Requirement of the Co-repressor Homeodomain-interacting Protein Kinase 2 for Ski-mediated Inhibition of Bone Morphogenetic Protein-induced Transcriptional Activation*

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Jun Harada, Kenji Kokura, Chie Kanei-Ishii, Teruaki Nomura, Md Matiullah Khan, Yongsook Kim, and Shunsuke Ishii

From theLaboratory of Molecular Genetics, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan and theLaboratory of Molecular Cardiology, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

Multiple co-repressors such as N-CoR/SMRT, mSin3, and the c-ski proto-oncogene product (c-Ski) mediate the transcriptional repression induced by Mad and the thyroid hormone receptor by recruiting the histone deacetylase complex. c-Ski also binds directly to Smad proteins, which are transcriptional activators in the transforming growth factor-β (TGF-β/bone morphogenetic protein (BMP)) signaling pathways, and inhibits TGF-β/BMP-induced transcriptional activation. However, it remains unknown whether other co-repressor(s) are also involved with Ski in the negative regulation of the TGF-β/BMP signaling pathways. Here, we report that the co-repressor homeodomain-interacting protein kinase 2 (HIPK2) directly binds to both c-Ski and Smad1. HIPK2 efficiently inhibited Smad1/4-induced transcription from the Smad site-containing promoter. A dominant negative form of HIPK2, in which the ATP binding motif in the kinase domain and the putative phosphorylation sites were mutated, enhanced Smad1/4-dependent transcription and the BMP-induced expression of alkaline phosphatase. Furthermore, the c-Ski-induced inhibition of the Smad1/4-dependent transcription was suppressed by a dominant negative form of HIPK2. The HIPK2 co-repressor activity may be regulated by an uncharacterized HIPK2 kinase. These results indicate that HIPK2, together with c-Ski, plays an important role in the negative regulation of BMP-induced transcriptional activation.

Transcriptional regulation in a variety of signaling pathways is regulated through the combined actions of many repressors and co-repressors. The products (c-Ski and Sno) encoded by the c-ski gene (1) and another member of the ski gene family, the sno (ski-related novel gene) gene (2), act as co-repressors and directly bind to two additional co-repressors N-CoR/SMRT and mSin3A, via their N-terminal cysteine-rich regions and C-terminal coiled-coil regions, respectively (3). N-CoR and SMRT, which share significant homology, were originally identified as co-repressors that mediate transcriptional repression by nuclear hormone receptors (4, 5) and form a complex with the class II histone deacetylases (HDACs) (6–9). mSin3A and mSin3B, which are orthologs of yeast Sin3, were originally shown to mediate transcriptional repression by bHLH proteins of the Mad family (10) and to form a complex with the class I HDACs (11, 12). Although these three co-repressors (N-CoR/SMRT, mSin3, and c-Ski/Sno) are not involved in the single macromolecular complex, they are needed for the transcriptional repression mediated by nuclear hormone receptors, Mad, and possibly other repressors (3, 13–17). In fact, c-Ski and Sno were demonstrated to be required for the transcriptional repression mediated by the tumor suppressor Rb (18).

In addition to N-CoR/SMRT, mSin3, and c-Ski/Sno, multiple other co-repressors have been identified recently. One of them is homeodomain-interacting protein kinase (HIPK2), which was originally identified as the protein bound to the homeodomain protein NK-3 (19). NK-3 belongs to the NK-2 family of proteins, which includes a large number of vertebrate homeodomain transcription factors that have important functions during embryonic development and organogenesis (reviewed in Ref. 20). HIPK2 directly binds to another co-repressor, Groucho, and also to HDAC1 (21). However, it has not been demonstrated whether HIPK2 can interact with other co-repressors, including c-Ski/Sno. The unique characteristic of HIPK2 is the possession of a kinase activity that is essential for mediating the NK-3-dependent transcriptional repression (19). HIPK2 localizes to nuclear speckles, and SUMO-1 modification of HIPK2 is required for this localization (22).

By recruiting the HDAC complex, c-Ski/Sno not only mediate the transcriptional silencing induced by various repressors, but also negatively regulate the transcriptional activation induced by Smad proteins (23–26). Members of the Smad group of proteins act as transcriptional activators and mediate TGF-β, BMP, and activin signaling from receptors to nuclei (for review see Refs. 27–29). In vertebrates, Smad1 is a substrate and mediator of the BMP receptor, whereas Smad2 and Smad3 are mediators of the TGF-β and activin signals (30–34). TGF-β, BMP, and activin first bind directly to the type II receptor, which leads to the formation of an oligomeric complex of the type I and type II receptors (35). Upon ligand binding, the C-terminal ends of these Smad proteins, which have bound directly to the type I receptor, are phosphorylated by the type I receptor. This results in their release (34) and hetero-oligomerization with Smad4, a common mediator of Smad (36–38). Hetero-oligomers of Smad move into the nucleus and directly participate in transcriptional activation via interactions with the transcriptional co-activator cAMP-response element-
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binding protein-binding protein/p300 (39–41). Drosophila Mad and mammalian Smads 1, 3, and 4 directly bind to the specific DNA sequence, CAGAC, although AGAC suffices (42–45). Such Smad binding elements are often present in the promoter region of TGF-β, activin, and BMP target genes. Smad2 and Smad4 interact with FAST-1, a member of the winged-helix transcription factor family, and mediate activin-dependent transcriptional activation (40, 41). Sk and Sno interact directly with Smad2, Smad3, and Smad4 on a TGF-β-responsive promotor element and repress their ability to activate TGF-β responses and ECL detection reagents (Amersham Biosciences).

