Interactions between PIA1 Proteins and SOX9 Result in an Increase in the Cellular Concentrations of SOX9

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We have identified PIA1 (protein inhibitor of activated STAT-1), -3, -xα, and -xβ as SOX9-associated polypeptides using the Gal4-based yeast two-hybrid system and a cDNA library derived from a chondrocytic cell line. These PIA1 proteins were shown to interact directly with SOX9 in two-hybrid, co-immunoprecipitation, and electrophoretic mobility shift assays. SOX9 was sumoylated in cotransfection experiments with COS-7 cells using PIA1 and SUMO-1 (small ubiquitin-like modifier-1) expression vectors. SOX9 was also sumoylated in vitro by PIA1 proteins in the presence of SUMO-1, the SUMO-activating enzyme, and the SUMO-conjugating enzyme. In COS-7 cells, PIA1 proteins stimulated the SOX9-dependent transcriptional activity of a Col2a1 promoter-enhancer reporter. This increase in reporter activity was paralleled by an increase in the cellular levels of SOX9. Cotransfection with a SUMO-expressing vector further enhanced the transcriptional activity of this SOX9-dependent Col2a1 reporter in COS-7 cells, and this additional activation was inhibited in the presence of either SUMO-1 mutants or PIA1 RING domain mutants or by coexpression of a desumoylation enzyme. Immunofluorescence microscopy of SOX9-transfected COS-7 cells showed that the subnuclear distribution of SOX9 became more diffuse in the presence of PIA1 and SUMO-1. Our results suggest that, by controlling the cellular concentrations of SOX9, PIA1 proteins and sumoylation may be part of a major regulatory system of SOX9 functions.

SOX9, a transcription factor of the SRY (sex-determining region, Y chromosome) family, is required for the establishment and differentiation of several cell lineages, including those of chondrocytes, Sertoli cells of male gonads, glial cells of the nervous system, and others. During chondrocyte differentiation, SOX9 is expressed abundantly in mouse chondroprogenitor cells and overtly differentiated chondrocytes (1) and regulates transcription of cartilage-specific extracellular matrix molecules such as collagen types II (2), IX (3), and XI (4) and aggrecan (5). Heterozygous mutations in the Sox9 gene result in dwarfism in mice (7).

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3 The abbreviations used are: HMG, high mobility group; E3, SUMO-protein isopeptide ligase; E1, SUMO-activating enzyme; E2, SUMO-conjugating enzyme; HA, hemagglutinin; GST, glutathione S-transferase; siRNAs, small interfering RNAs; GFP, green fluorescent protein.
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interacts with p53 (22, 23). PIAS proteins have been shown to act as SUMO (small ubiquitin-like modifier)-protein isopeptide ligases (E3) (23–25).

The SUMO conjugation system (sumoylation) of proteins presents similarities to the ubiquitin conjugation system (26, 27). Specifically, SUMO is activated in an ATP-dependent manner by a SUMO-activating enzyme (E1), consisting of an AOS1/UBA2 heterodimer. Activated SUMO is transferred to a SUMO-conjugating enzyme (E2), UBC9, and subsequently attached to the e-amino group of specific lysines in protein substrates. Although SUMO conjugation of substrates can be accomplished with only E1 and E2 enzymes, this process requires the presence of E3 ligase proteins to be performed efficiently and to confer target specificity. Recent studies show that sumoylation by PIAS family members is critical to target transcription factors such as p53 and c-Jun (22) and LEF1 (28, 29). PIAS proteins share a highly homologous central RING finger domain, which is required for their SUMO ligase activity. SOX9 has three potential sumoylation sites, Lys61, Lys253, and Lys398. In this work, we show that PIAS proteins directly interact with SOX9 and stimulate SOX9 sumoylation both in vitro and in intact cells. Our results indicate that, in COS-7 cells, both SOX9–PIAS interactions and the subsequent sumoylation of SOX9 stabilize SOX9 and may confer resistance against proteasomal degradation. The resulting increased cellular levels of SOX9 lead to an increase in the SOX9-dependent transcription of a Col2a1 reporter. We propose that SOX9 modification by SOX9 is part of a system that controls the cellular concentrations of SOX9.

MATERIALS AND METHODS

Yeast Two-hybrid cDNA Library Screen and Constructs—Full-length and truncated cDNAs of SOX9 were prepared as bait by PCR amplification and cloned into the pGBK7T vector, which contains the DNA-binding domain of Gal4. For screening, a cDNA library from the human chondrocytic cell line HCS-2/8 was constructed in the EcoRI site of the pGADT7 vector, which includes the Gal4 activation domain. A total of 5 × 10^6 independent cDNA clones with an average insert size of ~2.4 kb pairs (range of 2.0–4.0 kb pairs) were obtained. Aliquots (50 µg) of the resulting cDNA library were then individually transformed into AH105 yeast cells that had been transformed with a SOX9 bait containing amino acids 26–189 or 26–415 and screened on selection plates lacking Trp and Leu; equal numbers of cells were collected; and luciferase and ß-galactosidase internal control were measured 24 h after transfection. Cells were harvested in buffer containing 0.1 M potassium phosphate (pH 7.0), 20 mM KCl, and 1 mM MgSO₄. To measure the binding strength between PIAS1 and either full-length or truncated SOX9, the β-galactosidase activity of the reporter gene was measured, standardized to protein concentration, and subtracted from the β-galactosidase activity obtained after cotransformation of the pGADT7 empty vector with the same series of SOX9 polypeptides.

