Competition between Ski and CREB-binding Protein for Binding to Smad Proteins in Transforming Growth Factor-β Signaling*

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The family of Smad proteins mediates transforming growth factor-β (TGF-β) signaling in cell growth and differentiation. Smads repress or activate TGF-β signaling by interacting with corepressors (e.g. Ski) or coactivators (e.g. CREB-binding protein (CBP)), respectively. Specifically, Ski has been shown to interfere with the interaction between Smad3 and CBP. However, it is unclear whether Ski competes with CBP for binding to Smads and whether they can interact with Smad3 at the same binding surface on Smad3. We investigated the interactions among purified constructs of Smad, Ski, and CBP in vitro by size-exclusion chromatography, isothermal titration calorimetry, and mutational studies. Here, we show that Ski-(16–192) interacted directly with a homotrimer of receptor-regulated Smad protein (R-Smad), e.g. Smad2 or Smad3, to form a hexamer; Ski-(16–192) interacted with an R-Smad:Smad4 heterotrimer to form a pentamer. CBP-(1941–1992) was also found to interact directly with an R-Smad homotrimer to form a hexamer and with an R-Smad:Smad4 heterotrimer to form a pentamer. Moreover, these domains of Ski and CBP competed with each other for binding to Smad3. Our mutational studies revealed that domains of Ski and CBP interacted with Smad3 at a portion of the binding surface of the Smad anchor for receptor activation. Our results suggest that Ski negatively regulates TGF-β signaling by replacing CBP in R-Smad complexes. Our working model suggests that Smad protein activity is delicately balanced by Ski and CBP in the TGF-β pathway.

Transforming growth factor-β (TGF-β) is a member of a large family of cytokines that regulate a broad range of cellular functions, including cell growth, production of extracellular matrix components, differentiation, and apoptosis (1, 2). TGF-β signaling from the cell membrane to the nucleus is mediated by the Smad family of proteins, which are receptor-regulated Smad (R-Smad) and common Smad proteins (3–5). R-Smads are phosphorylated at the C-terminal SXS sequence by TGF-β type I receptor kinases (6, 7). For example, Smad2 and Smad3 are R-Smads that are phosphorylated by activin/TGF-β receptors. The common Smad protein Smad4 serves as a signaling partner to R-Smads. Phosphorylated R-Smad forms a functional signaling complex with Smad4 and then translocates into the nucleus to modulate expression of ligand-responsive genes. Structural analysis has shown that this functional signaling complex consists of two R-Smad subunits and one Smad4 subunit (Fig. 1A) (8).

Because of its critical role in determining cell fate, TGF-β signaling is subject to many levels of negative and positive regulation through both receptors and intracellular mediators (1, 9). As a negative regulator of Smad function, the nuclear oncoprotein Ski represses TGF-β signaling through interactions with Smad proteins (10–12). Human Ski is a 728-amino acid protein, and its N-terminal region is the most conserved region between Ski and its related gene product SnoN. Separate regions within Ski have been implicated in binding to Smad4 (amino acids 219–312) (13) and R-Smads (amino acids 17–45 and 125–131) (14–16). Biochemical analyses by Wu et al. (13) have shown that one domain of Ski (amino acids 17–212) interacts with Smad2 and that another domain of Ski (amino acids 200–323) interacts with Smad4; structural evidence from this group indicates that Ski-(219–312) binds to the L3 loop of Smad4 (13). Biochemical analyses by Ueki and Hayman (15) also demonstrated that Ski can use different domains to interact with both Smad3 and Smad4. Our previous structural analysis showed that a domain of Ski (amino acids 17–45) interacts with Smad3 at part of its Smad anchor for receptor activation (SARA)-binding surface (Fig. 1B) (16). SARA recruits Smad3 for phosphorylation by the receptor kinase (17). Upon phosphorylation, Smad3 dissociates from SARA and enters the nucleus, where its transcriptional activity can be repressed by Ski (16).
As positive regulators of Smad function, the transcriptional coactivators CREB-binding protein (CBP) and p300 are important components of TGF-β signaling and mediate the anti-oncogenic functions of Smad2 and Smad4 (18). These Smad proteins directly interact with the nuclear coactivator CBP/p300 to activate the transcription of TGF-β-responsive genes (18–21). The MH2 domain of R-Smads can interact with the C-terminal domain (amino acids 1891–2175) of CBP (18), and CBP directly acetylates the MH2 domain of Smad3 at Lys378 to positively regulate its transactivation activity (22).

Ski has been shown to interfere with the interaction between Smad3 and CBP in vivo (12). However, the interactions among Smad, Ski, and CBP have not been completely characterized. It is unclear whether Ski-(16–192) or CBP-(1941–1992) can interact with Smad3 at the SARA-binding surface and whether there is direct competition between CBP and Ski for binding to Smads.