body (M2; Sigma) or with mouse normal IgG. The immunocomplex was washed with 0.2% BSA in PBS and then with 1% skim milk. Immunoprecipitation was performed with an anti-FLAG antibody (C-15; Santa Cruz Biotechnology, Inc.) for Western blotting were used. Anti-Myc and anti-HA rabbit polyclonal antibodies. The signals were analyzed by confocal microscopy (LSM510; Zeiss). To investigate the co-localization of HIPK2 and Smad1, CV-1 cells (2 x 10^6 cells/6-cm dish) were transfected with the FLAG-HIPK2 expression plasmid (1.5 µg) and the c-Ski expression plasmid (1.5 µg). Forty-eight h after transfection, the cells were fixed with 2% paraformaldehyde and then permeabilized with 0.1% Triton X-100/phosphate-buffered saline. After incubation in the blocking solution, the cells were incubated with an anti-HIPK2 rabbit polyclonal antibody, which was raised against GST-HIPK2 protein containing the region between amino acids 860 and 1141 of HIPK2, and anti-c-Ski monoclonal antibodies, followed by incubation with FITC- or rhodamine-conjugated secondary antibodies. The signals were analyzed by confocal microscopy (LSM510; Zeiss). To investigate the co-localization of HIPK2 and Smad1, CV-1 cells were transfected with a mixture of the FLAG-HIPK2 expression plasmid (1.5 µg), the Myc-Smad1 expression plasmid (0.75 µg), and the Smad4 expression plasmid with or without the ALK3QD expression plasmid (0.5 µg). Immunostaining was performed similarly to that described above using an anti-HIPK2 rabbit polyclonal antibody and anti-Myc monoclonal antibody (Santa Cruz Biotechnology, Inc.).

Lucefrer Reporter Assays—In the experiments using the (CAGA)_{42}, MLP-Luc reporter, C2C12 cells (2 x 10^5 cells/6-cm dish) were transfected with mixture of the (CAGA)_{42} and ALK3QD plasmids, using LipofectAMINE PLUS. For the control of 1st immunoprecipitation, the plasmid to express HIPK2 lacking tag was used. Thirty-six h after transfection, the cells were lysed with SDS buffer, sonicated briefly, and centrifuged. The supernatant was incubated with anti-HIPK2 M2-agarose (100 µl) for 2 h at 4 °C. The beads were washed with Lysis Buffer A containing 150 mM NaCl three times, and then an SDS-FLAG-linked protein-A agarose (amplification reagent; Amersham Biosciences) was added. After lysis of FLAG linkage, the FLAG-linked proteins were eluted by adding Lysis Buffer A containing 250 mM NaCl and 3 x FLAG peptide (300 µg/ml) for 2 h at 4 °C. Second immunoprecipitation was performed using 150 µl of eluate and 350 µl of Lysis Buffer A containing 464 mM NaCl and 0.8 µg of anti-Ski (N-20; Santa Cruz Biotechnology, Inc.) or control IgG followed by addition of protein G-Sepharose.

To examine the co-immunoprecipitation of c-Ski and endogenous Smad1 and HIPK2, 293T cells were co-transfected with the plasmids that express c-Ski (16 µg) and ALK3QD (6 µg). Co-immunoprecipitation of c-Ski and endogenous Smad1 was performed as described above except for the use of Lysis Buffer B (50 mM HEPES, pH 7.8, 500 mM NaCl, 5 mM EDTA, 3 mM dithiothreitol, 1.0% Nonidet P-40, protease inhibitor mixture (Roche Applied Sciences)), Anti-Smad1/5/8 antibody (N-18; Santa Cruz Biotechnology, Inc.), or anti-c-Ski rabbit polyclonal antibody (H-329; Santa Cruz Biotechnology, Inc.) for immunoprecipitation and Western blotting, respectively. For co-immunoprecipitation of endogenous HIPK2 and endogenous Smad1, Lysis Buffer C (50 mM HEPES, pH 7.5, 500 mM NaCl, 0.2 mM EDTA, 10 µg/ml anti-Myc, 0.5% Nonidet P-40, protease inhibitor mixture (Roche Applied Sciences)) and anti-HIPK2 goat polyclonal antibody (C-15; Santa Cruz Biotechnology, Inc.) was used for immunoprecipitation, and anti-Smad1 rabbit polyclonal antibody (Upstate) for Western blotting were used. For co-immunoprecipitation of c-Ski and endogenous HIPK2, Lysis Buffer D (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.2 mM EDTA, 10 µg/ml anti-HA, 0.5% Nonidet P-40, protease inhibitor mixture (Roche Applied Sciences)) and anti-HIPK2 goat polyclonal antibody (C-15; Santa Cruz Biotechnology, Inc.) for immunoprecipitation and c-Ski rabbit polyclonal antibody (H-329; Santa Cruz Biotechnology, Inc.) for Western blotting were used.

