MOLECULAR AND CELLULAR BIOLOGY, Apr. 2007, p. 2661–2675 Vol. 27, No. 7
0270-7306/07/$08.00+0 doi:10.1128/MCB.01098-06

An Acetylation/Deacetylation-SUMOylation Switch through a Phylogenetically Conserved ψKXEP Motif in the Tumor Suppressor *HIC1* Regulates Transcriptional Repression Activity▽

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Received 19 June 2006/Returned for modification 18 July 2006/Accepted 16 January 2007

Tumor suppressor *HIC1* (*h*ypermethylated *i*n *c*ancer 1) is a gene that is essential for mammalian development, epigenetically silenced in many human tumors, and involved in a complex pathway regulating P53 tumor suppression activity. *HIC1* encodes a sequence-specific transcriptional repressor containing five *Krüppel*-like C_2H_2 zinc fingers and an N-terminal BTB/POZ repression domain. Here, we show that endogenous HIC1 is SUMOylated in vivo on a phylogenetically conserved lysine, K314, located in the central region which is a second repression domain. K314R mutation does not influence HIC1 subnuclear localization but significantly reduces its transcriptional repression potential, as does the mutation of the other conserved residue in the ψKXE consensus, E316A, or the overexpression of the deSUMOylase SSP3/SENP2. Furthermore, HIC1 is acetylated in vitro by P300/CBP. Strikingly, the K314R mutant is less acetylated than wild-type HIC1, suggesting that this lysine is a target for both SUMOylation and acetylation. We further show that HIC1 transcriptional repression activity is positively controlled by two types of deacetylases, SIRT1 and HDAC4, which increase the deacetylation and SUMOylation, respectively, of K314. Knockdown of endogenous SIRT1 by the transfection of short interfering RNA causes a significant loss of HIC1 SUMOylation. Thus, this dual-deacetylase complex induces either a phosphorylation-dependent acetylation-SUMOylation switch through a ψKXEXXSP motif, as previously shown for MEF2, or a phosphorylation-independent switch through a ψKXEP motif, as shown here for HIC1, since P317A mutation severely impairs HIC1 acetylation. Finally, our results demonstrate that HIC1 is a target of the class III deacetylase SIRT1 and identify a new posttranslational modification step in the P53-HIC1-SIRT1 regulatory loop.

HIC1 (*h*ypermethylated *i*n *c*ancer 1) is located in 17p13.3 in a region frequently hypermethylated or deleted in many types of prevalent human tumors (59). *HIC1* is a tumor suppressor gene since its enforced expression by stable transfection in various cancer cell lines results in a significant decrease in their clonogenic survival levels (59) and since heterozygous *Hic1*$^{+/-}$ mice develop, after 70 weeks, many different spontaneous malignant tumors (13). Moreover, animal models using *Hic1* and *p53* double-heterozygous-knockout mice have shown that the epigenetically silenced gene *Hic1* cooperates with the mutated tumor suppressor gene *p53* in determining cancer prevalence, progression, and spectrum (11). Indeed, *HIC1* is a direct P53 target gene through a P53-responsive element recently identified (7). Finally, a regulatory feedback loop between HIC1 and P53 has recently been deciphered in which HIC1 directly represses the transcription of *SIRT1*, which deacetylates and thereby inactivates P53 (12).

HIC1 is essential for normal mammalian development and could also be implicated in a contiguous-gene syndrome, the Miller-Dieker syndrome, a severe form of lissencephaly accompanied by developmental anomalies (61). Together with displaying perinatal death and a reduction in overall size, *Hic1*$^{-/-}$ mouse embryos display other developmental anomalies resembling those found in patients with Miller-Dieker syndrome (9).

The HIC1 protein is a sequence-specific transcriptional repressor containing three main functional domains: a conserved protein-protein interaction domain called BTB/POZ (Broad Complex, Tramtrack, and bric à brac/poxviruses and zinc finger) at the N terminus, five *Krüppel*-like C_2H_2 zinc fingers near its C terminus, and a central region which is not well conserved among the HIC1 proteins from various species (Fig. 1) (14, 16). HIC1 binds specifically to DNA through its zinc finger domain that recognizes the consensus sequence 5′-$^{C}/_{G}$NG^{C}/

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▽ Published ahead of print on 5 February 2007.