A SOX9 expression vector (pcDNA/HAS-SOX9) was kindly provided by Dr. Shunichi Murakami (M. D. Anderson Cancer Center). Full-length SOX9 linked to aimerized hemagglutinin (HA) tag at its N terminus was cloned into the pcDNA-5’UT vector (2). SOX9 mutants were generated in the pcDNA/HAS-SOX9 vector with three sets of oligonucleotides for the K61A substitution (SOX9/K61A5’, 5’-CCGATCTGGCCAAAGGAGGCCAGGAGGA-3’; and SOX9/K61A3’, 5’-CCGATCTGTCGTCTTCTGGCCAGATCGGGGTTCCG-3’), for the K253A substitution (SOX9/K253A5’, 5’-CAAGGCTGACCTGGCCAGAGAGGAGGAGGAGGGC-3’; and SOX9/K253A3’, 5’-GCCGCCCCCTCTGCTGAGGCAGGGTCAGGCTTCCGTTGTGCCTG-3’), and for the L398A substitution (SOX9/L398A5’, 5’-GTCAGCCTTG-3’; and SOX9/L398A3’, 5’-GGGACCTGACCTGTCGTCCGTTATGTGCGCTG-3’). Generation of pEGFP-PIAS1 (either wild-type or C350A), pEGFP-PIAS3, pEGFP-PIASxxa (either wild-type or C362A), pEGFP-PIASβ (either wild-type or C362A), pFLAG-SUMO-1, pFLAG-SUMO-1ΔG, and pEGFP-SENP2 (either wild-type or C549A) was previously described (25).

Purification of Reconstituent Proteins and Production of Antibody—For preparation of purified reconstituted SOX9 protein, an HA tag was coupled to the SOx9 gene by PCR and subcloned into the pBac-gus-2cp vector, which carries a His tag and an S-Tag, and transfected into SF9 insect cells for expression of reconstituted proteins. The expressed His-S-HA-SOX9 protein was purified with nickel-nitrilotriacetic acid-agarose (Qiagen Inc., Valencia, CA). Recombinant UBC9 and SUMO-1 were expressed in E. coli strain BL21 (DE3)pLysS and purified as described previously (27). Glutathione S-transferase (GST)-SU/A/His-UBA2 and PIAS proteins were expressed in SF9 cells and purified with glutathione-Sepharose 4B beads (Amersham Biosciences, Uppsala, Sweden) as described (23, 25, 27). Reconstituent SOX9 protein was also generated in E. coli for production of antibody. The SOX9 cDNA containing a FLAG tag was amplified by PCR; subcloned into the pET23b vector, which provides a His tag; and transformed into E. coli strain BL21 (DE3)pLysS. The expressed SOX9 protein was purified with nickel-nitrilotriacetic acid-agarose and by anti-FLAG affinity chromatography (Sigma). For antibody preparation, rabbits were immunized three times with 50 µg of purified SOX9 protein each. Serum was collected, and IgG was purified with a HiTrap protein G HP column (Amersham Biosciences).

Cell Culture and DNA Transfections—COS-7 monkey kidney cells, RCS rat chondrosarcoma cells, and HCS-2/8 human chondrosarcoma cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transient transfection experiments were performed using FuGENE 6 (Roche Applied Science). Knockdown experiments were performed with small interfering RNAs (siRNAs) for UBC9, SUMO-1, and SUMO-2 (kindly supplied by FASMAC Co., Tokyo, Japan). UBC9, SUMO-1, and SUMO-2 siRNAs (derived from human sequences) also recognize the corresponding monkey sequences. The sequences of the top strands of the siRNAs were as follows: UBC9, 5’-CAUGUCAUGUGUCACAU-3’; SUMO-2, 5’-CAUGUCAUGUGUCACAU-3’; and control, 5’-CAUGUCAUGUGUCACAU-3’. Transfection of siRNA was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Luciferase Reporter Gene Assay—The activity of the 4 × 48-p89 Col2a1 promoter-luciferase reporter and that of the pCMV/β-galactosidase internal control were measured 24 h after transfection. Cells were harvested in buffer containing 0.1 M potassium phosphate (pH 7.8), 1 mM dithiothreitol, and 0.2% Triton X-100, and luciferase and β-galactosidase activities were measured as described previously (31).
**Immunoblotting and Immunoprecipitation**—For both immunoblotting and immunoprecipitation, cells were washed with ice-cold phosphate-buffered saline and lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride). After brief sonication, the lysates were centrifuged at 4 °C, and protein concentration was measured. For immunoblotting, the cell lysates were boiled in 2× SDS sample buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 2% mercaptoethanol, and 0.02% bromphenol blue). For immunoprecipitation, the lysates were incubated with antibody and protein G-Sepharose CL-4B (Amersham Biosciences) for 4 h at 4 °C. The immunocomplexes were washed five times with lysis buffer, and the immunoprecipitated proteins were removed from protein G-agarose by boiling in 2× SDS sample buffer and loaded onto SDS-polyacrylamide gels. Polypeptides were transferred to polyvinylidene difluoride membranes, and the membranes were treated with a primary antibody and then with a horseradish peroxidase-conjugated secondary antibody. Bound antibody was detected by enhanced chemiluminescence (ECL, Amersham Biosciences) and exposed to x-ray film.

**In Vitro Sumoylation Assay**—The in vitro sumoylation assay was performed in a 20-μl reaction mixture containing 50 mM Tris-HCl (pH 7.4), 2 mM dithiothreitol, 3 mM ATP, 600 ng of His-S-HA-SOX9, 160 ng of GST-SUA1/GST-UBA2, 20 or 200 ng of UBC9, 200 ng of SUMO-1, and 8 or 40 ng of GST-PiAs proteins. After incubation for 30 min at 30 °C, the reaction mixture was fractionated by SDS-PAGE and immunoblotted with anti-HA monoclonal antibody (12CA5, Roche Applied Science). The reaction mixtures were also immunoprecipitated with anti-His polyclonal antibody (Oncogene Science, Cambridge, MA), and sumoylated proteins were detected with anti-SUMO-1 antibody (Zymed Laboratories Inc., South San Francisco, CA) (data not shown).