Subcellular Localization of HIPK2, c-Ski, and Smad1/C—CV 1 cells (2 x 10^6 cells/6-cm dish) were transfected with the FLAG-HIPK2 expression plasmid (1.5 µg) and the c-Ski expression plasmid (1.5 µg). Forty-eight h after transfection, the cells were fixed with 2% paraformaldehyde and then permeabilized with 0.1% Triton X-100/phosphate-buffered saline. After incubation in the blocking solution, the cells were incubated with an anti-HIPK2 rabbit polyclonal antibody, which was raised against GST-HIPK2 (upstate) containing the region between amino acids 860 and 1141 of HIPK2, and anti-c-Ski monoclonal antibodies, followed by incubation with FITC- or rhodamine-conjugated secondary antibodies. The signals were analyzed by confocal microscopy (LSM510; Zeiss). To investigate the co-localization of HIPK2 and Smad1, CV-1 cells were transfected with a mixture of the FLAG-HIPK2 expression plasmid (1.5 µg), the Myc-Smad1 expression plasmid (0.75 µg), and the Smad4 expression plasmid with or without the ALK3QD expression plasmid (0.5 µg). Immunostaining was performed similarly to that described above using an anti-HIPK2 rabbit polyclonal antibody and anti-Myc monoclonal antibody (Santa Cruz Biotechnology, Inc.).

Luciferase Reporter Assays—In the experiments using the (CAGA)_{42}, MLP-Luc reporter, C2C12 cells (2 x 10^5 cells/6-cm dish) were transfected with the mixture of the (CAGA)_{42} and ALK3QD plasmids, using LipofectAMINE PLUS. For the control of 1st immunoprecipitation, the plasmid to express HIPK2 lacking tag was used. Thirty-six h after transfection, the cells were lysed with SDS buffer, sonicated briefly, and centrifuged. The supernatant was incubated with anti-HIPK2 M2-agarose (100 µl) for 2 h at 4 °C. The beads were washed with Lysis Buffer A containing 150 mM NaCl three times, and then an SDS-FLAG-linked protein-A agarose (amplification reagent; Amersham Biosciences) was added. After lysis of FLAG linkage, the FLAG-linked proteins were eluted by adding Lysis Buffer A containing 250 mM NaCl and 3 x FLAG peptide (300 µg/ml) for 2 h at 4 °C. Second immunoprecipitation was performed using 150 µl of eluate and 350 µl of Lysis Buffer A containing 464 mM NaCl and 0.8 µg of anti-Ski (N-20; Santa Cruz Biotechnology, Inc.) or control IgG followed by addition of protein G-Sepharose.

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activity in the lysate was measured at 37 °C in a buffer containing 0.1 M HEPES and then grown in the presence of G418 (500 μg/ml). To examine the effects of BMP-2 on muscle and osteoblast differentiation, C2C12 cells were incubated for 36–40 h, and firefly luciferase activity was measured, together with Renilla luciferase activity (for an internal control), using a dual luciferase assay system.

Effect of Dominant Negative Form of HIPK2 on the BMP-induced Expression of Alkaline Phosphatase (ALP)—The retroviral expression plasmid for the FLAG-tagged dominant negative form of HIPK2 (FLAG-HIPK2-K221R-STY) was constructed using the Moloney murine leukemia virus-based retrovirus vector pDON-AI (Takara). To generate C2C12 cell pools that express FLAG-HIPK2-K221R-STY, together with the neomycin-resistance gene, C2C12 cells were infected with viruses and then grown in the presence of G418 (500 μg/ml). To examine the effects of BMP-2 on muscle and osteoblast differentiation, C2C12 cells were incubated at a density of 2 × 10^5 cells/cm². After an overnight incubation, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and various concentration of BMP-2 and then the cells were cultured for an additional 3 days. Cell lysates were prepared using ALP lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% Triton X-100), and ALP activity was determined as described previously by Katagiri et al. (49). ALP activity in the lysate was measured at 37 °C in a buffer containing 0.1 M 2-amino-2-methyl-1-propanol (Sigma) and 2 mM MgCl₂, pH 10.5, for 30 min using p-nitrophenol phosphate as the substrate. The enzyme activity was expressed as micromoles of p-nitrophenol produced per min per mg of protein. Protein concentration of the cell lysates was determined with the Bradford reagent (Bio-Rad).

RESULTS

HIPK2 Directly Binds to c-Ski in Vitro—During the search for various c-Ski-interacting proteins, we found that HIPK2 binds directly to c-Ski. The wild-type HIPK2 protein translated in vitro efficiently bound to the GST-c-Ski fusion protein in a pull-down assay (Fig. 1). The HIPK2 protein contains, from the N terminus, the kinase domain, the homeodomain protein-interacting domain, the PEST domain, which is thought to regulate protein stability, and the tyrosine- and histidine-rich domain (Fig. 1A, top). The HIPK2 mutant lacking the homeodomain protein-interacting domain did not bind to c-Ski, whereas the HIPK2 fragment containing only the homeodomain protein-interacting domain (amino acids 539–844) efficiently interacted with c-Ski. Thus, the region containing the homeodomain protein-interacting domain of HIPK2 binds directly to c-Ski.