To resolve the stoichiometric identity of Ski and CBP for binding to Smads and to provide insights into the regulation of TGF-β signaling, size-exclusion chromatography and isothermal titration calorimetry were used in vitro interactive assays to measure the binding of purified Ski-(16–192) and CBP-(1941–1992) to Smads. Here, we present biochemical and calorimetric evidence that Ski and CBP directly interact with R-Smad and that Ski competes with CBP for binding to Smad.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Mutagenesis**—The cDNA of human Ski-(16–192) was generated by PCR and subcloned into the pGEX-6P-1 vector (Amersham Biosciences). The Ski protein was expressed with a glutathione S-transferase (GST) tag, extracted with glutathione–Sepharose, and released with PreScission protease (GE Healthcare). Eluted protein was dialyzed in DEAE buffer (20 mM Tris (pH 7.3), 10 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride). Protein was purified using a DEAE-Sepharose column equilibrated with DEAE buffer and eluted with a gradient of 10–300 mM NaCl. Site-directed mutants of Ski-(16–192), and S3LC-(145–424) were generated using the QuickChange kit (Stratagene) and confirmed by sequencing.

The cDNAs of CBP-(1911–1992), CBP-(1931–1992), and CBP-(1941–1992) were generated by PCR and subcloned into the pGEX-6P-1 vector. The CBPs were expressed with a GST tag (GST-CBP-(1911–1992), GST-CBP-(1931–1992), and GST-CBP-(1941–1992)) and extracted with glutathione–Sepharose. Eluted proteins were dialyzed in DEAE buffer. Proteins were purified using an SP-Sepharose column equilibrated with DEAE buffer and eluted with a gradient of 10–300 mM NaCl.

The phosphorylated Smad2 and Smad3 constructs used to analyze Smad/Ski interactions in this study include their linker and MH2 domains. The phosphorylated Smad2 construct extends from residues 186 to 467 and is referred to as S2LC-2P. The phosphorylated Smad3 construct extends from residues 145 to 425 and is referred to as S3LC-2P. The Smad4 construct comprises its MH2 domain and part of its linker domain, extending from residues 273 to 552, and is referred to as S4AF (23). S2LC-2P and S3LC-2P were prepared using the intein-mediated phosphopeptide ligation method as described previously (16, 24). S4AF was purified as described previously (23). The Smad heteromeric S4AF/S3LC-2P complexes and S4AF/S2LC-2P were purified as described previously (8).

To form the heteromeric complexes S2LC-2P-Ski-(16–192) and S3LC-2P-Ski-(16–192) or S2LC-2P-GST-CBP-(1941–1992) and S3LC-2P-GST-CBP-(1941–1992), Ski-(16–192) or GST-CBP-(1941–1992) was added in 2-fold molar excess to S2LC-2P or S3LC-2P, respectively, at 4 °C for 24 h. Each heteromeric complex (S2LC-2P-Ski-(16–192), S3LC-2P-Ski-(16–192), S2LC-2P-GST-CBP-(1941–1992), or S3LC-2P-GST-CBP-(1941–1992)) was purified on a Superdex 200 HR 16/60 size-exclusion column (GE Healthcare) by fast protein liquid chromatography (FPLC). Uncomplexed Ski-(16–192) and GST-CBP-(1941–1992) were eluted in fractions 31–34 and 27–31, whereas the heteromeric complexes S2LC-2P-Ski-(16–192), S3LC-2P-Ski-(16–192), S2LC-2P-GST-CBP-(1941–1992), and S3LC-2P-GST-CBP-(1941–1992) eluted in fractions 22–25.

**Isothermal Titration Calorimetry**—To measure the binding of purified Ski-(16–192) and GST-CBP-(1941–1992) with Smads, isothermal titration calorimetry (ITC) was performed (25) using a VP-ITC calorimeter (MicroCal, LLC, Northampton, MA). All samples were dialyzed against ITC buffer (20 mM HEPES (pH 7.3), 100 mM NaCl, 0.1 mM EDTA, and 1 mM tris(2-carboxyethyl)phosphine) and degassed prior to titration. Experiments were performed at 15 °C. Isothermal interactions between proteins were measured by titrating over 55 injections using 1.4 ml of 40 μM protein/complex against 300 μl of 400 μM Ski-(16–192) or GST-CBP-(1941–1992). The proteins/comp-
plexes tested were phosphorylated Smad2, Smad3, the heterotrimer of S2LC-S4AF, and the heterotrimer of S3LC-S4AF. The heats of dilution for Ski-(16–192) or GST-CBP-(1941–1992) were measured by titrating Ski-(16–192) or GST-CBP-(1941–1992) into ITC buffer; these values were subtracted for data analysis. Data were analyzed with Origin 7.0 software (MicroCal, LLC) using a single-site binding model. ΔH, ΔS, and Kₐ values were determined experimentally, and ΔG was calculated from the following equation: ΔG = −RT ln Kₐ.