**GST Pulldown Assay**—Full-length and truncated SOX9 cDNAs were transcribed and translated in vitro in 50 μl of reaction mixture and in the presence of [35S]methionine using the Tnt T7 quick coupled transcription/translation kit (Promega Corp., Madison, WI). 10% of the reaction mixtures were mixed with SDS sample buffer and directly analyzed by SDS-PAGE. The rest of the reaction mixtures were then used for pulldown assay with glutathione-Sepharose-bound purified GST-PIAS1 (100 μg). Reactions were performed in 10 volumes of immunoprecipitation buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride) for 4 h at 4 °C. The agarose gel was washed five times with immunoprecipitation buffer, and [35S]-labeled SOX9 proteins were released by boiling with 2× SDS sample buffer and fractionated by SDS-PAGE.

**Electrophoretic Mobility Shift Assays**—An oligonucleotide probe binding to the HMG box (HMG-responsive element) was made with complementary oligonucleotides: 5’-ggACACTGAGAACAAAGCGCTCT-3’ and 5’-ggGTGTAGACGCCGTTTGGTCTAGGT-3’. The upperase sequence contains the consensus binding site for HMG protein, including SOX9, and gg residues were added at the 5’-end of each oligonucleotide for labeling with [32P]dCTP. DNA/protein reactions were carried out as described previously (32). Assays with recombinant proteins were carried out with 1 ng of poly(dG-dC) and 20 μg of bovine serum albumin, and those with cell extracts were carried out with 1.4 μg of poly(dG-dC) and 10 μg of bovine serum albumin.

**Indirect Immunofluorescence and Fluorescence Deconvolution Microscopy**—Cells transiently transfected with different expression vectors were first fixed with 4% formaldehyde/phosphate-buffered saline for 15 min at room temperature. The cells were then permeabilized with 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20 containing 5% skim milk for 30 min at room temperature and incubated with primary antibodies for 90 min at 4 °C. The dilutions of the primary antibodies were as follows: anti-SOX9 antibody, 1:100; and anti-FLAG monoclonal antibody M2 (Sigma), 1:100. After washing three times, the cells were incubated with secondary antibodies and 4’,6-diamidino-2-phenylindole dihydrochloride (2 μg/ml). The dilutions of the secondary antibodies were as follows: Alexa Fluor 555-conjugated goat anti-rabbit IgG, 1:5000, and Alexa Fluor 647-conjugated goat anti-mouse IgG, 1:5000. Green fluorescent protein (GFP)-tagged PIAS1 directly was detected. Images were obtained with a Zeiss deconvolution microscope.

**RESULTS**

**PIAS Polypeptides Interact with SOX9**—PIASx, -xβ, -1, and -3 were identified as SOX9-binding proteins in a yeast two-hybrid screen of a HCS-2/8 human chondrosarcoma cDNA library. PIASx and PIAS1 mRNAs were broadly expressed in all tissues examined, whereas PIAS3 showed relatively strong expression in lung, cartilage, kidney, and testis, but less in heart and liver (data not shown). The presence of PIAS1 and PIAS3 proteins in chondrocytes was also examined by Western blotting using specific antibodies in several cultured cells, including human chondrosarcoma cells (HCS-2/8), mouse primary chondrocytes, and rat chondrosarcoma cells (RCS). This confirmed results obtained by reverse transcription-PCR and Northern hybridization. The data indicate that PIAS1 and PIAS3 were strongly expressed in all chondrocytic cells, as well as in COS-7 cells (data not shown).

To confirm the direct interactions between SOX9 and PIAS proteins, we used several methods. First, to identify the PIAS1 interaction sites in SOX9, full-length SOX9 or N-terminal, C-terminal, or internal fragments were expressed in AH105 yeast cells as Gal4 DNA-binding domain fusion proteins (Fig. 1A), whereas PIAS1 was coexpressed as a Gal4 transactivation domain fusion protein. Interaction strength between PIAS1 and SOX9 fragments was estimated by measuring β-galactosidase activity, reflecting the activity of the reporter containing Gal4-binding sites (Fig. 1B). Maximal binding to PIAS1 was achieved by SOX9 fragments that included residues 182–509, whereas the shorter fragments that included residues 409–509 or 26–304 bound less efficiently.

Full-length SOX9 and truncated fragments were synthesized by in vitro transcription/translation and radiolabeled with [35S]methionine, and their association with PIAS1 was determined by binding to immobilized GST-PIAS1 (Fig. 1C). These experiments largely confirmed the two-hybrid assays in yeast, although they showed some quantitative differences. For instance, whereas the SOX9-(26–415) fragment did not show strong interactions with PIAS1 in the yeast two-hybrid assay, the in vitro translated fragment was pulled down with GST-PIAS1 beads (Fig. 1C). The results of the pulldown assays suggest that the C-terminal transactivation domain of SOX9 (residues 409–509), which contains one of the three putative sumoylation sites, is important for interaction with PIAS1. Additional segments of SOX9 that contain two other putative sumoylation sites also contribute to full binding (Fig. 1A).

We also examined the interaction between SOX9 and PIAS1 by coimmunoprecipitation of the proteins in transfected COS-7 cells. Fig. 1D shows that PIAS1 was immunoprecipitated with anti-SOX9 antibody in both the presence and absence of cotransfected SUMO-1. Like other members of the PIAS family, PIAS1 has been reported to act as a SUMO ligase.