To identify the specific region in the c-Ski protein that interacts with HIPK2, we next performed similar GST pull-down assays using various forms of c-Ski translated in vitro and the GST-HIPK2 fusion protein containing the homeodomain protein-interacting domain (Fig. 2, A and B). The results indicated that the two regions in the c-Ski protein bind to HIPK2, the C-terminal coiled-coil region (amino acids 556–728) and the region overlapping the cysteine-rich N-CoR binding domain (amino acids 197–330).

In Vivo Interaction between HIPK2 and c-Ski—The in vivo interaction between HIPK2 and c-Ski was investigated with a co-immunoprecipitation assay (Fig. 3A). The expression vectors for both c-Ski and FLAG-linked HIPK2 were transfected into 293T cells, and whole-cell lysates were prepared from the transfected cells and used for co-immunoprecipitation. The antibody against FLAG co-precipitated c-Ski, but the normal IgG control did not. The expression levels of the c-Ski and HIPK2 proteins did not affect each other.

To further confirm the in vivo association between c-Ski and HIPK2, we then examined the subcellular localization of c-Ski and HIPK2 in transfected CV-1 cells (Fig. 3B). As reported previously (3, 22), both HIPK2 and c-Ski were localized in a nuclear dot-like structure when they were expressed alone (Fig. 3B, upper panels). However, the nuclear dots containing HIPK2 were bigger than those containing c-Ski, and there were fewer HIPK2 dots than c-Ski dots. When c-Ski was co-expressed with HIPK2, the c-Ski signals completely overlapped the HIPK2 signals, and all of the c-Ski signals were localized in the big dot-structures. Thus, co-expression of HIPK2 with c-Ski recruits the c-Ski proteins to the HIPK2-containing dot-like structures. These results strongly suggest an in vivo associa-
plexes were analyzed by Western blotting with anti-Ski (Fig. 4C). The Smad family of proteins contains two conserved regions separated by a linker region. The N-terminal MH1 domain has DNA binding activity, whereas the C-terminal MH2 drives translocation into the nucleus and has transcriptional regulatory activity. In vitro translated HIPK2 bound to neither of two mutants that lacked either the MH2 domain (CT) or both the MH1 domain and the linker region (NT1). In contrast, the mutant lacking only the MH1 domain (NT2) efficiently bound to Smad1. Thus, both the MH2 domain and the linker region of Smad1 are required for efficient interaction with HIPK2.

That both c-Ski and Smad1 bind to the homedomain protein-interacting domain of HIPK2 suggests c-Ski and Smad1 could compete in their HIPK2 binding. To address this, we investigated the binding of in vitro-translated [35S]-HIPK2 to the GST-Smad1 resin in the presence of increasing amounts of in vitro translated c-Ski (Fig. 4D). The addition of c-Ski to the binding reaction increased the amount of HIPK2 bound to GST-Smad1, suggesting that c-Ski and Smad1 cooperatively bind to the homedomain protein-interacting domain of HIPK2 rather than compete in their binding.

In Vivo Interaction between HIPK2 and Smad1—We then investigated the in vivo interaction between HIPK2 and Smad1 using a co-immunoprecipitation assay (Fig. 5A). The expression vectors for both FLAG-linked HIPK2 and Myc tag-linked Smad1 were transfected together into 293T cells, along with the HA tag-linked Smad4 expression vector, and whole-cell lysates were prepared from the transfected cells and used for co-immunoprecipitation. The antibody against the Myc-tag coprecipitated both the FLAG-HIPK2 and HA-Smad4 proteins, whereas the normal IgG control precipitated neither of them, indicating that a complex between HIPK2 and Smad1-Smad4 had formed in vivo. Co-transfection of the expression vector for the constitutively active form of the BMP type Ia receptor (ALK3QD) and the plasmids described above affected neither the expression level of HIPK2 nor the co-immunoprecipitation of FLAG-HIPK2 and HA-Smad4 proteins. Further, co-expression of ALK3QD did not affect the amount of Smad1 proteins immunoprecipitated. In our present system, some proportion of the overexpressed Smad1/4 proteins in the transfected cells probably entered into the nucleus in the absence of BMP signaling.

We then confirmed that c-Ski forms a complex with HIPK2 in the presence of the BMP signaling using co-immunoprecipitation assays (Fig. 5B). The expression vectors for both FLAG-linked HIPK2 and c-Ski were transfected together into 293T cells, along with the expression vector for ALK3QD, and whole-cell lysates were prepared from the transfected cells and used for co-immunoprecipitation. The antibody against FLAG coprecipitated c-Ski, whereas the normal IgG control did not, indicating that a complex between HIPK2 and c-Ski had formed in vivo, even in the presence of BMP signaling.