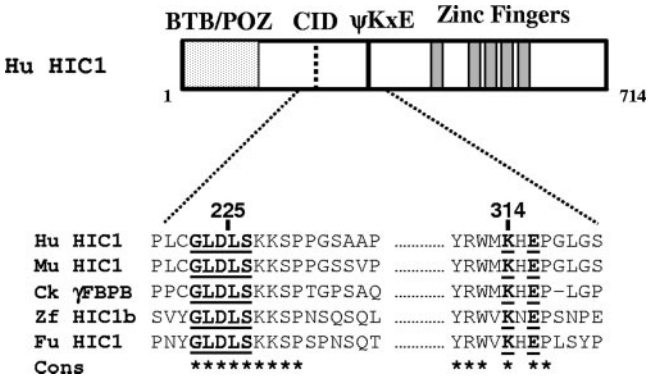

FIG. 1. Identification of a new conserved motif in the central repression domain of HIC1: a SUMOylation consensus. Schematic structure of the human HIC1 protein. The BTB/POZ domain and the five C_2H_2 zinc fingers are represented as dotted and gray boxes, respectively. The evolutionarily conserved CtBP-interaction domain (CID) (16) and the SUMOylation motif identified in this study (ψKXE) are represented as dotted and solid lines, respectively. Sequences from the various HIC1 proteins (starting at the conserved BTB/POZ domain) were aligned with CLUSTAL/Jalview (EMBL) and default parameters. Only the regions surrounding the CtBP interaction domain and the newly defined conserved region are shown. In the consensus lane (Cons), identical residues are shown as asterisks under the aligned sequences. The residues conforming to the CID consensus (GLDLS) and the SUMOylation consensus (ψKXE) are bold and underlined. Notably, the adjacent proline-directed phosphorylation site (SP) found in the recently defined PDSM or SAS ψKXEXXSP is not present in HIC1 (26). Hu, human; Mu, murine; Ck, chicken; Zf, zebrafish (*Danio rerio*); Fu, *Fugu rubipres*.

$_{G}$GGGCA$^{C}/_{A}$CC-3′ centered on a GGCA core motif (42). The BTB/POZ domain is a conserved structural motif found mainly in transcription factors, actin binding proteins, and substrate-specific adapters of CUL-3-based ubiquitin ligases (54). Crystal structures of the PLZF and BCL6 BTB/POZ domains have demonstrated that this domain is a tightly intertwined obligate dimer with a conserved dimerization interface (54). The homo-/heterodimerization as well as the oligomerization properties of the BTB/POZ domain are essential for the biological function of these proteins. The HIC1 BTB/POZ domain negatively regulates DNA binding of the full-length protein to a single site, whereas the oligomerization of the protein via this domain mediates cooperative DNA binding to multiple sites (42). Finally, the BTB/POZ domain is essential for the function of transcriptional repressors by directly recruiting nuclear corepressor (SMRT, N-CoR, or B-CoR)/histone deacetylase (HDAC) complexes, as shown for the human PLZF and BCL6 proteins (19, 38). Previously, we have shown that the HIC1 BTB/POZ domain is an autonomous transcriptional repression domain insensitive to trichostatin A (TSA), a specific inhibitor of class I and class II HDACs (14). The HIC1 central region is also an autonomous transcriptional repression domain that contains the short phylogenetically conserved sequence GLD LSKK, is involved in the recruitment of the CtBP corepressor (Fig. 1), and represses transcription in a TSA-sensitive manner (2, 16).

During the last 5 years, SUMOylation has emerged as a new and important versatile modification of numerous nuclear proteins (23, 28, 49, 57). SUMO (small ubiquitin-related modifier) is an 11-kDa polypeptide structurally related to ubiquitin and covalently conjugated to lysine residues within target proteins in the consensus ψKXE motif, where ψ is a large hydrophobic residue and X is any amino acid. This modification involves coordinated action, in a manner similar to the ubiquitination, of an E1 SUMO-activating enzyme, a unique E2-conjugating enzyme (Ubc9), and an E3 ligase, which promotes the transfer of SUMO from Ubc9 to the target protein. The protein inhibitor of activated STAT (PIAS) family proteins (48), nucleoporin protein RanBP2 (41), and the Polycomb protein Pc2 (31) have been identified as SUMO E3 ligases with some substrates specificity. Recently, the histone deacetylase HDAC4 has been shown to promote SUMOylation of MEF2 transcription factors independently of its deacetylase activity (26, 27, 63). However, the underlying mechanisms for this remain elusive since HDAC4 has been proposed to be a bona fide E3 ligase in vivo (64) but not in vitro (27) or to promote the phosphorylation of MEF2 on an adjacent serine residue to upregulate SUMOylation (27, 63). SUMOylation is a highly dynamic modification, and multiple proteases (Sentrin-specific proteases [SENP]) are able to remove SUMO from its specific substrates (28). In contrast to ubiquitination, which generally marks proteins for rapid degradation, SUMOylation is involved in the regulation of DNA binding activity, transcriptional activity, nuclear subcellular localization, and assembly of multiprotein complexes (23, 49, 57). The essential role of SUMO modification in a number of biological processes has been well established. However, it must be stressed that even though a small proportion of the substrate is modified and SUMO can be rapidly deconjugated, SUMOylation affects the long-term fate of the modified substrate. Several models have been proposed to solve these SUMO enigmas (28). Lysine residues are subject to several posttranslational modifications, including acetylation, methylation, ubiquitination, and SUMOylation. In some instances, the same lysine residue can be competitively targeted by two different modifications, resulting in distinct functional outputs, such as protein stability for the ubiquitination/SUMOylation of IκBα (17) or transcriptional activity for the acetylation/SUMOylation of the Sp3 (46) or MEF2 transcription factors (27). Recently, bioinformatics analyses identified a subset of SUMO consensus sites called the PDSM (phosphorylation-dependent SUMOylation motif), which associates a classical SUMO consensus site with an adjacent proline-directed phosphorylation site, ψKXEXXSP (29, 63), as for example, in the major MEF2 isoforms (26). In the case of MEF2A, a phosphorylation-regulated SUMOylation-acetylation switch (SAS) of this motif is essential for postsynaptic differentiation (51).

