecipitates were analyzed for myc-Smad 3 by Western blotting with anti-myc antibodies. As demonstrated in Fig. 2, Smad 3 co-precipitated with anti-Xpress antibodies, but not when His/Xpress-p68 was omitted from the transfection. These results establish that Smad 3 interacts with p68 in vivo. The anti-Xpress antibody efficiently precipitated His/Xpress-p68, as demonstrated by subsequent Western blotting with anti-Xpress antibodies (Fig. 2).

Previously, it was demonstrated that human p68 interacted with CBP in vivo [11] and that this interaction enhanced the transactivation potential of CBP [12]. Since Smad requires CBP for gene activation [2], the presence of CBP in the p68 immunoprecipitate was examined by Western blotting with CBP-specific antibodies. Neither CBP nor the closely related p300 was detected in the p68 immunoprecipitate (data not shown), demonstrating that, in contrast to previous reports [11,12], p68 does not bind CBP under the experimental conditions employed.

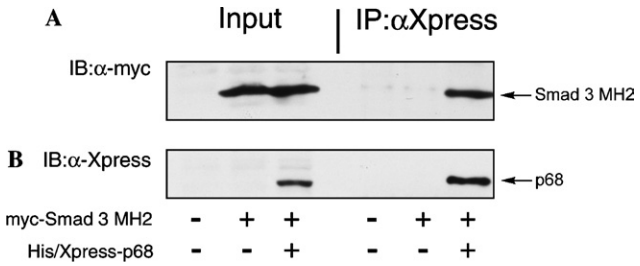

Fig. 2. In vivo interaction between Smad 3 and p68 RNA helicase. His/Xpress-tagged p68 RNA helicase and myc-tagged Smad 3 MH2 domain were cotransfected into Mv1Lu cells. Whole-cell lysates were prepared and subsequently immunoprecipitated with anti-Xpress antibodies and analyzed by Western blotting with anti-myc antibodies (A) and anti-Xpress antibodies (B). The samples marked "input" represent the signal from 5% of the amount of cell lysate used for each sample. His/Xpress-p68 was immunoprecipitated with anti-Xpress antibodies and myc-Smad 3 MH2 was co-precipitated only in the presence of His/Xpress-p68.

Functional interaction between p68 and Smad 3

p68 has been reported to enhance the transcriptional activity of CBP in reporter assays [12]. To determine if the interaction between p68 and Smad influences CBP-mediated gene transcription, a reporter assay was conducted using the DNA binding domain of Gal4 fused to the C-terminus of CBP (amino acids 1891–2441) and $GAL4_2$-tk80-luc, which contains 2 copies of the GAL4 consensus binding site controlling expression of firefly luciferase (Fig. 3). Little effect of p68 on Gal4-CBP-mediated activation of the reporter construct was observed, in contrast to the results reported by Rossow and Janknecht [12]. Overexpression of Smad 3, however, increased the luciferase activity when co-expressed with Gal4-CBP, but only in the presence of TGFβ, demonstrating that activation of Smad 3 via TGFβ leads to complex formation with CBP and transactivation. Overexpression of both Smad 3 and p68 led to a synergistic response compared to Smad 3 alone, suggesting that Smad 3 and p68 co-operate to enhance CBP-mediated transcriptional activation, but only after stimulation with TGFβ, suggesting that p68 is indeed a transcriptional cofactor for Smad-mediated signaling. The expression of both p68 and Smad was confirmed in this assay via Western blotting (data not shown).