To further examine whether Ski, HIPK2, and Smad1/4 form a complex, we performed the two-step co-immunoprecipitation (Fig. 5C). 293T cells were transfected with the expression plasmids for FLAG-HIPK2, Ski, and Smad1/4, and the prepared cell lysates were first immunoprecipitated with anti-FLAG antibody. This immunocomplex was eluted by anti-FLAG peptide and confirmed to contain Ski and Smad1/4, in addition to the homeodomain protein-interacting domain (ΔID) did not bind to Smad1, whereas the HIPK2 fragment containing only the homeodomain protein-interacting domain (amino acids 539–844) efficiently interacted with Smad1. Thus, the homeodomain protein-interacting domain of HIPK2 directly binds not only to c-Ski (Fig. 1) but also to Smad1.

To investigate which region of the Smad1 protein binds directly to HIPK2, GST fusion proteins containing some deletion mutants of Smad1 were used for in vitro binding assays (Fig. 4C). The Smad family of proteins contains two conserved regions separated by a linker region. The N-terminal MH1 domain has DNA binding activity, whereas the C-terminal MH2 drives translocation into the nucleus and has transcriptional regulatory activity. In vitro translated HIPK2 bound to neither of two mutants that lacked either the MH2 domain (CT) or both the MH1 domain and the linker region (NT1). In contrast, the mutant lacking only the MH1 domain (NT2) efficiently bound to Smad1. Thus, both the MH2 domain and the linker region of Smad1 are required for efficient interaction with HIPK2.

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To further examine whether Ski, HIPK2, and Smad1/4 form a complex, we performed the two-step co-immunoprecipitation (Fig. 5C). 293T cells were transfected with the expression plasmids for FLAG-HIPK2, Ski, and Smad1/4, and the prepared cell lysates were first immunoprecipitated with anti-FLAG antibody. This immunocomplex was eluted by anti-FLAG peptide and confirmed to contain Ski and Smad1/4, in addition to

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**Fig. 3. In Vivo interaction of c-Ski with HIPK2.** A, co-immunoprecipitation. Lysates from 293T cells, which were transfected with plasmids to express FLAG-linked HIPK2 or c-Ski as indicated above, were precipitated with the antibody shown above, and the immunocomplexes were analyzed by Western blotting with anti-FLAG antibodies (upper panel) or anti-FLAG antibodies (lower panel). In some lanes (Western), lysates were directly used for Western blotting. B, co-localization of c-Ski and HIPK2. Upper panels, CV-1 cells were transfected with each of the plasmids expressing FLAG-linked HIPK2 or c-Ski, and cells were then immunostained with anti-HIPK2 or anti-c-Ski antibodies. The HIPK2 and c-Ski stainings were visualized with FITC- and rhodamine-conjugated secondary antibodies, respectively. Lower panels, CV-1 cells were transfected together with the two plasmids to express FLAG-linked HIPK2 or c-Ski. In the left two panels, the HIPK2 and c-Ski stainings were visualized by FITC- and rhodamine-conjugated secondary antibodies, respectively. In the right most panel, the signals for both proteins are superimposed.
FIG. 4. Direct interaction between HIPK2 and Smad1. A, binding of HIPK2 to Smad1 with high affinity. Left panel, analysis of the GST-Smad fusion protein. The GST fusion proteins containing full-length Smads 1–4 that were bound by the glutathione-Sepharose resin were analyzed by 10% SDS-PAGE followed by Coomassie Brilliant Blue staining. Right panel, in vitro translated 35S-HIPK2 or 35S-c-Ski was mixed with the resins containing the GST-Smad fusion proteins indicated above. The bound proteins were analyzed on 10% SDS-PAGE, followed by autoradiography. In the input lanes, the amount of HIPK2 or c-Ski was 10% of that used for the binding assays. B, Smad1 binding domain in the HIPK2 protein. Left panel, schematic representation of the HIPK2 proteins used. The results of the binding assays shown in the right panel are indicated. The relative binding activities are designated + and −, which indicate the binding of 24–30 and less than 1%, respectively. Right panel, three forms of in vitro translated 35S-HIPK2 were mixed with the GST-Smad1 resin, and the bound proteins were analyzed as described above. In the input lanes, the amount of HIPK2 was 10% of that used for the binding assays. C, HIPK2 interaction domain in the Smad1 protein. Upper panel, schematic representation of the Smad1 proteins used. The results of the binding assays shown in the lower panel are indicated on the right. The relative binding activities are designated + and −, which indicate the binding of 39–42 and less than 1%, respectively. Lower left panel, analysis of the GST-Smad1 fusion protein. The GST fusion proteins containing the various forms of Smad1 indicated above that were bound by the glutathione-Sepharose resin were analyzed by 10% SDS-PAGE followed by Coomassie Brilliant Blue staining. Lower right panel, in vitro translated 35S-HIPK2 was mixed with the resins containing the GST-Smad1 fusion proteins indicated above, and the bound proteins were analyzed as described above. In the input lanes, the amount of HIPK2 or c-Ski was 10% of that used for the binding assays. D, c-Ski enhances the binding of HIPK2 to the GST-Smad1. Binding of in vitro-translated HIPK2 fragment containing the hemeoprotein-interacting domain to GST-Smad1 was examined in the presence of increasing amounts of in vitro translated c-Ski. The relative amount of HIPK2 bound to GST-Smad1 is shown below the figure.