In this paper, we demonstrate that HIC1 is SUMOylated in vivo and in vitro on a phylogenetically conserved M/VK^{314}XEP motif located in the central repression domain of HIC1. The mutation of this motif does not affect the punctate nuclear localization of HIC1 in transient transfection assays. In contrast, K314R or E316A mutations significantly impair the repression potential of HIC1, as does the overexpression of SSP3 deSUMOylase. Furthermore, HIC1 is acetylated by P300/CBP. Strikingly, the SUMOylation-deficient K314R mutant is acetylated at a significantly lower level than wild-type (wt) HIC1 is, suggesting that this lysine is a target for both SUMOylation and acetylation. HDAC4 promotes SUMOylation on this K314 residue of HIC1, whereas SIRT1, a class III HDAC, as well as other class I or II HDACs deacetylates it. In addition, SIRT1

knockdown by RNA interference results in a partial loss of HIC1 SUMOylation. Finally, the mutation of the proline residue in the ψKXEP motif has little effect on HIC1 SUMOylation but severely impairs its acetylation.

Thus, HIC1 transcriptional repression activity is positively controlled by two types of deacetylases, SIRT1 and HDAC4, which increase its deacetylation and SUMOylation, respectively, on the same phylogenetically conserved lysine residue. Since this lysine residue is not contained within a PDSM/SAS motif as it is in the MEF2 transcription factors (63), we thus propose that ψKXEP represents a core motif qualifying as a phosphorylation-independent SUMOylation/acetylation switch. In addition, we have shown for the first time that HIC1 is a target of the class III deacetylase SIRT1 and we have thus identified a new posttranslational modification step in the P53-HIC1-SIRT1 regulatory loop.

MATERIALS AND METHODS

DNA constructs. The pcDNA3-FLAG-HIC1 expression vector and the 5XHIRE reporter gene have been previously described (16, 42). The pcDNA3 FLAG-HIC1 K314R, E316A, and P317A were derived from the wild-type FLAG-HIC1 vector by PCR mutagenesis. Construction details on the wild-type and K314R, E316A BTB-CR-Gal4 nuclear localization signal-hemagglutinin (HA) chimeras are available upon request. All vectors were verified by nucleotide sequencing.

PIASXα/ARIP3, PIASXβ/Miz1, and PIASγ (40) were kindly provided by Merlin Crossley (University of Sydney, Sydney, New South Wales, Australia). pCMV5-Myc-PIAS1 (45) and pCMV-FLAG-Pc2 (32) were kindly provided by S. H. Lin (Hong Kong University of Science and Technology, Hong Kong, China) and D. Wotton (University of Virginia, Charlottesville, VA), respectively, through the courtesy of Merlin Crossley. The expression vector for the SUMO-specific protease SSP3 (24) was kindly provided by Ron Hay (University of St. Andrews, St. Andrews, Scotland). The expression vectors for wt SIRT1 and its catalytically inactive mutant (H363Y) (34) were kindly provided by Tony Kouzarides (University of Cambridge, United Kingdom), as were expression vectors for Gal4-P300, HA-CBP, and FLAG-PCAF.

Cell culture and transfections. Cos-7, DAOY, RK13 (rabbit kidney), and U2OS cell lines were maintained in Dulbecco's medium supplemented with 10% fetal calf serum. EcR-CHO clone 6, a stably transfected CHO cell line with inducible FLAG-HIC1 expression under the control of five modified ecdysone response elements, has been previously described (16). FLAG-HIC1 expression was induced by the ecdysone analogue ponasterone A (Invitrogen) at 10 μM for 48 h.