Size-exclusion Chromatography—To obtain the stoichiometry of Ski and CBP for binding to Smads, size-exclusion chromatography was performed on a Superdex 200 HR 10/30 column using the ÄKTA Explorer 10 FPLC system (GE Healthcare). All runs were performed at room temperature in FPLC buffer (20 mM HEPES (pH 7.3), 0.1 mM EDTA, 100 mM NaCl, and 1 mM dithiothreitol). Prior to loading onto the column, protein samples were incubated in 1 mM tris(2-carboxyethyl)phosphine for 60 min at room temperature. FPLC operation and data analysis were done with UNICORN software. The column was calibrated with blue dextran (to determine the void volume) and molecular mass standards (ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), and ferritin (440 kDa)). Elution fractions (0.5 ml) were collected at room temperature at a flow rate of 0.5 ml/min.

GST Pulldown Assay—The GST fusion form of a protein was immobilized onto glutathione-Sepharose beads, which were then cleaned with wash buffer (20 mM Tris (pH 7.4), 0.1 mM EDTA, 10 mM NaCl, and 1 mM dithiothreitol). A second protein purified without a GST tag was then added to the beads and incubated at 4 °C for 1 h. The beads were washed again and analyzed by SDS-PAGE, and the gels were stained with Coomassie Blue. The standard conditions were as follows: 100 µl of beads, 100 µg of GST fusion protein, and 200 µg of binding partner. Unless stated otherwise, the beads were washed six times (each time with 1 ml of wash buffer), rapidly mixed, and centrifuged, and the buffer was removed.

RESULTS

Homotrimeric S2LC-2P or S3LC-2P Interacts with Ski to Form a Heterohexamer—To determine whether Smads and Ski-(16–192) interact directly in the purified state, S2LC-2P (phosphorylated Smad2-(186–467)) or S3LC-2P (phosphorylated Smad3-(145–425)) and Ski-(16–192) were incubated overnight at 4 °C and passed over a size-exclusion column. Ski-(16–192) was found to interact directly with S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 2, A and B). When mixed at a 2:1 molar ratio, a significant portion of Ski-(16–192) co-eluted in the same fractions as the heteromeric S3LC-2P-S4AF or S2LC-2P-S4AF complex. The remaining uncomplexed Ski-(16–192) also eluted in a monomeric form. The elution peak of the S2LC-2P-S4AF-Ski-(16–192) or S3LC-2P-S4AF-Ski-(16–192) complex shifted 1.0 fraction forward relative to the peak of trimERIC S2LC-2P-S4AF or S3LC-2P-S4AF, respectively.

Likewise, to further determine the interactions between heteromeric R-Smad-Smad4 and Ski-(16–192), ITC was used to analyze the thermodynamics and stoichiometry of heteromeric R-Smad-Smad4-Ski-(16–192) complex formation. The ITC results are shown in Fig. 3C and Table 1. Ski-(16–192) titrated into both S2LC-2P-S4AF and S3LC-2P-S4AF at a relative molar ratio of 0.67, meaning that two molecules of Ski-(16–192) can interact with three molecules of S2LC-2P-S4AF or S3LC-2P-S4AF. This result suggests that Ski-(16–192) interacts only with the subunits of S2LC-2P or S3LC-2P in the R-Smad-Smad4 heterotramer.

Again, we found that the affinities of S2LC-2P-S4AF and S3LC-2P-S4AF for Ski-(16–192) were very close. The apparent Kₐ values for S2LC-2P-S4AF-Ski-(16–192) and S3LC-2P-S4AF-Ski-(16–192) were 1.5 and 1.6 µM, respectively, corresponding to ΔG values for complex formation of −7.7 kcal/mol, respectively (Table 1).

Ski-(16–192) interacted with S2LC-2P or S3LC-2P as shown by co-elution with an apparent molecular mass larger than that of S2LC-2P or S3LC-2P alone (Fig. 2, A and B). Ski-(16–192) also interacted with the S2LC-2P-S4AF or S3LC-2P-S4AF heterotrimer as shown by co-elution of all three species with an apparent molecular mass larger than that of the trimERIC S2LC-2P-S4AF or S3LC-2P-S4AF complex (Fig. 3, A and B). To confirm that Ski-(16–192) does not interact with Smad4, size-exclusion chromatography and ITC experiments were also carried out. The results suggest that Smad4 alone or within the
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(A) [Graph showing peak analysis with labels 158 K, 67 K, 43 K, 25 K, and S2LC-2P/Ski on the X-axis with Y-axis ranging from 0 to 1600 mAU (280 nm).]

(B) [Graph showing band analysis with labels 45 K, 31 K, 21 K, and S2LC-2P/Ski on the X-axis with Y-axis ranging from 0 to 1600 mAU (280 nm).]

(C) [Graph showing calorimetric analysis with labels S2LC-2P ↔ Ski on the X-axis with Time (min) ranging from -100 to 900 and Y-axis with µcal/sec ranging from -1.2 to 0.2, and kcal/mole of injectant ranging from -10 to -1.]

(D) [Diagram showing structure of Ski, Smad2/3, and Smad2/3 phosphorylated forms with an arrow indicating the change.]

