Glucocorticoids play pivotal roles in the maintenance of homeostasis but, when dysregulated, may also have deleterious effects. Smad6, one of the transforming growth factor β (TGFβ) family downstream transcription factors, interacts with the N-terminal domain of the glucocorticoid receptor (GR) through its Mad homology 2 domain and suppresses GR-mediated transcriptional activity in vitro. Adenovirus-mediated Smad6 overexpression inhibits glucocorticoid action in rat liver in vivo, preventing dexamethasone-induced elevation of blood glucose levels and hepatic mRNA expression of phosphoenolpyruvate carboxykinase, a well known rate-limiting enzyme of liver gluconeogenesis. Smad6 suppresses GR-induced transactivation by attracting histone deacetylase 3 to DNA-bound GR and by antagonizing acetylation of histone H3 and H4 induced by p160 histone acetyltransferase. These results indicate that Smad6 regulates glucocorticoid actions as a corepressor of the GR. From our results and known cross-talks between glucocorticoids and TGFβ family molecules, it appears that the anti-glucocorticoid actions of Smad6 may contribute to the neuroprotective, antitumoral and pro-wound healing properties of the TGFβ family of proteins.

Physiologically important, whereas others may also be associated with pathologic processes (5, 6).

The actions of glucocorticoids are mediated by the ubiquitous intracellular glucocorticoid receptor (GR), which functions as a hormone-activated transcription factor of glucocorticoid target genes (3, 7). The GR consists of three domains, the N-terminal or "immunogenic" domain, the central, DNA-binding domain (DBD), and the C-terminal, ligand-binding domain. The functions of the latter two domains have been studied extensively, whereas those of the immunogenic domain are less well known (7). In the unliganded state, the GR is located primarily in the cytoplasm (7). After binding to its agonist ligand, the GR undergoes conformational changes and translocates into the nucleus. Ligand-activated GR then binds to the glucocorticoid response elements (GREs) as a dimer and attracts several so-called coactivators and chromatin-remodeling factors to the promoter region through its two transactivation domains, activation function (AF)-1 and AF-2 (7, 8).

Among them, the p160 type histone acetyltransferase coactivators play an essential role in GR-induced transcriptional activity, being attracted to the promoter region in an early phase of transcriptional activation and facilitating access of other transcription-related molecules on the chromatin through acetylation of lysine residues located in several histone tails, such as those of histone H3 and H4 (8–12). In contrast, corepressors, such as the nuclear receptor corepressors and the silencing mediator for retinoid and thyroid hormone receptor, and associated histone deacetylases cause deacetylation of histones, silencing gene transcription by preventing access of cis-acting molecules to the promoter region (8).

Members of the Smad family of proteins transduce signals of transforming growth factor β (TGFβ) superfamily members, such as TGFβ, activin, and bone morphogenetic proteins (BMPs), by associating with the cytoplasmic side of the type I cell surface receptors of these hormones (13, 14). Nine distinct vertebrate Smad family members have been identified, and they have been classified into three groups: receptor-regulated Smads (R-Smads), such as Smad1, -2, -3, -5, and -8; a common partner Smad (Co-Smad), Smad4; and inhibitory Smads (I-Smads) like Smad6 and Smad7 (13).
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All Smads have two characteristic domains, the Mad homology domains 1 and 2 (MH1 and -2), in their N-terminal and C-terminal portions, respectively, separated by a proline-rich linker region (13). The MH1 domain of R- and Co-Smads is important for complex formation with other Smads, transcriptional activation and repression, and interaction with other transcription factors and target DNA sequences (13). The MH2 domain of R-Smads mediates their interaction with cell surface receptors (13, 15), whereas the highly conserved MH2 domain of I-Smads interacts with type I receptors and is sufficient for their inhibitory activity (14).

Upon binding of TGFβ, activin, or BMP to their receptors, cytoplasmic R-Smads are phosphorylated by the receptor kinases, translocate into the nucleus, and stimulate the transcriptional activity of TGFβ, activin-, or BMP-responsive genes by binding to their response elements located in their promoter regions as a heterotrimer with Co-Smad (13). I-Smads, such as Smad6 and Smad7, act as inhibitory molecules in the TGFβ family signaling by forming stable associations with activated type I receptors, which prevent the phosphorylation of R-Smads (13). Smad6 also competes with Smad4 in the heteromeric complex formation induced by activated Smad1 (16). In addition, I-Smads directly suppress the transcriptional activity of TGFβ family signaling by binding to promoter DNA and attracting histone deacetylases and/or the C-terminal binding protein (17–19). Since I-Smads are produced in response to activation of TGFβ family signaling (20), they literally function in the negative feedback regulation of the Smad signaling pathways. Smad6 preferentially inhibits BMP signaling, whereas Smad7 is a more general inhibitor, repressing TGFβ and activin signaling in addition to that of BMP (14).