SOX9 contains an HMG box domain that binds directly to a specific sequence in the minor groove of DNA. In electrophoretic mobility shift assays using a DNA probe containing an HMG box consensus sequence, addition of recombinant PIAS1 to recombinant SOX9 produced a
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FIGURE 1. Interaction between SOX9 and PIAS1. A, schematic line diagrams of PIAS1 and SOX9. PIAS1 contains a putative chromatin-binding SAP domain and a COG5222 zinc finger RING domain. SOX9 deletion mutants were made to analyze the interaction with PIAS1 in a yeast two-hybrid assay. SOX9 contains an HMG consensus DNA-binding domain, a C-terminal transactivation domain (CT), and three putative sumoylation sites (Lys61, Lys253, and Lys398). SOX9 has four ATG codons in the N-terminal end, all in the same reading frame; the fourth ATG codon was considered as the start codon of SOX9 in this experiment. Numbers in the diagrams indicate the amino acid positions of the respective protein.
FIGURE 2. PIAS proteins enhance SOX9 transcriptional activation of a multimerized Col2a1 enhancer-promoter in transiently transfected COS-7 cells. A: left panel, COS-7 cells were transfected with 0.77 mg of 4×48-p89 Col2a1 reporter together with an expression constructs encoding β-galactosidase (3 ng for normalization); SOX9 (100 ng); and PIAS1, -3, or -xα, or -xβ (300 ng). Right panel, COS-7 cells were transfected with the same reporter construct with SOX9 (100 ng) and increasing amounts of PIASxα as indicated. In A and B, the levels of luciferase activity were normalized to β-galactosidase activity. All experiments were carried out at least three times. B, shown are the results from Western blot (WB) analysis of a lysate of COS-7 cell(100 μg of protein per lane) transfected with SOX9 (100 ng) or PIAS1, -3, -xα, or -xβ (300 ng). Coexpression of PIAS proteins increased SOX9 levels in COS-7 cells. C, shown are the results from Western blot analysis of COS-7 cells transfected with 1 μg of SOX9 or PIAS1 (1 μg) expression vector. The proteasome inhibitor MG132 (final concentration of 10 μM) was added 12 h after the start of transfection, and the cells were further incubated for another 12 h. Cycloheximide (final concentration of 100 μg/ml) was added 18 h after the start of transfection, and the cells were further incubated for 6 h.

Members of the PIAS Family Act as E3 Ligases for SOX9—Conjugation of proteins to SUMO increases the molecular mass by ~20 kDa and thus causes the appearance of a slower migrating species on SDS-polyacrylamide gel. To determine whether SOX9 is sumoylated, we coexpressed multimerized HA-SOX9, multimerized FLAG-SUMO-1, and GFP-PIAS proteins in COS-7 cells. In the absence of PIAS proteins, the vast majority of SOX9 extracted from transfected cells migrated as a 75-kDa species on SDS-polyacrylamide gel. The mobility of a minor species migrating at ~95 kDa was consistent with a slower migrating DNA-protein complex and increases the total amount of complex formed (Fig. 1E). The presence of both SOX9 and GST-PIAS1 in the DNA-protein complexes was verified by addition of anti-SOX9 or anti-GST antibody. Both antibodies gave rise to a supershift (Fig. 1E). Purified PIAS1 did not bind to the DNA probe, but enhanced the binding of SOX9. These experiments support a direct interaction between SOX9 and PIAS1.

PIAS1, -xα, -xβ, and -3 Enhance SOX9-dependent Transactivation of a Reporter Containing a Multimerized Enhancer Sequence of Col2a1—In cotransfection experiments with COS-7 cells, PIAS proteins increased the activity of a SOX9-dependent Col2a1 reporter construct by 2.5–4-fold. This increase was concentration-dependent. In the absence of SOX9, PIAS proteins did not affect the activity of the reporter (Fig. 2A). Western blot analysis indicated that coexpression of SOX9 and PIAS significantly increased the protein levels of SOX9 (Fig. 2B), suggesting that the interactions between SOX9 and PIAS proteins lead to stabilization of the SOX9 polypeptide. Thus, the increase in the activity of the SOX9-dependent reporter in COS-7 cells is likely due in large part to stabilization of SOX9, although increased binding of SOX9 to DNA might also have a role. To examine whether the postulated stabilization of SOX9 by PIAS is due to resistance to proteasomal degradation, we tested whether MG132, an inhibitor of proteasomal degradation, would further increase the levels of SOX9. As shown previously (33), MG132 increased the levels of SOX9 in the absence of PIAS. This increase was abolished by addition of cycloheximide, an inhibitor of protein synthesis. In the presence of PIAS, MG132 did not further increase the enhanced levels of SOX9, and cycloheximide had little effect. These results suggest that the interactions between SOX9 and PIAS inhibit the normal degradation of SOX9 by the proteasome pathway.