References and Further Details:

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[Additional scientific data and methodologies may be included in the full text document.]
heterotrimeric Smad complex does not interact with Ski-(16–192). This lack of interaction is supported by five observations. First, S4AF and Ski did not form a complex on the size-exclusion column (data not shown). Second, Ski-(16–192) did not interact with Smad4 upon ITC analysis (data not shown). Third, the S2LC-2P-Ski-(16–192) or S3LC-2P-Ski-(16–192) complex eluted with an apparent molecular mass larger than that of the S2LC-2P-S4AF-Ski-(16–192) or S3LC-2P-S4AF-Ski-(16–192) complex (Figs. 2A and 3A). Fourth, the molar ratio of Ski-(16–192) titrated into S2LC-2P-S4AF or S3LC-2P-S4AF was smaller than that of Ski-(16–192) titrated into S2LC-2P or S3LC-2P. Fifth, the $K_d$ for the S2LC-2P-S4AF-Ski-(16–192) or S3LC-2P-S4AF-Ski-(16–192) complex was greater than that for the S2LC-2P-Ski-(16–192) or S3LC-2P-Ski-(16–192) complex, respectively (Table 1). This result is consistent with a previous report that the Smad4/Ski interaction involves a region of Ski (residues 219–312) other than another region of Ski similar to that used in this study (residues 17–192) (13). Taken together, these results suggest that the complex is a heteropentamer with a preferred stoichiometry of two subunits of S2LC-2P or S3LC-2P, two subunits of Ski-(16–192), and one subunit of S4AF (Fig. 3D).

The analysis above showed that Ski-(16–192) bound to homotrimeric S2LC-2P and S3LC-2P as well as to heterotrimeric S2LC-2P-S4AF and S3LC-2P-S4AF. To compare the binding of Ski and CBP to Smads, the same conditions used for Ski-(16–192) were used for CBP-(1941–1992) to test its interaction with Smads.

A previous study showed that the MH2 domain of R-Smads can interact with the C-terminal domain (amino acids 1891–2175) of CBP (18). Furthermore, Wu et al. (13) identified a 26-residue sequence motif of CBP/p300 (amino acids 1955–1980) that is both necessary and sufficient to form a stable complex with R-Smads. To confirm these results, we carried out in vitro binding assays using CBP-(1911–1992), CBP-(1931–1992), and CBP-(1941–1992). An in vitro GST-mediated pulldown assay was used to confirm that CBP-(1941–1992) is sufficient to format a stable complex with the S3LC-2P homotrimer and the S3LC-2P-S4AF heterotrimer (data not shown).

Because CBP-(1941–1992) was unstable, GST was required to stabilize it. Before testing the interaction of GST-CBP-(1941–1992) with Smads, it was necessary to test whether GST alone interacts with Smad2. Our results show that GST did not interact with Smad2 and Smad3 (Fig. 4B, upper panels). To further investigate whether Smads and GST-CBP-(1941–1992) interact directly in the purified state, size-exclusion chromatography and ITC were performed. GST-CBP-(1941–1992) interacted directly with S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 4, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted in the same fractions as trimeric S2LC-2P or S3LC-2P. The remaining uncomplexed GST-CBP-(1941–1992) eluted in a dimeric form, presumably through GST dimerization. The elution peak of the S2LC-2P-GST-CBP-(1941–1992) or S3LC-2P-GST-CBP-(1941–1992) complex shifted 2.0 fractions forward relative to the peak of trimeric S2LC-2P or S3LC-2P, respectively. The ITC results for formation of the heteromeric Smad-GST-CBP-(1941–1992) complex are shown in Fig. 4C and Table 1. GST-CBP-(1941–1992) co-eluted into both S2LC-2P and S3LC-2P at a relative molar ratio of 1.0, meaning that a single molecule of GST-CBP-(1941–1992) can interact with one molecule of S2LC-2P or S3LC-2P. Thus, these results suggest that the heteromeric Smad-GST-CBP-(1941–1992) complex is a heterohexamer with a preferred stoichiometry of three subunits of S3LC-2P and three subunits of GST-CBP-(1941–1992) (Fig. 4D).

Similar to their binding to Ski-(16–192), S2LC-2P and S3LC-2P had almost equal affinities for CBP-(1941–1992), but these affinities were slightly weaker than those for Ski-(16–192). The apparent $K_d$ values for S2LC-2P-GST-CBP-(1941–1992) and S3LC-2P-GST-CBP-(1941–1992) were 1.4 and 1.3 $\mu M$, respectively, corresponding to $\Delta G$ values for complex formation of $-7.7$ and $-7.8$ kcal/mol, respectively (Table 1).