In this study, we found that Smad6 interacts with the GR and suppresses the latter’s transcriptional activity by attracting histone deacetylase HDAC3. HDAC3 antagonized histone acetylation induced by p160 type histone acetyltransferase coactivators. This inhibitory effect of Smad6 was present in vivo, suppressing glucocorticoid-stimulated gluconeogenesis in the rat liver. It is likely that Smad6 functions as a target tissue regulator of glucocorticoid action.

MATERIALS AND METHODS

Plasmids—pLexA-GR-(263–419) was described previously (21). pLexA-GR-(263–319), pLexA-GR-(263–367), pLexA-GR-(319–367), and pLexA-GR-(367–427) were constructed by subcloning the coding sequences of the corresponding human GR fragments into pLexA (Clontech, Palo Alto, CA). pB42AD-GR-(2–419), pB42AD-MR-(2–603), pB42AD-PR-A-(2–567), pB42AD-AR-(2–559), and pB42AD-ERE-(2–180) were constructed by inserting the coding sequences of the indicated portions of the human GRα, mineralocorticoid receptor (MR), progesterone receptor-A (PR-A), androgen receptor (AR), and estrogen receptor α (ERα) into pB42AD (Clontech). pLexA-Smad6-(1–496), (1–330), and (331–496) were constructed by subcloning the indicated portions of Smad6 coding sequences into pLexA. pB42AD-Smad6-(318–496) is a clone obtained in the original yeast two-hybrid screening using GR-(263–419) as bait (21). pB42AD-G2B-(55–226) was described previously (21). pDEFFlag(N)-mSmad6WT, -mSmad7WT, -mSmad6N, -mSmad6C, -mSmad76, and -mSmad764 are all kind gifts from Dr. K. Miyazono (University of Tokyo, Tokyo, Japan) (22). pRShGrα and pRSrGrα-(Δ262–404), which express the full-length human GRα and its fragment that lacks amino acids 262–404, respectively, were generous gifts from Dr. R. M. Evans (Salk Institute, La Jolla, CA). pMMTV-Luc, which expresses the luciferase under the control of the full-length mouse mammary tumor virus (MMTV) promoter that contains four functional GREs (23), was kindly provided by Dr. G. L. Hager (National Institutes of Health, Bethesda, MD). pSG5-GRIP1 was a generous gift from Dr. M. R. Stallcup (University of Southern California, Los Angeles, CA). pCDNA1/Amp-MR, pSHBHAR-A, and pRc/C MV-hp53, which express the human MR, AR, and p53, respectively, are kind gifts from Drs. N. WARRIER (Centre Recherche Hôtel-Dieu Québec and Laval University, Quebec, Canada), E. R. Barrack (Henry Ford Health Sciences Center, Detroit, MI), and P. Chumakov (Princeton University, Princeton, NJ), respectively. pSVPRA, NE0, pRSV-RelA, pRc/ RSV-CREB341, and pRSV-PKA, which express the human PR-A, ERα, the p65 component of NF-κB, the full-length CRE-binding protein (CREB), and a constitutive active form of the protein kinase A, respectively, were described previously (24). pERE-E1B-Luc was also described previously (24). pG13-Py-Luc, which expresses the luciferase under the control of the p53-responsive elements, was a kind gift from Dr. B. Vogelstein (The Johns Hopkins University, Baltimore, MD). (κB)3-Luc, which contains three κB-responsive elements upstream of the luciferase gene, was described previously (25). pCRE-Luc, which has a CREB-response element in front of the luciferase gene, was purchased from Clontech. HA-HDAC3-expressing plasmid was kindly provided by Dr. M. S. Featherstone (McGill University, Montréal, Canada). FLAG-tagged HDAC1, -4, -5, and -6 were generous gifts from Dr. S. L. Schreiber (Harvard University, Cambridge, MA). pSG5, pCDNA3, pMAM-neo-Luc, and pSV40-β-Gal are purchased from Stratagene (La Jolla, CA), Invitrogen, Clontech, and Promega (Madison, WI), respectively.