Asterisks indicate immunoprecipitated PIAS1 proteins. Numbers on the left indicate the molecular masses of protein markers in kilodaltons. In the right panels, SOX9 proteins bound to immobilized GST-PIAS1. The in vitro translated SOX9 proteins that bound to GST-PIAS1 were pulled down by GST-immobilized beads and resolved by SDSPAGE. Asterisks indicate SOX9 fragments that were pulled down. GST alone did not bind to SOX9 fragments. D, in vivo association of SOX9 and PIAS1. HA epitope-tagged SOX9 and GFP-tagged PIAS1 proteins were transiently expressed in COS-7 cells with or without coexpression of FLAG-tagged SUMO-1. Equivalent amounts of total cellular proteins were immunoprecipitated (IP) with anti-SOX9 polyclonal antibody. GFP-PIAS1 proteins coimmunoprecipitated with SOX9 were detected by Western blotting (WB) with anti-GFP monoclonal antibody (upper panel). Asterisks indicate immunoprecipitated PIAS1 proteins associated with SOX9 protein. HA-SOX9 in the precipitated fraction was detected with anti-HA monoclonal antibody (lower panel). E, gel shift assay using recombinant SOX9, PIAS1 proteins, and their antibodies (Abs). Recombinant SOX9 (40 ng) and GST-PIAS1 (200 ng) proteins were incubated with a 32P-labeled HMG probe (5 fmol); for supershift experiments, anti-SOX9 (2.5 μg of IgG) or anti-GST (2.5 μg of IgG) antibody was added. PIAS1 by itself did not bind to the HMG probe; but in the presence of SOX9 and PIAS1, there was an additional slower migrating DNA-protein complex. Both antibodies to SOX9 as well as to GST caused a supershift. Asterisks indicate various DNA-protein complexes.
low extent of sumoylation of SOX9 produced by endogenous enzymes (Fig. 3A). Coexpression of PIAS1 or PIASx caused a very significant enhancement of the 95-kDa band and the appearance of additional higher molecular mass species that may represent conjugates of several SUMO-1 molecules and SOX9 (Fig. 3A). Other PIAS proteins that were identified during our yeast two-hybrid screening showed similar effects (data not shown). To confirm the conjugation of SUMO-1 to SOX9, the cell lysates were immunoprecipitated with anti-SOX9 polyclonal antibody and incubated with anti-FLAG monoclonal antibody. Wild-type PIASx and PIASx and RING domain mutants were cotransfected with wild-type or mutant FLAG-SUMO-1 and HA-SOX9 in COS-7 cells. FLAG-SUMO-1-modified SOX9 proteins were immunoprecipitated with anti-SOX9 polyclonal antibody and detected with anti-FLAG monoclonal antibody. Wild-type PIASx and PIASx and RING domain mutants of PIASx and PIASx bearing an alanine substitution for Cys362 in the RING domain as well as FLAG-SUMO-1ΔGG (mutant SUMO-1 with the C-terminal GG residues deleted) were inactive. SOX9 is sumoylated mainly through Lys398. Wild-type (WT) HA-SOX9 or mutant HA-SOX9 with three different lysine-to-alanine mutations was cotransfected into COS-7 cells with GFP-PIAS1 and FLAG-SUMO-1. Equal amounts of total cell lysates were loaded on the gel, and wild-type or mutant HA-SOX9 was detected with anti-HA monoclonal antibody. Actin labeling was used for the loading control. E, sumoylated SOX9 is digested by the human desumoylation enzyme SENP2. Cells were cotransfected with HA-SOX9, wild-type or mutant GFP-PIAS1, FLAG-SUMO-1, and wild-type or mutant (mut) FLAG-SENP2 (human). Sumoylated SOX9 (*) from total cell lysates was detected with anti-HA monoclonal antibody. Sumoylated SOX9 was weakly expressed by two PIAS1 RING domain mutants and abolished upon coexpression of the desumoylation enzyme SENP2, but not of a mutant of SENP2.

Because PIAS proteins with mutations in the RING domain have a reduced SUMO-1 ligase activity, we also examined the effect of RING domain mutations in PIAS proteins on sumoylation of SOX9. Fig. 3C shows that coexpression of PIASα(C362A) or PIASβ(C362A) with SOX9 and SUMO-1 did not support SUMO-1 conjugation to SOX9 compared with wild-type PIAS proteins. Also, cotransfection with SUMO-1 and the inactive RING domain mutants of PIAS1 (PIAS1(C350A) and PIAS1(C350S)) decreased the intensity of the 95-kDa species of sumoylated SOX9 (Fig. 3E). In parallel experiments, the use of a SUMO-1 mutant unable to undergo conjugation (SUMO-1ΔGG) prevented the appearance of the 95-kDa species and hence the SUMO modification of SOX9 (Fig. 3C).

To determine whether SUMO modification of SOX9 takes place at the putative lysine sumoylation sites in SOX9, SOX9 mutants harboring mutations K61A, K253A, and K398A in different combinations were coexpressed with PIAS1 and SUMO-1. All combinations containing the K398A substitution of SOX9 were not conjugated to SUMO-1, indicating that Lys398 is the dominant sumoylation residue (Fig. 3D). Some
reduction of SOX9 sumoylation was seen after transfection with the 
Lys253 substitution, suggesting a minor role for Lys253 in sumoylation (Fig. 3D).

We also further examined the effect of the desumoylation enzyme 
SENP2 in COS-7 cells. Cotransfection of SENP2 and SUMO-1 with 
PIAS1 and SOX9 decreased the intensity of the 95-kDa species of 
sumoylated SOX9, whereas a known inactive SENP2 mutant had no 
effect (Fig. 3E). This efficient sumoylation of SOX9 requires PIAS pro-

teins with an active RING domain, a SUMO polypeptide that has the ability to be conjugated, and a specific site in SOX9.

SOX9 Is Sumoylated by PIAS1, -x, and -3 In Vitro—Next, we carried out in vitro sumoylation experiments using purified recombi-

nant SOX9 protein as a substrate to determine whether PIAS1, -x, -xβ, and -3 are able to conjugate SUMO-1 to SOX9. Tagged PIAS1, -x, -xβ, or -3 His-S-HA-tagged SOX9 was expressed in a baculovirus vector as a recombinant protein in S9 cells; purified by affinity chromato-
graphy on glutathione-Sepharose or nickel-agarose, respectively; and then used for sumoylation of SOX9 in the presence of purified recombi-

nant E1 (SU1A1/human UBA2 heterodimer) and E2 (human UBC2). In the absence of PIAS proteins, if E1, E2, or SUMO-1 was missing from the reaction, no sumoylation of SOX9 was detected (Fig. 4). In the absence of PIAS proteins, a sumoylated SOX9 species was detected with excess E2 protein (Fig. 4, lane 5), whereas no SOX9 was sumoylated with 10-fold less E2 (lane 6). Addition of PIAS1, -x, -xβ, or -3 strongly enhanced sumoylation of SOX9 in a dose-dependent manner, confirming the results of SOX9 sumoylation by PIAS1 proteins in transfec-
ted cells.