The heterotrimeric S2LC-2P-S4AF or S3LC-2P-S4AF complex interacts with GST to form a heterohexameter—GST-CBP-(1941–1992) also interacted directly with the S3LC-2P-S4AF or S2LC-2P-S4AF complex as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B). When mixed at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted with trimeric S2LC-2P or S3LC-2P as judged by co-elution on a size-exclusion column (Fig. 5, A and B).
FIGURE 3. Ski interacts with the S3LC-2P-S4AF or S2LC-2P-S4AF complex. A, the elution peak of the S2LC-2P-S4AF-Ski (left panel) or S3LC-2P-S4AF-Ski (right) complex shifted 1.0 fraction forward relative to the peak of trimeric S2LC-2P-S4AF or S3LC-2P-S4AF, respectively. Interactions between Ski-(16–192) and S2LC-2P-S4AF or S3LC-2P-S4AF are shown by their elution profiles upon size-exclusion chromatography. Ski-(16–192) and S2LC-2P-S4AF or S3LC-2P-S4AF were loaded as 2:1 molar mixtures onto a size-exclusion column, and their interactions are indicated by co-elution as a heteromeric complex with a peak around fraction 14. Excess uncomplexed Ski-(16–192) eluted as a monomer with a peak around fraction 21. mAU, milli-absorbance units. B, Ski co-eluted with trimeric S2LC-2P-S4AF (left panels) or S3LC-2P-S4AF (right panels). Constructs (indicated to the right of each panel) were injected into a size-exclusion column, and eluted fractions were analyzed by SDS-PAGE. Molecular mass standards are shown above and to the left; fraction numbers are shown below. Gels were stained with Coomassie Blue. C, Ski titrated into both S2LC-2P-S4AF and S3LC-2P-S4AF at a relative molar ratio of 0.67. ITC analyses are shown for the interaction between S3LC-2P-S4AF and Ski-(16–192) and between S2LC-2P-S4AF and Ski-(16–192). D, Ski interacted with the heterotrimer of Smad2-Smad4 or Smad3-Smad4 to form a heteropentamer of Smad2-Smad4-Ski or Smad3-Smad4-Ski.
in the same fractions as heteromeric S3LC-2P-S4AF or S2LC-2P-S4AF. The remaining uncomplexed GST-CBP-(1941–1992) eluted in a dimeric form. The elution peak of the S2LC-2P-S4AF-GST-CBP-(1941–1992) or S3LC-2P-S4AF-GST-CBP-(1941–1992) complex shifted 1.6 fractions forward relative to the peak of heterotrameric S3LC-2P-S4AF or S2LC-2P-S4AF, respectively.

GST-CBP-(1941–1992) alone eluted as a dimer, and GST-CBP did not directly interact with Sma(d4 (data not shown). GST-CBP-(1941–1992) interacted with S2LC-2P or S3LC-2P as shown by co-elution with an apparent molecular mass larger than that of S2LC-2P or S3LC-2P alone (Figs. 4, A and B). GST-CBP-(1941–1992) also interacted with the S2LC-2P-S4AF or S3LC-2P-S4AF heterotrimer as shown by co-elution of all three species with an apparent molecular mass larger than that of the trimeric S2LC-2P-S4AF or S3LC-2P-S4AF complex (Figs. 5, A and B). Only two bands were seen in all three species of proteins (Fig. 5B, lower panels) because the molecular mass of S4AF is almost equal to that of GST-CBP-(1941–1992). Three observations suggest that Sma(d4 alone or within the heterotrimeric Smad complex does not interact with GST-CBP-(1941–1992) (Fig. 5B). First, S4AF and GST-CBP-(1941–1992) did not form a complex on the size-exclusion column (data not shown). Second, GST-CBP-(1941–1992) did not interact with Sma(d4 upon ITC analysis (data not shown). Third, the S2LC-2P-GST-CBP-(1941–1992) or S3LC-2P-GST-CBP-(1941–1992) complex eluted with an apparent molecular mass larger than that of the S2LC-2P-S4AF-GST-CBP-(1941–1992) or S3LC-2P-S4AF-GST-CBP-(1941–1992) complex, respectively (Figs. 4A and 5A). Again, taken together, the results suggest that the complex is a heteropentamer with a preferred stoichiometry of two subunits of S2LC-2P or S3LC-2P, two subunits of GST-CBP-(1941–1992), and one subunit of S4AF (Fig. 5C).

Ski Competes with GST-CBP for Binding to S3LC-2P—The above experiments showed that Ski-(16–192) and GST-CBP-(1941–1992) both directly interacted with homotrimeric S2LC-2P and S3LC-2P as well as with heterotrimeric S2LC-2P-S4AF and S3LC-2P-S4AF. The binding affinity of Ski-(16–192) for Smad was slightly stronger than that of GST-CBP-(1941–1992) for Smad (Table 1). To further investigate whether Ski-(16–192) can directly compete with GST-CBP-(1941–1992) for binding to Smad, in vitro binding assays were carried out with the S3LC-2P-GST-CBP-(1941–1992) and S3LC-2P-Ski-(16–192) complexes.