Yeast Two-Hybrid Screening and Assay—The yeast two-hybrid screening was performed using GR-(263–419) as bait in the human Jurkat cell cDNA library with the LexA system (Clontech). For a yeast two-hybrid assay, yeast strain EGY48 (Clontech) was transformed with pOP8-LacZ and the indicated pLexA- and pB42AD-based plasmids. β-Galactosidase activity was then measured in the cell suspension as previously described (26). The β-galactosidase activity was normalized for A600. -Fold induction was calculated by the ratio of adjusted β-galactosidase values of transformed cells cultured in the presence of galactose/raffinose versus those in the medium containing glucose.

Cell Cultures and Transfection—Human colon carcinoma HCT116 and uterine cervical carcinoma HeLa cells were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in McCoy’s 5A or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 units of penicillin, and 50 μg/ml streptomycin. HCT116/MMTV cells, which were stably transformed with pMAM-neo-Luc that has the full-length MMTV promoter upstream of the luciferase gene, were maintained in the McCoy’s medium containing 0.2 mg/ml neomycin and the same supplements. Rat hepatoma HTC cells were described previously (21). HCT116 and HCT116/MMTV cells do not contain functional GR, whereas HeLa and HTC cells express fully active GR.

HCT116 and HCT116/MMTV cells were transfected as previously described (21). For the experiments using pMMTV-Luc or other reporter genes for indicated nuclear receptors and transcription factors, different amounts of Smad6- or Smad7-related plasmids were cotransfected with 0.5 μg/well of the indicated nuclear receptor- or transcription factor-expressing plasmid, 1.5 μg/well of luciferase-expressing reporter plasmid, and 0.5 μg/well of pSV40-β-Gal. For stimulation of p53, CREB, or NF-κB transcriptional activity, 0.5 μg/well of pRc/C MV-hp53, RSV-PKA, or pRSV-RelA was cotransfected as well. Empty vectors were used to maintain the same amounts of transfected DNA. 10–6 M dexamethasone or progesterone, or 10–8 M aldosterone, dehydrotestosterone, or estradiol was added to the medium after 24 h of transfection. The indicated concentrations of tricostatin A (TSA) (Sigma), the his-
Smad6 Is a Corepressor of GR

In Smad6, the N-terminal domain consists of 420 amino acids and accounts for over half of the entire molecule (7). Although it contains the AF-1 domain at amino acid positions 77–261, through which the GR communicates with components of the transcriptional machinery (7), the functions of the rest of the N-terminal domain are yet unknown. Thus, we performed a yeast two-hybrid screening assay using bait a GR fragment spanning amino acids 263–419, located between the AF-1 domain and the DBD in a Jurkat cDNA library. Among over 85 independent interactors, we found two independent clones containing the C-terminal portions of the human Smad6 coding sequence (data not shown). In a reconstituted yeast two-hybrid assay, GR-(263–419) expressed as a fusion with LexA-DBD, but not with a Smad6 fragment containing amino acids 1–330 (Fig. 1A). These results indicate that GR-(263–419) interacts with the C-terminal portion of Smad6, which corresponds to the MH2 domain of this molecule. Since we have also found that the WD repeat proteins, the guanine nucleotide-binding proteins, and Smad6 interact with each other, the WD repeats of Smad6 may function as a molecular switch that connects the guanine nucleotide-binding proteins to the glucocorticoid receptor. In this study, we demonstrated that Smad6 is a corepressor of GR-(263–419) through its C-terminal portion.

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Smad6 Interacts with GR-(263–419) through Its C-terminal Portion—The N-terminal domain of the human GR consisting of 420 amino acids accounts for over a half of the entire molecule (7). Although it contains the AF-1 domain at amino acid positions 77–261, through which the GR communicates with components of the transcriptional machinery (7), functions of the rest of the N-terminal domain are yet unknown. Thus, we performed a yeast two-hybrid screening assay using bait a GR fragment spanning amino acids 263–419, located between the AF-1 domain and the DBD in a Jurkat cDNA library. Among over 85 independent interactors, we found two independent clones containing the C-terminal portions of the human Smad6 coding sequence (data not shown). In a reconstituted yeast two-hybrid assay, GR-(263–419) expressed as a fusion with LexA-DBD, but not with a Smad6 fragment containing amino acids 1–330 (Fig. 1A). These results indicate that GR-(263–419) interacts with the C-terminal portion of Smad6, which corresponds to the MH2 domain of this molecule. Since we have also found that the WD repeat proteins, the guanine nucleotide-binding proteins, and Smad6 interact with each other, the WD repeats of Smad6 may function as a molecular switch that connects the guanine nucleotide-binding proteins to the glucocorticoid receptor. In this study, we demonstrated that Smad6 is a corepressor of GR-(263–419) through its C-terminal portion.