FIG. 5. In Vivo association of HIPK2 with Smad1. A, co-immunoprecipitation of HIPK2 and Smad1. Lysates from 293T cells, which were transfected with plasmids to express the proteins indicated above, were precipitated with the antibody shown above, and the immunocomplexes were analyzed by Western blotting with the antibodies indicated on the right. In some lanes (Western), lysates were directly used for Western blotting. B, co-immunoprecipitation of c-Ski and HIPK2 in the BMP signal receiving cells. Co-immunoprecipitation was performed as described for Fig. 3A except for the co-expression of the constitutively active form of the BMP receptor (ALK3QD). C, two-step co-immunoprecipitation of the complex containing HIPK2, Ski, and Smad1/4. Lysates from 293T cells, which were transfected with plasmids to express FLAG-HIPK2 (or no tag-linked HIPK2), Ski, and Smad1/4, were first precipitated with anti-FLAG antibody and eluted with FLAG peptide, and eluates were analyzed by Western blotting with the antibodies indicated on the left. In some lanes (Western), lysates were directly used for Western blotting. The eluates were then immunoprecipitated with anti-Ski antibody or control IgG, followed by Western blotting. IP, immunoprecipitation. Co-IP, co-immunoprecipitation. D, co-immunoprecipitation of c-Ski and endogenous HIPK2 and Smad1 proteins. Lysates from 293T cells, which were transfected with plasmids to express c-Ski and ALK3QD, were precipitated with the antibody shown above, and the immunocomplexes were analyzed by Western blotting with the antibodies indicated below. E, co-localization of HIPK2 and Smad1. Left panel, CV-1 cells were transfected with a mixture of the plasmids to express Myc-Smad1, HA-Smad4, and ALK3QD. The cells were then immunostained with an anti-Myc antibody to detect Myc-Smad1, followed by a rhodamine-conjugated secondary antibody, and analyzed by confocal microscopy. Right panels, CV-1 cells were transfected with a mixture of the plasmids to express the proteins on the left. In the left two panels, the HIPK2 and Myc-Smad1 stainings were visualized by FITC- and rhodamine-conjugated secondary antibodies, respectively. In the right most panel, the signals for both proteins are superimposed.

FLAG-HIPK2. The control lysates, which were prepared from the cells transfected with no tag-linked HIPK2 expression plasmid, contained neither Ski nor Smad1/4. The FLAG-peptide
eluate was then immunoprecipitated with anti-Ski or control IgG. The anti-Ski immunocomplex contained Smad1/4, but the IgG complex did not. Thus, Ski, HIPK2, and Smad1/4 can form a complex.

We also performed the co-immunoprecipitation of endogenous proteins in 293T cell lysates. It was reported that the degradation of c-Ski protein is induced by TGF-β signaling (50, 51). In addition, to date there was no report of the co-immunoprecipitation of endogenous c-Ski and Smad1 proteins in the BMP-treated cells. We thus used the 293T cells transfected with the plasmids that express c-Ski and ALK3QD and prepared the cell lysates from the transfected cells. Anti-HIPK2 antibody co-precipitated endogenous Smad1, whereas the normal IgG did not (Fig. 5D). Furthermore, anti-HIPK2 and anti-Smad1 antibodies co-precipitated the c-Ski proteins.

To further confirm the in vivo association between HIPK2 and Smad1, we examined their subcellular localization in the transfected CV-1 cells (Fig. 5E). The Smad1 proteins were localized in the nuclear fine microspeckle structures when they were expressed with ALK3QD (Fig. 5E, left most panel). When Smad1 was co-expressed with HIPK2 and ALK3QD, the Smad1 signals completely overlapped the HIPK2 signals, and all of the Smad1 signals were localized in the various sizes of dot-like structures (Fig. 5E, right three panels). Thus, co-expression of HIPK2 with Smad1 recruits the Smad1 proteins to the HIPK2-containing dot-like structures. These results strongly suggest the in vivo association between Smad1 and HIPK2 in the nucleus.

**Inhibition of Smad1-induced Transcriptional Activation by HIPK2**—The physical association between HIPK2 and Smad1 suggested that HIPK2 may negatively regulate the BMP-induced transcriptional activation mediated by Smad1. The identification of the AGAC motif as a binding site for Smad3 and Smad4 had been obtained by the screening of random sequences (44). The crystal structure of Smad3 bound to the AGAC motif has been determined, and this sequences was also reported to bind to Smad1 with low affinity (52). However, another report found that Smad1 did not bind to this AGAC sequence (44). Although the reason for this difference is not clear, a mutation in the AGAC motif in the BMP-responsive Xenopus Vent-2 promoter abrogated the BMP responsiveness (53). Therefore, to examine the effect of HIPK2 on the Smad1-induced transcriptional activation, we first used a luciferase reporter containing the AGAC motif (Fig. 6A, left). Co-transfection of the Smad1/4 expression vectors with this reporter into C2C12 cells stimulated the luciferase expression 5.2- and 7.8-fold in the absence and presence of ALK3QD, respectively. Co-expression of the wild-type HIPK2 strongly inhibited this Smad1/4-induced transcriptional activation. In contrast, co-expression of the kinase inactive mutant form of HIPK2 (HIPK2-K221R), which has a mutation in the active site of the kinase domain (subdomain II; Lys to Arg at position 221), did not significantly enhance the Smad1/4-induced transcriptional activation. The region between subdomains VII and VIII (the so-called activation loop) of multiple members of the kinase superfamily contains serine, threonine, and tyrosine residues, which are phosphorylated by upstream kinases such as mitogen-activated protein kinase kinase (Fig. 6B). HIPK2 also contains serine, threonine, and tyrosine residues in the corresponding region, leading to the possibility that phosphorylation of these residues by an uncharacterized kinase leads to activation of HIPK2. We generated the vector to express an HIPK2 mutant (HIPK2-K221R-STY) that contains the mutations of both the active site of the kinase domain (K221R) and the putative phosphorylation sites in the activation loop (STY at amino acids 352–354 to AAF). Co-expression of this HIPK2 mutant stimulated the Smad1/4-induced transcriptional activation more effectively than HIPK2-K221R mutant (Fig. 6A).