Ectopically Expresssed SUMO-1 Further Enhances the PIAS1-, -x, and -xβ- and -3-Induced Increase in the Activity of a SOX9-dependent Col2a1 Reporter—Addition of a SUMO-1 expression vector in cotransfection experiments with COS-7 cells together with SOX9 and PIAS proteins produced a further reproducible increase in the activity of the Col2a1 reporter (Fig. 5A). This increase above the level of activation of the reporter by PIAS proteins and SOX9 was inhibited by a SUMO-1 mutant unable to undergo ligation to the protein substrate (SUMO-1ΔGG). Similarly, in cells cotransfected with mutant forms of PIASxα and PIASβ in which the RING motif had been mutated (PIASxα(C362A) and PIASxβ(C362A), respectively), no SUMO-
dependent increase in transcription of the Col2a1 reporter occurred, although the increase in reporter activity with the PIAS mutants with-

out SUMO-1 was almost the same as that with the wild-type PIAS proteins (Fig. 5B). This strongly suggests that the SUMO ligase activity of PIAS proteins is not required to increase the activity of the SOX9-de-
pendent reporter. This view was further supported by the use of an expression vector for the desumoylation enzyme SENP2. SENP2 expression inhibited the increase in reporter activity due to sumoyla-
tion, whereas a SENP2 mutant unable to desumoylate did not inhibit reporter activity (Fig. 5C). Thus, these experiments indicated that, in COS-7 cells, the increase in SOX9-dependent reporter activity caused by PIAS proteins was not due to sumoylation of SOX9 by endogenous SUMO proteins. Fig. 5D shows that the observed effect of SOX9 coex-
pressed with PIAS1 and/or SUMO-1 on transcriptional activation of the Col2a1 reporter depended on SOX9 binding to DNA. Indeed, with a 
Col2a1 reporter in which the SOX9-binding sequence was mutated ((Mab)×4-p89-luciferase) or in which the enhancer was missing (p89-
luciferase), coexpression of SOX9 and/or PIAS1 and SUMO-1 completely failed to activate the Col2a1 reporter. This experiment also showed that addition of SUMO-1 further increased the cellular levels of 
SOX9. This increase in the levels of SOX9 was in parallel with the increase in activity of the wild-type Col2a1 reporter.

In the Western blot analysis of COS-7 cells in which SOX9 was coex-
pressed with wild-type PIAS or RING domain mutants, the levels of 
SOX9 were increased (Fig. 6A), suggesting that the SUMO ligase activity of PIAS is not required for the apparent stabilization of SOX9. To fur-
ther support the view that PIAS proteins increased the activity of the SOX9-dependent Col2a1 reporter in the absence of sumoylation, siRNAs were used to inhibit sumoylation (Fig. 6B and C). Cotransfac-
tion of siRNA to E2 (UBC9), which depleted UBC9 protein levels almost completely in COS-7 cells (Fig. 6B), blocked the increase in 
reporter activity due to SUMO-1 modification, but did not inhibit the relative increase in reporter activity due to PIAS1. Similarly, with 
siRNA to SUMO-1 or SUMO-2, the relative increase in reporter activity due to PIAS1 was largely maintained (Fig. 6C). Similar results were obtained upon addition of siRNAs to both SUMO-1 and 
SUMO-2 (Fig. 6C). The combination of siRNAs to SUMO-1, -2, and -3 showed similar results (data not shown), suggesting that PIAS1 acts on the SOX9-dependent reporter independently of sumoylation.

SOX9 Mutants Containing Mutations in the Sumoylation Sites Show Enhanced Transactivation of a Col2a1 Promoter-Reporter—SOX9 mutants containing mutations of three putative sumoylation sites (Lys61, Lys253, and Lys396) (Fig. 7A) did not undergo sumoylation (Fig. 3D). In trans-
fection of COS-7 cells, mutant SOX9 stimulated the SOX9-dependent reporter significantly more than did wild-type SOX9 even if the levels of mutually Sox9 were lower than those of wild-type SOX9 (Fig. 7B). This suggests the possibility that mutant SOX9 may have a higher intrinsic transcriptional activity compared with wild-type SOX9. Cotransfection of mutant SOX9 with PIAS further increased the activity of the SOX9-dependent reporter (Fig. 7B). In these experiments, the levels of mutant SOX9 increased in parallel with the increase in wild-type SOX9 levels produced by PIAS. These experiments suggested that the sumoylation
FIGURE 5. Increased activity of the SOX9-dependent Col2a1 reporter by PIAS proteins and by sumoylation. A, effect of coexpression of PIAS proteins, SOX9, wild-type SUMO-1, and SUMO-1ΔGG on the activity of the 4×48-p89 Col2a1 reporter. COS-7 cells were transfected with 0.77 μg of the Col2a1 reporter together with expression constructs encoding β-galactosidase (3 ng) for normalization and SOX9 (100 ng); PIAS1, -3, -x, or -β (300 ng); and wild-type SUMO-1 or SUMO-1ΔGG (200 ng) as indicated. *, p = 0.008; ***, p = 0.031; *, p = 0.15; *4, p = 0.070; *5, p = 0.007; *6, p = 0.023; *7, p = 0.022; *, p = 0.009. A, PIASx and PIASxβ enhance the activity of the SOX9-dependent Col2a1 reporter through the RING domain in the presence of SUMO-1. COS-7 cells were transfected with 0.77 μg of the Col2a1 reporter together with expression constructs encoding β-galactosidase (3 ng); SOX9 (3 ng); PIASx or PIASβ (300 ng) or RING domain-mutated (mut) PIASx(C362A) or PIASβ(C362A); and SUMO-1 (200 ng) as indicated. *, p = 0.0069; ***, p = 0.0836. C, the desumoylation enzyme inhibits the increased activity of the SOX9-dependent Col2a1 reporter due to PIASβ and SUMO-1. COS-7 cells were transfected with the Col2a1 reporter (0.77 μg) together with expression constructs encoding β-galactosidase (3 ng); SOX9 (3 ng), PIASβ (300 ng), SUMO-1 (200 ng), and wild-type or mutant SMT3IP2 (mouse origin; 300 ng) as indicated. *, p = 0.06. hSENP2WT, human wild-type SENP2. D, the increased activity of the Col2a1 reporter by PIAS1 and SUMO-1 depends on SOX9-DNA binding. COS-7 cells were transfected with the Col2a1 reporter (0.77 μg), a mutant reporter with mutations in each of the SOX9-binding sites [(Mab)X4 p89 Luc], or a minimal promoter reporter (p89 Luc) together with expression constructs encoding β-galactosidase (3 ng). Wild-type SOX9 (100 ng), PIAS1 (300 ng), and SUMO-1 (500 ng).
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FIGURE 6. PIAS proteins increase the activity of a SOX9-dependent reporter independently of sumoylation. A, RING domain mutants of PIAS1 stabilize SOX9. COS-7 cells were cotransfected with SOX9 expression vectors and with vectors expressing wild-type (wt) PIAS1, PIAS1(C350A), or PIAS1(C350S) (1 μg). SOX9 or actin in cell lysates was detected by Western blotting. B, depletion of the E2 UBC9 does not affect enhancement of SOX9-dependent reporter activity by PIAS1. COS-7 cells were transfected with human UBC9 siRNA (20 nM); and after a 48-h incubation, the cells were used for transient transfection of the 4×48-p89 COL2A1 reporter (0.77 μg) together with expression constructs encoding β-galactosidase (3 ng), SOX9 (100 ng), PIAS1 (300 ng), and SUMO-1 (200 ng) as described in the legend to Fig. 2. Depletion of UBC9 by siRNA in cell lysates is shown by Western blot (WB) analysis with anti-UBC9 antibody. C, depletion of SUMO-1 and SUMO-2 does not affect enhancement of SOX9-dependent reporter activity by PIAS1. COS-7 cells were transfected with human SUMO-1 and/or human SUMO-2 siRNA (20 nM each); and after a 48-h incubation, cells were used for transient transfection of the 4×48-p89 COL2A1 reporter together with expression constructs encoding β-galactosidase (3 ng), SOX9 (100 ng), and/or PIAS1 (300 ng) as indicated. Depletion of SUMO-1 and/or SUMO-2 in cell lysates was shown by Western blot (WB) analysis as described above. cont, control.