The C-terminal portion of Smad6 contains three WD repeats (amino acids 311–330) and a coiled-coil region (amino acids 417–419). The C-terminal portion of Smad6 interacts with GR-(263–419) through its C-terminal portion.

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protein β (Gβ), and Rack1 (receptor for activated protein kinase C 1) interact with GR (263–419) in addition to Smad6 (21), we examined whether Smad6 and Gβ2 interact with different portions of GR-(263–419) in a yeast two-hybrid assay. Several plasmids expressing three different portions of GR-(263–419) expressed as fusions with the LexA-DBD were constructed, and their binding activity to B42 AD-fused Smad6-(319–496) and Gβ2-(55–226) was tested. The latter corresponds to blades 1–4 of Gβ2 and demonstrated strong interaction with GR-(263–419) in our previous examination (21) (Fig. 1B). Smad6 interacted with GR-(263–419) strongly but not with the other shorter fragments of this portion of the GR. In contrast, Gβ2 bound to GR-(263–319) and GR-(263–367) as well as to GR-(263–419), but not with GR-(319–367) and GR-(367–427). These results indicate that the entire GR-(263–419) is required for Smad6 to interact with this GR fragment, whereas the N-terminal fragment GR-(263–319) is sufficient for supporting the interaction of Gβ2, suggesting that Smad6 and Gβ2 bind to different surfaces of the GR, although these proteins were found as GR-interacting molecules in the same yeast two-hybrid screening.

**Smad6 Suppresses GR-induced Transcriptional Activity**—We next examined the effects of overexpressed Smad6 on GR-induced transcriptional activity of the glucocorticoid-responsive MMTV promoter in HCT116 cells (Fig. 2). Increasing amounts of Smad6-expressing plasmid dose-dependently suppressed the transcriptional activity of the GR (Fig. 2A). Smad6 also suppressed GR transcriptional activity on the integrated MMTV promoter, which was introduced into the chromosome of HCT116/MMTV cells by a standard stable transfection procedure (Fig. 2B). Smad6 strongly suppressed the transcriptional activity of the wild type GR, whereas it did not influence that of GR-(Δ262–404), which is devoid of an interaction domain for Smad6 (Fig. 2C).

We also tested Smad6 on a well known endogenous glucocorticoid-responsive gene, the rat TAT. Glucocorticoids increase TAT enzymatic activity by stimulating the transcriptional rate of this gene via tandem GREs located in its promoter region (30). In rat HTC cells, 10^-6 M dexamethasone increased the TAT activity by 7-fold, whereas transfection of siRNAs for Smad6 significantly enhanced dexamethasone-stimulated TAT activity (Fig. 2D). The siRNA transfection reduced mRNA abundance of Smad6 in these cells (Fig. 2E). These results indicate that endogenous Smad6 acts as a negative regulator of GR transactivation on the endogenous glucocorticoid-responsive gene.

**Smad6, but Not Smad7, Suppresses GR Transcriptional Activity via Its C-terminal MH2 Domain**—To define a domain of Smad6, which is responsible for its suppression of GR transcriptional activity, we employed Smad7, several chimeras of Smad6 and Smad7, and their fragments, and tested them in the GR-induced transactivation of the MMTV promoter in HCT116 cells (Fig. 3, A and B). Although Smad7 shares high similarity with Smad6 and has several overlapping activities (14), Smad7 did not suppress GR transcriptional activity at all. Among tested chimeras and fragments, those harboring the C-terminal MH2 domain of Smad6, such as Smad6C and Smad7/6, preserved the wild type’s suppressive effect on GR transactivation. Thus, the MH2 domain of Smad6 is critical for suppression of GR transactivation, possibly by supporting physical interaction with the GR. Although Smad7 has a similar MH2 domain, it has no effect on GR transactivation.

**Smad6 Antagonizes Dexamethasone-stimulated Gluconeogenesis in Rat Liver by Suppressing PEPCk Gene Induction**—We next examined the effect of Smad6 overexpression in the rat liver on circulating glucose levels and mRNA abundance of the PEPCk gene in order to verify Smad6-induced suppression of GR transactivation in vivo. We focused on glucocorticoid-induced gluconeogenesis, since it is a well known biological activity of glucocorticoids mediated by their transactivation property (5, 31). PEPCk is a major rate-limiting enzyme of gluconeogenesis through which glucocorticoids stimulate glucose production in the liver (5). This organ is also known to express substantial amounts of Smad6 (32).