To further confirm the effect of HIPK2 on the Smad1-induced transcriptional activation, we next used the luciferase reporter containing the tandem repeats of BMP-responsive Smad-binding element (12XGCGG) (59) (Fig. 6A, right). Co-expression of Smad1/4 with this reporter in C2C12 cells stimulated the luciferase expression 6.8- and 15-fold in the absence and presence of ALK3QD, respectively. Co-expression of the wild-type HIPK2 inhibited this Smad1/4-induced transcriptional activation. In contrast, co-expression of either of two HIPK2 mutants (HIPK2-K221R or HIPK2-K221R-STY) slightly enhanced the Smad1/3-induced transcriptional activation in the presence of ALK3QD. Thus, HIPK2 suppresses the Smad1-induced transcriptional activation, and two kinase-inactive forms of HIPK2 appeared to act as dominant negative forms.
We then asked whether HIPK2 is required for the Ski-induced inhibition of the Smad1/4-dependent transcription in a similar assay system (Fig. 6C). Co-expression of low levels of c-Ski moderately inhibited the Smad1/4-induced transcriptional activation from the 12XGCGG reporter in the absence and presence of ALK3QD. Co-expression of HIPK2 with c-Ski enhanced the HIPK2-mediated inhibition. Furthermore, HIPK2-K221R mutant suppressed the c-Ski-dependent inhibition of transcription. These results suggest that the kinase activity of HIPK2 is at least partly required for the inhibition of the BMP-induced transcriptional activation by c-Ski.

We also investigated whether HIPK2 suppresses the BMP action. BMP signaling induces ALP activity in a number of cellular systems including C2C12 cells (49). Therefore, we tested whether HIPK2 could repress BMP-induced ALP expression in C2C12 cells. We generated the C2C12 cell pools expressing the dominant negative form of HIPK2 (HIPK2-K221R-STY) by infecting the retrovirus vector. The C2C12 cells harboring the HIPK2-K221R-STY expression vector or the control empty vector were treated with various concentration of BMP-2, and the ALP activity was measured. The ALP levels in the cells expressing HIPK2-K221R-STY were much higher that that of control cells (Fig. 6D). Especially, low concentration of BMP-2 (50 or 100 ng/ml) was not able to induce the ALP activity in the control C2C12 cells, whereas an induction of significant levels of ALP activity were observed in the cells expressing HIPK2-K221R-STY. Thus, HIPK2 negatively regulates the BMP-induced ALP expression.

DISCUSSION

We have demonstrated that the co-repressor HIPK2 directly binds to both Smad1 and c-Ski and is required for the c-Ski-mediated inhibition of Smad1/4-dependent transcription. c-Ski directly interacts with two co-repressors, N-CoR/SMRT and mSin3A, and recruits their associated type I and II HDACs to the target transcription factor (3). HIPK2 also interacts with type I HDAC and another co-repressor, Groucho (21). Therefore, it is likely that HIPK2 and c-Ski synergistically recruit HDACs to Smad1/4 to negatively regulate transcription.

So far, two reports have described the interaction between Smad1 and c-Ski, with inconsistent results. Akiyoshi et al. (25) reported that interaction between c-Ski and Smad1 was not detected by yeast two-hybrid and co-immunoprecipitation assays using COS-7 cells. In contrast, Wang et al. (46) reported a direct interaction between Smad1 and c-Ski1 in 293T cells. The effect of c-Ski on Smad1/4-dependent transcriptional activation was also inconsistent between the two reports by Akiyoshi et al. (25) and Wang et al. (46). Our results indicated that c-Ski directly binds to Smad1, but the affinity between them is much weaker than that between c-Ski and Smad3 (Fig. 4A). One possibility is that HIPK2 stabilizes the interaction between Smad1 and c-Ski by directly binding to both of them. Because our results demonstrate that HIPK2 is required for the c-Ski-mediated inhibition of the Smad1/4-dependent transcriptional activation, the effect of c-Ski on Smad1/4-dependent transcriptional activation might depend on the amount of HIPK2 in the cells. However, we could not find the significant difference in the levels of HIPK2 in COS7 and 293T cells. Therefore, it remains unknown why Ski differently affects the Smad1-dependent transcriptional activation in those two types of cells. Another uncharacterized factor that acts together with HIPK2 might resolve the reported discrepancy of the role of c-Ski in BMP-induced transcriptional activation.