Discussion

The TGFβ superfamily of secreted growth and differentiation factors controls diverse developmental processes such as embryonic axis determination, cellular proliferation/differentiation/apoptosis, and extracellular matrix production [1]. The role of TGFβs in adult tissues is underscored by the observation that the TGFβ signaling pathway is frequently dysregulated in tumors,

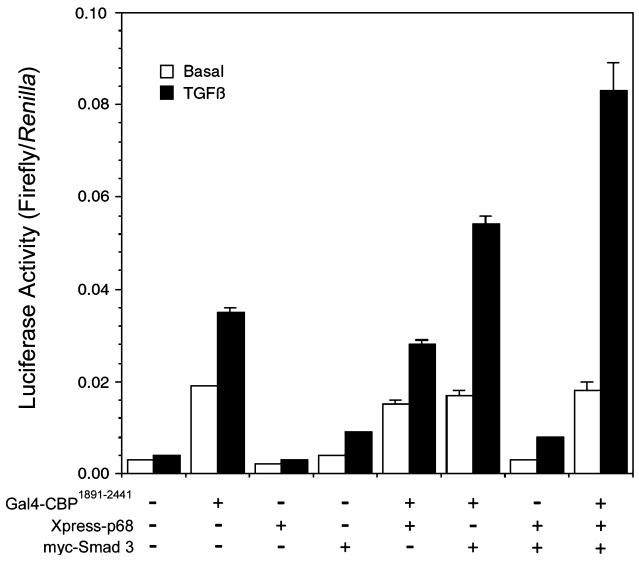

Fig. 3. Cooperation between p68, Smad, and CBP in transcriptional activation. Mv1Lu cells were transfected with the $Gal4_2$-tk80-Luc reporter plasmid containing two copies of the Gal4 DNA binding consensus sequence and various combinations of His/Xpress-p68, myc-Smad, and Gal4-$CBP^{1891–2441}$ as indicated. Twenty-four hours post-transfection, cells were stimulated with 1 ng/ml TGFβ and incubated for an additional 24 h. Luciferase activity from $Gal4_2$-tk80-Luc was detected only in the presence of Gal4-$CBP^{1891–2441}$. This activity was increased in each case by addition of TGFβ to the cell cultures. Overexpression of p68 had little effect on reporter activity, however, overexpression of Smad 3 led to enhanced TGFβ-induced activity, which was further stimulated in the presence of both Smad 3 and p68 ($P < 0.01$, one-way ANOVA).

leading to escape of the cells from the growth inhibitory action of TGFβ and tumor suppressors [18,19]. TGFβ's role in embryonic development has been demonstrated by gene ablation studies in which a crucial role for TGFβ2 and TGFβ3 in craniofacial development was noted [20–22]. More recently, a conditional knockout of the type II TGFβ receptor in Wnt-1-expressing embryonic cells led to various anomalies such as a complete cleft of the secondary palate and improper calvarial development [23].

TGFβ signaling through cell-surface receptors leads to phosphorylation of intracellular Smad proteins which heterodimerize and translocate to the nucleus where they act as transcriptional regulators by binding to promoters of target genes. Smads bind DNA through their conserved MH1 domains. They activate transcription, however, by recruiting additional proteins (e.g., CBP and p300) through their MH2 domains, or inhibit transcription via recruitment of nuclear co-repressors such as c-Ski and SnoN. The DNA binding specificity of the MH1 domain was demonstrated to encompass the core nucleotide sequence of GTCT [4]. Scanning of the genome reveals that this tetranucleotide sequence is found throughout. Clearly, additional regulatory mechanisms that confer promoter specificity to Smads must be active since binding to the GTCT consensus sequence alone would lead to widespread indiscriminate DNA binding by Smads. Therefore, it is unlikely that Smads solely are responsible for the pleiotropic effects of TGFβ. This notion has led to the search for additional, cell-specific proteins that bind to Smads and aid in the regulation of gene transcription. In several model systems, investigators have identified proteins that interact not only with Smads but also with the promoters of TGFβ target genes resulting in specific gene activation. For example, the proteins OAZ and FAST2 are required for Smad-dependent developmental processes by forming transcriptional complexes with Smads in the transcription of *Xvent-2* and *goosecoid*, respectively [5,6]. Yeast two-hybrid studies have identified proteins that bind to Smads, some in the role of transcriptional cofactors such as SIP1 [24] and others whose function in TGFβ signaling remains unclear such as Dishevelled, Erbin, and Par-3 [8]. In the current report, p68 RNA helicase was identified as a Smad binding protein using a yeast two-hybrid screen of an embryonic mouse orofacial cDNA expression library with Smad 3 as the bait.