We injected the FLAG-Smad6-expressing adenovirus or the control adenovirus that expresses LacZ into the peritoneal cavity. After 24 h, we...
FIGURE 2. Smad6 suppresses GR transcriptional activity. A, Smad6 overexpression dose-dependently suppresses GR transcriptional activity on the MMTV promoter in HCT116 cells. HCT116 cells were transfected with the indicated amounts of Smad6-expressing plasmids together with pRSHGRα, pMMTV-Luc, and pSV40-β-Gal. The bars represent mean ± S.E. values of luciferase activity normalized for the β-galactosidase activity in the absence or presence of 10⁻⁶ M dexamethasone. **, p < 0.01, compared with the base line. B, Smad6 overexpression suppresses GR transcriptional activity on the integrated MMTV promoter in HCT116 cells. HCT116/MMTV cells, which contain the integrated MMTV promoter-driven luciferase gene, were transfected with the Smad6-expressing plasmid together with pRSHGRα and pSV40-β-Gal. The bars represent mean ± S.E. values of luciferase activity normalized for β-galactosidase activity in the absence or presence of 10⁻⁶ M dexamethasone. **, p < 0.01, compared with the base line. C, GR-(Δ262–404) has greater transcription activity than the wild type GR, and Smad6 loses its suppressive effect on GR-(Δ262–404)-induced transactivation in HCT116 cells. HCT116 cells were transfected with the Smad6-expressing plasmid and pRSHGRα or pRSHGRα-(Δ262–404), together with pMMTV-Luc and pSV40-β-Gal. The bars represent mean ± S.E. values of luciferase activity normalized for the β-galactosidase activity in the absence or presence of 10⁻⁶ M dexamethasone. *, p < 0.01; n.s., not significant, compared with the base line. D and E, abrogation of endogenous Smad6 by Smad6 siRNA enhances dexamethasone-stimulated tyrosine aminotransferase activity in HTC cells. HTC cells were transfected with control or Smad6 siRNAs and were treated with 10⁻⁶ M dexamethasone for 24 h. Cell lysate and total RNA were harvested, and the TAT activity (D) and Smad6 mRNA abundance (E) were determined. The bars represent mean ± S.E. values of the TAT activity (D) or fold induction of Smad6 mRNA (E) in the absence or presence of 10⁻⁶ M dexamethasone. *, p < 0.01; n.s., not significant, compared with the base line.

FIGURE 3. Smad6-(331–496) is responsible for Smad6-induced suppression of GR transcriptional activity. In contrast to Smad6, Smad7 does not suppress GR transactivation. A, HCT116 cells were transfected with the indicated Smad6- or Smad7-related molecule-expressing plasmids together with pRSHGRα, pMMTV-Luc, and pSV40-β-Gal. The bars represent mean ± S.E. values of luciferase activity normalized for β-galactosidase activity in the absence or presence of 10⁻⁶ M dexamethasone (A). *, p < 0.05; **, p < 0.01; n.s., not significant, compared with the base line. B, linearized Smad6 and -7 and related molecules. This figure is adapted from Ref. 22.
We next examined the effects of known HDACs on the GR-induced transactivation of the MMTV promoter in HCT116 cells (TABLE ONE). In the absence of Smad6, overexpressed HDAC1, -3, -4, and -5, but not HDAC6, demonstrated significant suppressive effect on dexamethasone-stimulated GR transactivation. Among them, HDAC3 showed the strongest effect. Smad6 effectively suppressed GR transactivation, and only HDAC3, but not the other tested HDACs, further suppressed GR transactivation in a statistically significant fashion. These results may indicate that Smad6 suppresses GR transcriptional activity of the MMTV promoter by primarily cooperating with HDAC3.

We further examined association of GR with Smad6 and HDAC3 on the MMTV GREs in HCT116/MMTV cells by using ChIP assays (Fig. 5, B–D). FLAG-Smad6 was attracted to GREs with the wild type GR, whereas such attraction almost disappeared with GR-Δ262–404 that lacks most of the Smad6 interaction domain (Fig. 5B). HA-tagged HDAC3 was successfully co-precipitated with GREs only when FLAG-Smad6 was transfected and attracted to GREs (Fig. 5C). Further, HA-HDAC3 was co-precipitated with GREs in the presence of the wild type FLAG-Smad6, but not in the presence of FLAG-Smad6N that does not have a C-terminal MH2 domain that interacts with GR (Fig. 5D).