HIPK2 has a kinase domain, and an HIPK2 mutant that contains a mutation in the ATP-binding site did not inhibit the Smad1/4-induced transcriptional activation, indicating that the kinase activity is required for the co-repressor activity. Requirement of the kinase activity of HIPK2 has also been reported for NK-3-dependent transcriptional repression (19). HIPK2 has an activation loop that contains the putative phosphorylation sites (Fig. 6B). The HIPK2 mutant (K221R) that contains a mutation in the ATP-binding site of the kinase domain enhanced the Smad1/4-dependent transcriptional activation as a dominant negative form (Fig. 6A). Interestingly, the HIPK2 mutant (K221R-STY) that has mutations in both the ATP-binding site and the putative phosphorylation sites in the activation loop enhanced the Smad1/4-dependent transcriptional activation more effectively than the K221R mutant. These results suggest that HIPK2 kinase activity is positively regulated by uncharacterized kinase, which directly phosphorylates the specific residues in the activation loop of HIPK2. In Western blots, the wild-type HIPK2 gives rise to a higher molecular weight band than the K221R mutant, suggesting that wild-type HIPK2 is autophosphorylated. The HIPK2-STY mutant generated the both phosphorylated and unphosphorylated bands in Western blot analysis, suggesting that at least one residue among the three mutated amino acid residues in the STY mutant is phosphorylated. At present, it remains unknown how the kinase activity of HIPK2 is involved in the co-repressor activity. HIPK2 phosphorylates neither Smad1 nor c-Ski at least in in vitro assays, although HIPK2 phosphorylates histones as reported that the Dyrk kinases, which have the striking homology with HIPK2, phosphorylate histones (54). It is also unknown what kind of upstream kinase phosphorylates the activation loop of HIPK2. Further analyses on these points will clarify more precisely the mechanisms of the negative regulation of BMP signaling.

HIPK2 effectively suppressed Smad1/4-induced transcriptional activation in C2C12 cells. In contrast, Smad3/4-dependent transcription was only slightly inhibited by HIPK2 in the TGF-β-responsive HepG2 cells, although Smad3 interacts with HIPK2 with a similar affinity as Smad1. Further, the dominant negative form of HIPK2 (K221R-STY) only slightly enhanced the Smad3/4-dependent transcriptional activation at least in HepG2 cells. These results suggest that HIPK2 is an important regulator of BMP-induced transcriptional activation but not of TGF-β-induced transcriptional activation at least in HepG2 cells. The molecular mechanism for this is unknown at present, but one possibility is that the amount of some HIPK2-interacting factors such as Groucho, which is needed for the HIPK2-mediated silencing, is low in HepG2 cells. The TGF-β-specific Smad, Smad3, has a high affinity with c-Ski, whereas the BMP-specific Smad, Smad1, has a low affinity with c-Ski. Therefore, in the case of Smad3-dependent transcriptional activation, c-Ski alone could be sufficient to negatively regulate the transcription. Thus, the affinity between each Smad and its co-repressors may affect the role of each co-repressor in negative regulation.

We demonstrated previously (55) that multiple co-repressors including c-Ski directly associate with PML, a component of nuclear bodies. Recently, HIPK2 was also reported to co-localize in nuclear bodies, together with p53 (57, 58). However, as shown in Fig. 3B, the nuclear dots containing HIPK2 were bigger than those containing c-Ski. We have observed that exogenously expressed HIPK2 partially associates with PML and increases the size of nuclear body. Similar observations

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3 K. Kokura and S. Ishii, unpublished results.

4 C. Kanei-Ishii, unpublished results.

5 J. Harada, unpublished results.

6 J. Harada and S. Ishii, unpublished results.

7 T. Nomura and S. Ishii, unpublished results.
Role of HIPK2 in Negative Regulation of BMP Signaling

have been reported recently (60), and it was speculated that HIPK2 modiﬁes nuclear body structure by interacting with SUMO-1 modiﬁcation pathway. PML has an important role for the c-Ski function to mediate the Mad-dependent transcrip-
tion (55). Therefore, it is interesting whether PML has any role in the negative regulation of Smad1-dependent transcription, which is mediated by both c-Ski and HIPK2.

Recently (57, 58), HIPK2 was shown to induce the phospho-
ylation of p53 at Ser-46, followed by apoptosis, in response to UV irradiation. Thus, HIPK2 is thought to inhibit cell growth and increase apoptosis by activating the p53 activity. Consist-
ent with this, we observed that overexpression of HIPK2 ap-
parently inhibits the proliferation of multiple cell lines.6 How-
ever, the results in this study suggest that HIPK2 enhances cellular proliferation by negatively regulating the Smad1/4-de-
pendent transcriptional activation, together with Ski. These results suggest that HIPK2 functions as either a positive or a negative regulator for cellular proliferation, probably depend-
ing on the cell types. In the BMP-responsive cells, HIPK2 may enhance the cell growth, whereas it may inhibit the growth of p53-expressing cells. Similar opposite functions of the co-re-
pressors Ski/Sno in growth regulation are also known. Ski acts
p53-expressing cells. Similar opposite functions of the co-
repressors Ski/Sno in growth regulation are also known. Ski acts

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