p68 is a protein originally identified based on its cross-reactivity with an antibody to SV40 large T antigen [25]. p68 was subsequently cloned and revealed to have significant homology to eIF-4A, the prototypical member of the DEAD box family of ATP-dependent RNA helicases [10]. DEAD box proteins have a core of eight conserved amino acid motifs thought to be involved in RNA binding and unwinding, including the Asp-Glu-Ala-Asp (D-E-A-D) sequence for which the family is named. Homologs of p68 have been identified in yeast, chickens, frogs, humans, and mice, and display remarkable sequence conservation. For example, the human and mouse p68 proteins share 98% amino acid identity. Most DEAD box proteins possess an ATP-dependent RNA unwinding (helicase) activity and function in diverse processes such as transcription initiation [26,27], RNA splicing [28], ribosome biogenesis [29], and embryonic development [30]. Interestingly, Smad 3 has been demonstrated to interact with another protein involved in RNA splicing, Sf3b2 [7]. SF3b2 is involved in formation of the U2 snRNP complex, whereas p68 recently has been demonstrated to modulate dissociation of the U1 snRNP complex from the 5′ splice site [31].

p68 displays broad distribution in adult tissues but more restrictive patterns of expression in the developing embryo [30,32]. Of note, *Xenopus* migrating cranial neural crest displayed strong p68 expression, suggesting a role for p68 in neural crest migration and/or differentiation during craniofacial development [32]. However, this pattern of expression may not be widespread among vertebrates since p68 is only weakly expressed in chick cranial neural crest [32].

p68 binds to CBP in vivo leading to enhanced CBP-mediated transcription [11]. Rossow and Janknecht

[12] characterized the interaction between p68 and CBP, demonstrating that p68 preferentially binds the C-terminus of CBP and that p68 RNA helicase increased the transcriptional activity of CBP through a mechanism that required the ATPase activity of p68. In the studies detailed in the present report, no binding of CBP to p68 or activation of CBP by p68 was detected. However, a consistent effect of p68 on Smad/CBP-mediated transcriptional activity was observed, suggesting that p68 alone had no direct effect on CBP, but rather was part of a complex composed of Smad and CBP. Increased transcriptional activity was observed only when the TGFβ pathway was activated. Thus, p68 could be functioning as a transcriptional coactivator, along with CBP, to promote accessibility of the transcriptional machinery. Such a role is similar to that for the Snf/Swi family of proteins, which are related to RNA helicases.

It is unclear why p68 had no effect on CBP-mediated transcription (Fig. 3) or why a physical interaction between p68 and CBP was not detected. One explanation could result from cell-specific effects. p68 has been reported to demonstrate dramatic cell-specific differences in potentiating ERα AF-1 activity [11]. Alternatively, the His/Xpress tag fused to the amino terminus of p68 may alter its function by interfering either with nuclear translocation or interaction with other critical proteins. This is unlikely, however, since a myc-tagged version of human p68 displayed predominantly nuclear localization in Mv1Lu cells and an HA-tagged p68 demonstrated synergism with CBP in luciferase reporter assays [12].

In the present report, the interaction between Smad and p68 was confirmed through GST pull-down assays with GST fusion proteins of representative Smad family members. Interaction of p68 with additional Smad family members demonstrates a broad specificity of binding and suggests other pathways that may utilize p68 RNA helicase (e.g., BMPs).

In summary, results of the present study have identified p68 RNA helicase as a Smad 3 binding protein that leads to enhanced TGFβ signaling which is mediated by CBP. p68 is involved in diverse cellular activities and its ATP-dependent helicase activity is central to its function. Thus, the identification of p68 in the complex of transcription factors utilizing CBP suggests that control of nucleic acid secondary structure through p68 helicase activity, and control of chromatin structure through the histone acetylase activity of CBP, are important, and possibly coordinated, processes in TGFβ-dependent transcriptional activation.

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