Smad6 Antagonized p160 Histone Acetyltransferase Coactivator–Induced Histone Acetylation and Enhancement of GR Transcriptional Activity—GR stimulates the transcriptional activity by attracting transcriptional intermediate molecules, such as coactivators and cofactors, which modulate the chromatin structure and further communicate with the transcriptional machinery, including general transcription factors and the RNA polymerase II (8). Among them, histone acetyltransferase coactivators, such as p160 type nuclear receptor coactivators and p300/CREB-binding protein, plays an essential role in GR-induced transcription activation by acetylating specific lysine residues of the histone tail (33). Once histones are acetylated at their N-terminal tails, they allow accumulation of transcription factors, cofactors, and the RNA polymerase II on the promoter region (34). Since Smad6 suppresses GR transactivation by attracting HDAC3, we examined whether Smad6 antagonizes to dexamethasone- and p160 coactivator-induced acetylation of histones using ChIP assays with specific antibodies against acetylated histone H3 and H4 (Fig. 6, A–D). Histone H3 and H4 are well suppressed by Smad6 Antagonized p160 Histone Acetyltransferase Coactivator–Induced Histone Acetylation and Enhancement of GR Transcriptional Activity—GR stimulates the transcriptional activity by attracting transcriptional intermediate molecules, such as coactivators and cofactors, which modulate the chromatin structure and further communicate with the transcriptional machinery, including general transcription factors and the RNA polymerase II (8). Among them, histone acetyltransferase coactivators, such as p160 type nuclear receptor coactivators and p300/CREB-binding protein, plays an essential role in GR-induced transcription activation by acetylating specific lysine residues of the histone tail (33). Once histones are acetylated at their N-terminal tails, they allow accumulation of transcription factors, cofactors, and the RNA polymerase II on the promoter region (34). Since Smad6 suppresses GR transactivation by attracting HDAC3, we examined whether Smad6 antagonizes to dexamethasone- and p160 coactivator-induced acetylation of histones using ChIP assays with specific antibodies against acetylated histone H3 and H4 (Fig. 6, A–D). Histone H3 and H4 are well suppressed by

We next examined the effects of known HDACs on the GR-induced transactivation of the MMTV promoter in HCT116 cells (TABLE ONE). In the absence of Smad6, overexpressed HDAC1, -3, -4, and -5, but not HDAC6, demonstrated significant suppressive effect on dexamethasone-stimulated GR transactivation. Among them, HDAC3 showed the strongest effect. Smad6 effectively suppressed GR transactivation, and only HDAC3, but not the other tested HDACs, further suppressed GR transactivation in a statistically significant fashion. These results may indicate that Smad6 suppresses GR transcriptional activity of the MMTV promoter by primarily cooperating with HDAC3.

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known natural targets of the acetylation reaction promoted by p160 proteins (33). We used HTC cells, which express endogenous GR and the glucocorticoid-responsive TAT gene that has two GREs at its promoter region (30). Acetylated histone H3 and H4 appeared on the TAT GREs in response to dexamethasone addition, and overexpression of GRIP1 further potentiated their appearance. Expression of the wild type Smad6 reduced acetylation of H3 and H4 induced by dexamethasone and strongly attenuated GRIP1-potentiated accumulation of acetylated histones on GREs. Smad6N, which does not have the MH2 domain, lost all of these effects. In agreement with these results, Smad6 wild type, but not Smad6N, almost abolished GRIP1-induced enhancement of GR transcriptional activity on the MMTV promoter (Fig. 6E). These results

FIGURE 5. Smad6 suppresses GR transcriptional activity by attracting histone deacetylase HDAC3. A, the histone deacetylase inhibitor TSA abolishes the suppressive effect of Smad6 on GR-induced transactivation of the MMTV promoter in HCT116 cells. HCT116 cells were transfected with the Smad6-expressing or the control plasmid together with pRSVrLuc, pMMTV-Luc, and pSV40-β-gal. The cells were subsequently treated with the indicated concentrations of TSA. The bars represent mean ± S.E. values of luciferase activity normalized for β-galactosidase activity in the absence or presence of 10⁻⁶ M dexamethasone. **, p < 0.01; n.s., not significant, compared with the base line. B, Smad6 is attracted to GREs through the GR in HCT116/MMTV cells. HCT116/MMTV cells were transfected with FLAG-Smad6- and GR-expressing plasmids, and ChIP assays were performed with anti-FLAG, anti-GR, or control antibodies. Lane 1 indicates the molecular weight marker. C and D, HDAC3 is attracted to GREs through Smad6 in HCT116/MMTV cells. HCT116/MMTV cells were transfected with HA-HDAC3-, FLAG-Smad6-, and GR-expressing plasmids, and ChIP assays were performed with anti-HA, anti-FLAG, anti-GR, or control antibodies. Lane 1 indicates the molecular weight marker.
thus indicate that Smad6 facilitates deacetylation of histone H3 and H4, whose acetylation was induced by GR activation and subsequent attraction of histone acetyltransferases including p160 proteins.