sites of SOX9 were not needed for the enhanced stabilization of SOX9 by PIAS. The reason for the apparent increased intrinsic transcriptional activity of mutant SOX9 is not understood, but the results indicate that the higher transcriptional activity of mutant SOX9 occurs without stabilization of mutant SOX9.

SOX9 Localization Diffuses after Sumoylation—SUMO proteins have been shown to localize to specific subnuclear structures known as PML-containing nuclear bodies (34, 35). These structures, which contain PML, Sp100, and other proteins, are associated with the nuclear matrix (36). To examine the effect of PIAS1 and sumoylation on the subnuclear localization of SOX9, we performed a four-color immunofluorescence analysis of transfected COS-7 cells. Both SOX9 and PIAS1 showed mainly a punctate nuclear staining pattern when transfected alone; SOX9 was present more densely around the nucleoli (Fig. 8A). When PIAS1 and SOX9 were cotransfected, both proteins were at least partially co-localized, showing a somewhat more diffuse pattern (Fig. 8B). Coexpression of PIAS1 and SUMO-1 resulted in complete co-localization and punctate staining. In contrast, the transfected SUMO-1 mutant SUMOΔGG did not localize to the nucleus (Fig. 8B). Coexpression of SOX9, PIAS1, and SUMO-1 showed a striking overlapping distribution of all three proteins in a distinctly diffuse pattern, which was not observed with the SUMO-1 mutant (Fig. 8C). To exclude the possibility that the observed distribution pattern might be due to artificial effects caused by the expression tags, the tags were exchanged. However, after the tags had been changed, the subnuclear distributions of SOX9 after coexpression of PIAS1 and SUMO-1 were similar to that shown in Fig. 8C (data not shown).

To exclude the possibility that the observed pattern of SOX9 distribution was not a result of overexpression, the distribution of endogenous SOX9 was examined in the chondrocytic cell line HCS-2/8, which contains endogenous SOX9 and PIAS1 (data not shown). Immunofluorescence analysis of cultured HCS-2/8 cells revealed a nuclear, but more diffuse, distribution of endogenous SOX9 (Fig. 8D).

SOX9 mutants lacking all three sumoylation sites did not show the diffuse pattern, but a more punctate subnuclear distribution when coexpressed with PIAS1 and SUMO-1 (Fig. 8E). Similarly, coexpression of the desumoylation enzyme SENP2 retained SOX9 in a more punctate subnuclear distribution in the presence of PIAS1 and SUMO-1, whereas coexpression of a SENP2 mutant did not show this effect (Fig. 8F).

DISCUSSION

The SUMO ligase proteins PIAS1, -α, -β, and -3 were identified in a yeast two-hybrid screen as SOX9-interacting proteins. Several assays provided additional evidence for a direct interaction between SOX9 and
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does not establish that a direct interaction exists between SOX9 and PIAS proteins. A two-hybrid assay in yeast showed that the C-terminal transcriptional activation domain of SOX9 was a major interaction segment, but that other segments could also participate in these interactions. The SOX9-PIAS interactions were further supported by in vitro pulldown assays, by co-immunoprecipitation of SOX9 and PIAS1 after cotransfection of COS-7 cells, by their co-localization in the nucleus of these cells as detected by immunofluorescence, and by electrophoretic mobility shift assays demonstrating the existence of a DNA-protein complex containing both SOX9 and PIAS1.

Our experiments also demonstrated that, both in intact cells and in vitro, PIAS proteins enhanced the sumoylation of SOX9, acting as SUMO ligases, as shown previously for other target proteins (23, 25). Indeed, sumoylation of SOX9 produced a slower migrating species of SOX9 on SDS gels, corresponding to an increase of ~20 kDa. This species was not present when either a SUMO mutant unable to be ligated to substrate protein or a PIAS mutant defective in SUMO ligase activity was used. SOX9 contains three potential sumoylation sites, but Lys$^{398}$ is the preferred site of SUMO modification. Indeed, the K398A mutation in SOX9 strongly inhibited sumoylation of SOX9.