Ski-(16–192) interacted directly with the S3LC-2P-GST-CBP-(1941–1992) complex and replaced GST-CBP-(1941–1992) as judged by co-elution on a size-exclusion column (Fig. 6, upper left panels). When incubated overnight at 4 °C at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted in the same fractions as heteromeric S3LC-2P-Ski-(16–192). The remaining uncomplexed GST-CBP-(1941–1992) eluted in a dimeric form (elution profile not shown). Analysis of eluted fractions by SDS-PAGE indicated that two subunits of GST-CBP-(1941–1992) were replaced by two subunits of Ski-(16–192) in the heterohexamer of Smad3-CBP (Fig. 6, upper right panel). In turn, GST-CBP-(1941–1992) interacted directly with the S3LC-2P-Ski-(16–192) complex and replaced Ski-(16–192) as judged by co-elution on a size-exclusion column (Fig. 6, lower left panels). When mixed overnight at 4 °C at a 2:1 molar ratio, a significant portion of GST-CBP-(1941–1992) co-eluted in the same fractions as heteromeric S3LC-2P-Ski-(16–192). The remaining uncomplexed GST-CBP-(1941–1992) eluted in a dimeric form. Ski-(16–192) that dissociated from the S3LC-2P-Ski-(16–192) complex eluted in a monomeric form (elution profile not shown). Gel analysis of fractions indicated that two subunits of Ski-(16–192) were replaced by two subunits of GST-CBP-(1941–1992) in the heterohexamer of Smad3-Ski (Fig. 6, lower right panel).

In addition, purified Ski-(16–192) did not disrupt the purified complex of S3LC-2P-S4AF or S2LC-2P-S4AF in this study. To exclude effects of other cellular components in cell extracts, purified Ski-(16–192) was tested to determine whether it directly disrupts the purified complex of R-Smad-Sma(d4. S4AF interacted directly with the S2LC-2P-Ski-(16–192) or S3LC-2P-Ski-(16–192) complex and formed a heteromeric S2LC-2P-S4AF-Ski-(16–192) or S3LC-2P-S4AF-Ski-(16–192) complex as judged by co-elution on a size-exclusion column (Fig. 7A). When S4AF and S2LC-2P-Ski-(16–192) or S3LC-2P-Ski-(16–192) were mixed overnight at 4 °C at a 4:1 molar ratio, a significant portion of S4AF co-eluted in the same fractions as the heteromeric S2LC-2P-Ski-(16–192) or S3LC-2P-Ski-(16–192) complex. The remaining uncomplexed S4AF eluted in a monomeric form. Ski-(16–192) that dissociated from the S3LC-2P-Ski-(16–192) complex also eluted in a monomeric form (elution profile not shown). When S4AF replaced one subunit of homotrimeric S2LC-2P, one-third of Ski-(16–192) dissociated from this complex because S4AF did not interact with Ski-(16–192). Our results show that when Ski-(16–192) was used to directly interact with the R-Smad-Sma(d4 complex (Fig. 3, A and B) or when Sma(d4 was used to directly interact with the R-Smad-Ski complex (Fig. 7A), the R-Smad-Sma(d4 complex was stable.

Ski and CBP Interact with Sma(d3 at a Portion of the SARA-binding Surface—Our results described above show that Ski-(16–192) competed with GST-CBP-(1941–1992) for binding to S2LC-2P and S3LC-2P. To investigate whether Ski-(16–192) and GST-CBP-(1941–1992) can interact with Sma(d3 at the same binding site, binding experiments were carried out with site-specific mutations of Sma(d3. Our results show that Ski-(16–192) interacted with S3LC at the SARA-binding surface (Fig. 7B, left panel), consistent with a previous report that Ski-(17–45) interacts with Sma(d3 at the SARA-binding surface (16). Site-specific mutations of several Sma(d-binding domain (SBD)-binding residues in S3LC abolished or reduced its interactions with Ski-(16–192). These residues included Trp385 and Tyr725, which bind the β structure of SBD, as well as Phe383 and Tyr733, which bind the helical structure of SBD (Fig. 1B). However, the binding determinants for SARA and Ski are not identical, as mutating Asn383 and Val723, which participate in SBD binding, had no effect on Ski-(16–192) interactions. This result is consistent with a previous study in which these mutations were analyzed using GST-Ski-(17–45) (16). Meanwhile, CBP-(1941–1992) also interacted with S3LC at a portion of the SARA-binding surface (Fig. 7B, right panel). Site-specific mutations of two SBD-binding residues in S3LC, Trp385 and Tyr735, abolished or reduced its interaction with CBP-(1941–
Ski and CBP Compete for Binding to Smad Proteins

A

B

C

D

S2LC-2P → GST-CBP

S3LC-2P → GST-CBP

158 K 67 K 43 K

Fraction: 11 12 13 14 15 16 17 18 19 20 21 22 23

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1992). However, the binding determinants for Ski and CBP are not identical either, as mutations of Phe<sup>303</sup> and Tyr<sup>323</sup>, which abolished or reduced Ski-(16–192) interactions, had no effect on CBP-(1941–1992) interactions.

In addition, hydrophobic residues in Ski were involved in contact with Smad3 (Fig. 7C). Mutations of Leu<sup>21</sup> or Phe<sup>24</sup> in Ski abolished its interaction with S3LC or S3LC-2P, and mutation of Leu<sup>26</sup> dramatically reduced its interaction with S3LC-2P because the K<sub>d</sub> was 3-fold greater than that of the wild-type protein (Table 1).