Smad6 Suppresses the Transcriptional Activity of Several Steroid Hormone Receptors but Not That of the Estrogen Receptor and Has No Such Effect on the Activities of p53, NF-κB, and CREB—Since GR and other steroid hormone receptors share many characteristics in their structure, mechanisms of action, and use of coactivators and corepressors (8), we examined Smad6 on the transcriptional activity of other steroid hormone receptors (Fig. 7). We expressed steroid hormone receptors or other transcription factors with their responsive promoter-driven luciferase reporter genes in HCT116 cells (Fig. 7A). As expected, Smad6 suppressed mineralocorticoid receptor (MR)-, progesterone receptor-A (PR-A)-, and androgen receptor (AR)-induced transcription of their responsive genes in addition to that of GR. Smad6, however, did not affect transcriptional activity of ERα, p53, NF-κB, and CREB. Consistent with these functional analyses, N-terminal domains of MR, PR-A, and AR interacted with Smad6 (331–496) in a yeast two-hybrid assay similar to the GR, whereas that of ERα failed to associate with this portion of Smad6 (Fig. 7B).

DISCUSSION

Smad6 interacted with GR at amino acids 263–419, located between the AF-1 and DBD of the GR, using its C-terminal MH2 domain for this interaction. Smad6 suppressed GR-induced transcriptional activity of glucocorticoid-responsive genes, such as the transiently transfected and integrated MMTV promoter and the endogenous glucocorticoid-responsive TAT gene. Overexpressed Smad6 in the rat liver inhibited glucocorticoid-induced increase of blood glucose levels and induction of PEPCK gene expression, suggesting that Smad6 antagonizes glucocorticoid-induced gluconeogenesis in the liver in vivo. Although Smad7 is highly homologous to Smad6, it had no effect on GR-induced transcriptional activity.

### TABLE ONE

| Effect of Smad6 and HDAC expression on dexamethasone-stimulated GR transcriptional activity in HCT116 cells |
|-------------------------------------------------------|
| Smad6 (−) | Smad6 (+) |
| (−) | 100.0 ± 2.95 | 31.9 ± 3.82 |
| HDAC1 | 74.4 ± 8.18<sup>a</sup> | 26.6 ± 4.40 |
| HDAC3 | 48.9 ± 5.16<sup>b</sup> | 16.7 ± 0.46<sup>c</sup> |
| HDAC4 | 85.5 ± 2.43<sup>b</sup> | 28.8 ± 4.37 |
| HDAC5 | 78.6 ± 6.04<sup>b</sup> | 21.6 ± 2.63 |
| HDAC6 | 95.9 ± 4.97 | 31.5 ± 2.15 |

<sup>a</sup> *p* < 0.05 (*n* = 3), compared with base line in the absence or presence of Smad6 expression.

<sup>b</sup> *p* < 0.01 (*n* = 3), compared with base line in the absence or presence of Smad6 expression.

<sup>c</sup> *p* < 0.02 (*n* = 3), compared with base line in the absence or presence of Smad6 expression.
Although definitely less ubiquitous than the GR, Smad6 is widely expressed in the organism. Smad6 mRNA is normally found in the lung, kidney, liver, heart, and, in small amounts, also in the brain and the skeletal muscle (32). It is thus possible that Smad6 regulates glucocorticoid activity in these organs in addition to the liver, where it suppresses glucocorticoid-stimulated gluconeogenesis. Since TGFβ, activin, and BMP-7 regulate the expression level of Smad6 (20), it is likely that these TGFβ family members indirectly regulate glucocorticoid action in target tissues through Smad6. To emphasize the potential implications of our findings, the complex bidirectional interactions between the signaling systems of glucocorticoids and TGFβ family proteins are summarized in TABLES TWO and THREE. These mutual influences suggest that the antiglucocorticoid properties of Smad6 may be of major importance in maintaining some of the actions of glucocorticoids under restraint. The Smad6 pathway in the cell may thus contribute to the antineurotoxic, anticatabolic, and pro-wound healing properties of TGFβ family members, which oppose these negative actions of glucocorticoids.