In DNA cotransfections of COS-7 cells, all four PIAS proteins increased the SOX9-dependent activity of a Col2a1 reporter in which a short chondrocyte-specific enhancer was multimerized. This increase in reporter activity was paralleled by an increase in the cellular concentration of SOX9, suggesting the possibility that PIAS polypeptides might inhibit SOX9 degradation. These experiments were performed under conditions similar to those used for the co-immunoprecipitation experiments, suggesting that SOX9 and PIAS are part of the same complex. We acknowledge that these co-immunoprecipitation experiments did not establish that a direct interaction exists between SOX9 and PIAS polypeptides in transfected COS-7 cells. We noted an excellent correlation between the levels of SOX9 and the increase in reporter activity by the different PIAS polypeptides. This increase in reporter gene activity and the parallel increase in the levels of SOX9 took place in the absence of added SUMO. Indeed, with a SUMO mutant unable to be ligated to substrate protein or with a PIAS mutant defective in SUMO ligase activity, the increase in SOX9-dependent reporter activity still took place. Furthermore, it is unlikely that the increase in SOX9-dependent reporter activity was due to sumoylation of SOX9 mediated by endogenous SUMO because this enhanced SOX9-dependent reporter activity persisted even in presence of the desumoylation enzyme SENP2. The increase in reporter activity was, however, further enhanced by cotransfection with a SUMO-expressing vector.

Mutations in the three potential SOX9 sumoylation sites, which prevented the sumoylation of SOX9, increased the activity of the SOX9-dependent reporter, and a K398A mutation, the major site of SOX9 sumoylation, caused an increase in the activity of the SOX9-dependent reporter similar to that caused by the mutant with the three substitutions (data not shown). Lys$^{398}$ is also a major site of ubiquitination, as the same K398A mutation inhibits ubiquitination and produces a modest increase in the half-life of SOX9 (33). The increase in SOX9-dependent reporter activity produced by the mutant with mutations in the three potential sumoylation sites appears to be due to a higher intrinsic transcriptional activity of SOX9 and might be the result of better interactions of this mutant with coactivators or reduced interactions with corepressors. Thus, we believe that the increase in activity of the SOX9-dependent reporter, which was observed with the mutant that prevented sumoylation of SOX9, should not be interpreted as meaning that sumoylation inhibits the transcriptional activity of SOX9 given that all other experiments indicated that the PIAS polypeptides and sumoylation increased the levels of SOX9 and the activity of a SOX9-dependent reporter.

Normal turnover of SOX9 occurs, at least in part, through ubiquitination and proteasomal degradation. Indeed, in presence of an inhibitor of proteasomal degradation, the levels of SOX9 increased. In contrast, the same proteasome inhibitor did not further increase the cellular concentration of SOX9 found in cells cotransfected with PIAS. This suggests that PIAS proteins inhibit the proteasome-mediated degradation of SOX9. Furthermore, in the presence of the protein synthesis inhibitor cycloheximide, the cellular concentrations of transfected SOX9 decreased rapidly. This decrease was strongly inhibited in the presence of PIAS, supporting the hypothesis that PIAS proteins withdraw SOX9 from the normal pathway of proteasomal degradation and sequester SOX9 in a state resistant to degradation.

After transfection of COS-7 cells, SOX9 showed a punctate distribution in the nucleus. Cotransfection with PIAS1 showed significant co-localization of SOX9 and PIAS, with a somewhat more diffuse subnuclear distribution of SOX9. Cotransfection of SOX9, PIAS1, and SUMO-1 produced extensive co-localization of all three proteins in a distinctly diffuse nuclear distribution. This diffuse localization of all three proteins depended on the ability of SUMO-1 to be ligated and on intact sumoylation sites in SOX9. We speculate that the interactions of SOX9 with PIAS and SUMO changed the localization of SOX9 within the nucleus. This change to a diffuse distribution of SOX9 correlated with the highest activity of a SOX9-dependent reporter and with the highest cellular concentrations of SOX9. It should be noted that the
change in the subnuclear distribution of SOX9 was opposite the change in the distribution of LEF1, which, when cotransfected with PIAS, changed from a diffuse pattern to a punctate nuclear distribution. This change was associated with inhibition of the activity of a LEF1-dependent reporter in contrast to the increase in the activity of a SOX9-dependent promoter.

Fig. 9 summarizes our model for the role of PIAS in its interactions with SOX9 and for the role of sumoylation of SOX9. We postulate that SOX9, the SOX9-PIAS complexes, and sumoylated SOX9 are in equilibrium with each other; sumoylated SOX9 can be desumoylated through the enzyme SENP2. Our model implies that, when PIAS interacts with SOX9, it segregates SOX9, preventing its degradation through ubiquitination and the proteasome pathway. This would result in an increase in the cellular concentration of SOX9 and of transcriptionally active SOX9. Sumoylation of SOX9 also changes its subnuclear distribution.

We postulate that PIAS and the SUMO system have an important role in controlling the optimal cellular concentration of SOX9. Indeed, such an optimal SOX9 concentration is likely needed for the optimal function of SOX9. A 50% reduction in the cellular concentration of SOX9 as seen in patients with campomelic dysplasia, a genetic disease due to heterozygous mutations of SOX9, has severe consequences for skeleton formation and for sex determination. In mice, a modest increase in SOX9 of 20% in chondrocytes inhibits chondrocyte proliferation and causes dwarfism (7). In addition to the PIAS polypeptides and SUMO as well as the ubiquitination system, several other mechanisms are likely to participate in maintaining an optimal concentration of SOX9. PIAS and the SUMO pathway could also have a critical role in controlling the optimal cellular concentration of SOX9.
role in regulating the optimal subnuclear distribution of SOX9 needed to fulfill its function in controlling broad programs of cell differentiation and cell fate.

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