Several point mutations of Lys<sup>19</sup>, Glu<sup>22</sup>, Leu<sup>26</sup>, Phe<sup>38</sup>, Ile<sup>126</sup>, Leu<sup>127</sup>, or Leu<sup>131</sup> in Ski had no significant effect on its interaction with S3LC or S3LC-2P.

**FIGURE 4.** GST-CBP interacts with S2LC-2P or S2LC-2P. A, the elution peak of the S2LC-2P-S4AF-GST-CBP (left panel) or S3LC-2P-S4AF-GST-CBP (right panel) complex shifted 1.6 fractions forward relative to the peak of trimeric S2LC-2P-S4AF or S3LC-2P-S4AF, respectively. Interactions between GST-CBP-(1941–1992) and S2LC-2P-S4AF or S3LC-2P-S4AF are shown by their elution profiles upon size-exclusion chromatography. GST-CBP-(1941–1992) and S2LC-2P-S4AF or S3LC-2P-S4AF were loaded as 2:1 molar mixtures onto a size-exclusion column, and their interactions are indicated by co-elution as a heteromeric complex with a peak around fraction 13. Excess uncomplexed GST-CBP-(1941–1992) eluted as a dimer with a peak around fraction 17. mAU, milli-absorbance units. B, GST-CBP co-eluted with trimeric S2LC-2P-S4AF (left panels) or S3LC-2P-S4AF (right panels). Constructs (indicated to the right of each panel) were injected into the size-exclusion column, and eluted fractions were analyzed by SDS-PAGE. Molecular mass standards are shown above and to the left; fraction numbers are shown below. Gels were stained with Coomassie Blue. C, CBP interacted with the heterotrimmer of Smad2-Smad4 or Smad3-Smad4 to form a heteropentamer with a peak around fraction 13. Excess uncomplexed GST-CBP-(1941–1992) eluted as a dimer with a peak around fraction 17. mAU, milli-absorbance units. D, CBP interacted with the homotrimer of Smad2 or Smad3-Smad4 to form a heterohexamer of Smad2-Smad4-CBP or Smad3-Smad4-CBP.

**FIGURE 5.** GST-CBP interacts with the S3LC-2P-S4AF or S2LC-2P-S4AF complex. A, the elution peak of the S2LC-2P-S4AF-GST-CBP (left panel) or S3LC-2P-S4AF-GST-CBP (right panel) complex shifted 1.6 fractions forward relative to the peak of trimeric S2LC-2P-S4AF or S3LC-2P-S4AF, respectively. Interactions between GST-CBP-(1941–1992) and S2LC-2P-S4AF or S3LC-2P-S4AF are shown by their elution profiles upon size-exclusion chromatography. GST-CBP-(1941–1992) and S2LC-2P-S4AF or S3LC-2P-S4AF were loaded as 2:1 molar mixtures onto a size-exclusion column, and their interactions are indicated by co-elution as a heteromeric complex with a peak around fraction 13. Excess uncomplexed GST-CBP-(1941–1992) eluted as a dimer with a peak around fraction 17. mAU, milli-absorbance units. B, GST-CBP co-eluted with trimeric S2LC-2P-S4AF (left panels) or S3LC-2P-S4AF (right panels). Constructs (indicated to the right of each panel) were injected into the size-exclusion column, and eluted fractions were analyzed by SDS-PAGE. Molecular mass standards are shown above and to the left; fraction numbers are shown below. Gels were stained with Coomassie Blue. C, CBP interacted with the heterotrimer of Smad2/Smad4 to form a heterohexamer.
Here, we have shown that both CBP and Ski directly interact with R-Smad proteins with slightly different affinities. These results are the first direct stoichiometric evidence from ITC and size-exclusion chromatography for interactions among Smad, CBP, and Ski. Taken together with previous reports (2, 6–10, 16, 18, 20, 26–28), the present evidence suggests a model of Smad protein regulation by Ski and CBP in the TGF-β pathway (Fig. 8). Upon phosphorylation at its C-terminal serines by the activated type I TGF-β receptor kinase, Smad2 or Smad3 is induced to form a homotrimer, which interacts with Smad4 to form a functional signaling heterotrimer that translocates into the nucleus. CBP directly interacts with R-Smad-Smad4 to activate the transcription of TGF-β-responsive genes, whereas Ski directly interacts with R-Smad-Smad4 to repress the transcription of TGF-β-responsive genes. Moreover, Ski can repress TGF-β signaling activity by displacing CBP from R-Smads. Similarly, CBP can activate TGF-β signaling activity by displacing Ski from R-Smads.