Smad6 knock-out mice develop multiple cardiovascular abnormalities, such as those caused by endocardial cushion transformation and aortic ossification and have elevated blood pressure in adulthood (35). We suggest that alterations of glucocorticoid actions in target organs may also be developed in Smad6 KO mice. Indeed, organ- or tissue-specific alterations of glucocorticoid sensitivity may produce diverse pathologies, including highly prevalent disorders, such as the metabolic syndrome and depression, that account for a major proportion of human morbidity and mortality (36, 37).

In addition to GR, Smad6 also suppressed the transcriptional activities of several other steroid hormone receptors, including the MR, PR-A, and AR (but not that of ERα or of the transcription factors p53, NF-kB, and CREB), possibly via direct interaction with their N-terminal domains, immunogenic domains. MR, AR, and PR-A are phylogenetically closer to GR than ERα, with relatively long N-terminal domains (38).

Smads other than Smad6 interact with members of nonsteroidal nuclear receptor family proteins. Thus, Smad3 interacts with the vitamin D receptor and enhances its transcriptional activity together with p160 coactivators (39, 40). Smad7, but not Smad6, suppresses this Smad3-induced enhancement of VDR activity (40). Smad3 also interacts with the GR and the AR, and activation of these receptors sup-

| TABLE TWO |
| Effect of glucocorticoids on tissue levels and biologic effects of TGFβ family molecules |
| Data are from Refs. 2 and 51–53. ↑, stimulation; ↓, suppression. |
| Tissue levels | BMPs | Activin/Inhibin |
| ↑ | ↑ | ↓ |
| ↓ | ↓ | ↑ |

FIGURE 7. Smad6 suppresses the transcriptional activity of several steroid hormone receptors through direct interaction with them on their responsive promoters. A, Smad6 suppresses the GR-, MR-, PR-A-, and AR-induced transcriptional activity of their responsive promoters, whereas it does not affect that of ERα, p53, NF-kB, and CREB. HCT116 cells were transfected with GR-, MR-, PR-A-, AR-, ERα-, p53-, NF-kB- (p65), or CREB-expressing plasmids together with their respective responsive promoter-driven luciferase genes in the presence or absence of Smad6-expressing plasmid. The cells were subsequently treated with the indicated hormones or stimulators. The bars represent mean ± S.E. values of luciferase activity normalized for β-galactosidase activity in the absence or presence of 10−6 M dexamethasone. **, p < 0.01; n.s., not significant, compared with the base line. Prog, progesterone; Aldo, aldosterone; DHT, dehydrotestosterone; E2, estradiol. B, Smad6-(331–496) interacts with the N-terminal domains of GR, MR, PR-A, and AR, but not with that of ERα, in a yeast two-hybrid assay. EGY48 yeast cells were transformed with pBOP-LacZ, pLexA-Smad6-(331–496), and p42AD plasmid encoding the N-terminal domain of the indicated steroid hormone receptors. The bars represent mean ± S.E. values of fold activation compared with the base line.
Smad6 Is a Corepressor of GR

| TABLE THREE
| Comparison of the biologic effects of glucocorticoids and TGFβ family molecules
| Data are from Refs. 2 and 54–61. Th1 and Th2, T-helper cells, subtype 1 and 2, respectively. |
| Glucocorticoids | TGFβ family molecules |
|-----------------|-----------------------|
| Central nervous system | Neurotoxic | Neuroprotective |
| Reproductive system | Suppressive | Both suppressive and stimulatory |
| Metabolic system | Catabolic | Anabolic |
| Immune system | Th1 to Th2 shift | Th1 to Th2 shift |
| Musculo-skeletal system | Suppression | Stimulation |
| Wound healing | Suppression | Stimulation |

FIGURE 8. Summary of Smad6 effect on GR-induced transactivation. Smad6 suppresses GR-induced transactivation by directly interacting with the GR and by attracting HDAC3 to the promoter region of a responsive gene. Smad6 may antagonize the acetylation of histones induced by histone acetyltransferase coactivators. This figure is based on the current results and information reported in Refs. 8, 12, 18, and 34.

Smad6 may play a physiologic role in the termination of GR transactivation initiated by the attraction of histone acetyltransferase coactivators, allowing GR to restart a new cycle of transcriptional activation (10–12), in contrast to classic corepressors, which play a role in the silencing of genes in the absence of ligand or presence of antagonists (8, 45–47). It is also possible that Smad6/HDAC3 deacetylate other GR-attracted transcriptional components, such as the p160 or p300/CBP histone acetyltransferases and possibly the GR itself. Through this mechanism, Smad6 might further regulate ligand-bound GR-induced transcriptional activity. Acetylation of these molecules regulates their influence on transcription, whereas Smad7 deacetylates itself via attracting HDACs, further regulating its own stability and activity (48–50).

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