Regulation of Smad proteins in the TGF-β signaling pathway can be controlled at many levels such as by coactivators/corepressors and by cell type. Because the affinities of Smad for Ski and CBP are almost equal (Table 1), the transcriptional activity of Smads in the nucleus is delicately balanced by steady-state levels of Ski and CBP. Moreover, the levels of Ski and CBP depend on cell-type specificity because Smad coactivators or corepressors may vary among different cell types (2). Ski is ubiquitously expressed in virtually all adult and embryonic tissues at low levels, but its expression is increased in many human tumor cells (29). Ski may play an important role in binding to Smads to depress TGF-β signaling activity in tumor cells. These observations are consistent with the finding that the recruitment of transcriptional coactivators or corepressors to target genes in TGF-β signals depends on cell type-specific partner proteins (30).
Ski and CBP Compete for Binding to Smad Proteins

To interact with R-Smads, Ski and CBP compete for similar binding sites. The MH2 domain of Smad3 is a versatile protein/protein interaction module. At the receptor complex, the MH2 domain of Smad3 interacts with SARA and the receptor kinase (17, 31–36). In the nucleus, the MH2 domain of Smad3 further interacts with Ski and CBP (11, 16, 18, 21, 22, 37). In this study, we therefore evaluated how the residues that bind SARA in the MH2 domain of Smad3 affect the interaction between Smad3 and Ski or CBP. Trp405 was identified in the MH2 domain of Smad3 as a key residue for interactions with both Ski and CBP (Fig. 7B). Trp405 resides in the conserved H5 helix of Smad3, which binds the β structure of SBD in SARA (Fig. 1B) (16), and is fully conserved among most Smads (23). Phe303 was also identified in the MH2 domain of Smad3 as a key residue for interaction with Ski, but not for interaction with CBP (Fig. 7B). This finding is consistent with those of Mizuide et al. (37) showing that amino acids 261–314 in the MH2 domain of Smad3 are important for interaction with c-Ski. Phe303 resides in the conserved β6 sheet of Smad3, which binds the helical structure of SBD in SARA (Fig. 1B) (16), suggesting that Ski and CBP can interact with Smad3 at similar binding sites on Smad3. This finding explains why both activation and repression of gene expression use the same set of activated Smad proteins (2).

We have found that Smad, Ski, and CBP could exist as a stable complex of Smad-Ski-CBP regardless of whether Ski was incubated with the Smad3-CBP heteromer or whether CBP was incubated with the Smad3-Ski heteromer. This result also provides evidence that both activation and repression of gene expression use the same set of activated Smad proteins. The Smad-Ski-CBP complex may provide a large platform for the binding of multiple Smad-coactivator or Smad-corepressor molecules. Consistent with our result, another group reported finding c-Ski and CBP in the same complex after stimulating mouse embryonic cells with TGF-β (38), suggesting that the TGF-β signaling pathway cooperatively regulates genes with Smad-binding element-containing promoters.

Purified proteins were used in our experiments to test whether Ski disrupts the formation of the R-Smad-Smad4 heterotrimer. Our results demonstrate that the R-Smad-Smad4 complex was stable whether Ski was used to directly interact with the R-Smad-Smad4 complex (Fig. 3B) or whether Smad4 was used to directly interact with the R-Smad-Ski complex (Fig. 7A). These results provide evidence that the binding of Ski still permits formation of the R-Smad-Smad4 heterotrimer. This result is consistent not only with previous results from our laboratory (16), but also with co-immunoprecipitation results presented by Ueki and Hayman (15) showing that Ski does not disrupt Smad3-Smad4 heteromer formation. They also demonstrated that Ski can use different domains to interact with Smad3 and Smad4 and that recruitment of Ski to the Smad3-Smad4 complex through binding to either Smad3 or Smad4 is both necessary and sufficient for repression. Wu et al. (13) also demonstrated that Ski can use different domains to interact with Smad3 and Smad4. Although their results indicated that Ski disrupts Smad3-Smad4 heteromer formation, they still proposed that Smad4 and Smad2:Smad3 remain bound to the Ski protein in an inactive conformation that no longer stimulates gene expression. In contrast to full-length Ski, which can interact with R-Smad and Smad4, the Ski domain that was used in our study can interact only with R-Smad.

On the basis of the report by Wu et al. (13), full-length Ski likely binds to both R-Smads and Smad4 in the complex. To better understand the role of Ski in repressing Smad activity, including the level of oligomerization when interacting with Smads, it would be necessary to use full-length Ski or a Ski construct that contains the domains for binding to both R-Smad and Smad4. Unfortunately, our attempts to express these constructs of Ski were unsuccessful. Thus, whether full-length Ski can interact with both R-Smad and Smad4 and whether CBP can compete with full-length Ski for binding to R-Smad remain unknown. Although Ski represses TGF-β signaling by multiple mechanisms, our results suggest that the R-Smad-Smad4 complex will still occupy the promoter of the target gene and that Smads will actively repress transcription when Ski is bound to Smads.

In summary, we have demonstrated that purified Ski and CBP directly interact with purified Smads and that Ski and CBP compete for binding to Smad with slightly different affinities. In addition, Ski and CBP interact with Smad at a portion of the
binding sites on Smad. Finally, our model suggests that Smad activity is delicately balanced by Ski and CBP in the TGF-β signaling pathway. To determine whether this model is also applicable to bone morphogenetic protein signaling, additional studies are required to investigate the interactions of Ski with Smad1 and Smad5.

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