Cells were transfected in OptiMEM (Gibco-BRL) by the polyethyleneimine method, as previously described (16), either in 100-mm dishes (in vivo interaction) with 2.5 μg of DNA or in 12-well plates (repression assay and confocal microscopy) with 500 ng of DNA. Cells were transfected for 6 h and then incubated in fresh complete medium for 48 h before being processed for the relevant assay.

In vitro and in vivo SUMOylation assays. In vitro SUMOylation assays were carried out as previously described (17, 50) by using 4 μl of a standard reticulocyte lysate (in vitro-translated substrate kit; Promega) per 20-μl reaction mixture.

The in vivo SUMOylation assay was carried out as previously described (50). Briefly, Cos-7 cells were transfected with 1.25 μg of pcDNA3, pcDNA3-FLAG HIC1, or pcDNA3-FLAG HIC1 K314R expression vectors and with 1.25 μg of pSG5-His_6-SUMO-1 or empty control vectors using the polyethyleneimine method. At 48 h posttransfection, the cells were washed in ice-cold phosphate-buffered saline (PBS), harvested directly in 1 ml of Gua8 buffer (6 M guanidine-HCl, 100 mM NaCl, 10 mM Tris, 50 mM NaH_2PO_4 [pH 8.0]), briefly sonicated, and centrifuged. Clarified extracts were incubated for 2 h with 15 μl (packed volume) of Ni-agarose affinity beads (TALON metal affinity resin; Clontech). Bound proteins were washed three times in Gua8 buffer, twice in Urea6.5 buffer (8 M urea, 100 mM NaCl, 50 mM NaH_2PO_4 [pH 6.5]), and once in cold phosphate-buffered saline before being eluted by boiling in Laemmli loading buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To detect FLAG-HIC1 proteins, Western blots were performed with the anti-FLAG M2 monoclonal antibody (Sigma). For the E3 ligase assays with limiting amounts of SUMO-1, 125 ng of pSG5-His_6-SUMO-1 and 125 ng of expression vectors for each E3 ligase were used (40).

For the SUMOylation assay in the presence of short interfering RNAs (siRNAs), 293T cells were first transfected in six-well plates with 2.2 pmol of SIRT1 siRNA (Ambion) or of negative control siRNA (Eurogentec) by using 8 μl of Interferin (Polyplus transfection) per well. Twenty-four hours after, cells were transfected for the SUMOylation assay with 900 ng of pcDNA3-FLAG-HIC1 and 900 ng of His-SUMO-1 expression vectors using 5 μl of Exgen 500 (Euromedex) transfection reagent per well. Finally, 24 h after this second transfection, cells were rinsed and harvested in 500 μl of cold PBS. Fifty microliters was directly lysed in Laemmli buffer (Input). After centrifugation, cells were resuspended in 1 ml of Gua8 buffer and the SUMOylation assay was performed as described above.

Immunoprecipitation assays. Cells were lysed directly in 1 ml of radioimmunoprecipitation assay buffer (RIPA; 150 mM NaCl, 20 mM Tris-HCl [pH 7.4], 1% Triton X-100, 0.1% SDS, 0.5% Na-deoxycholate) containing 20 mM *N*-ethylmaleimide (NEM; Sigma) to minimize deSUMOylation. After centrifugation, supernatants were incubated for 2 h with 4 μl of the anti-HIC1 (325) polyclonal antibody. Then, protein A-Sepharose beads (Amersham Biosciences) were added for 1 h. The beads were washed first with P1 buffer (RIPA) and then with P2 buffer (RIPA and TNE [100 mM Tris-HCl {pH 7.4}, 0.1 M NaCl, 1 mM EDTA], vol/vol), P3 buffer (TNE with NaCl 0.5 M), and P4 buffer (TNE). Finally, proteins were eluted by boiling in Laemmli loading buffer and separated by SDS-PAGE before Western blotting.

Western blotting and antibodies. Proteins were fractionated by SDS-PAGE and transferred onto nitrocellulose membranes. After 1 h of blocking in PBSM (PBS with 5% milk), the membranes were incubated overnight at 4°C with specific primary antibodies in PBSTM (PBS with 0.1% Tween 20 and 5% milk) and washed three times for 10 min with PBSN (PBS with 0.1% IGEPAL). The membranes were next incubated for 1 h at room temperature with secondary antibodies coupled to peroxidase (Amersham) (diluted 1/10,000) in PBSM, washed two times for 10 min with PBSN, rinsed with PBS, and revealed with a Western blot chemiluminescence reagent kit (Amersham).

Antibodies against various portions of the C-terminal part of HIC1 (325 and 2563) have been previously described (16). To detect SUMO-1, we used the monoclonal antibody 21C7 (catalog no. 33-2400; Zymed) raised against full-length SUMO-1. Anti-FLAG M2 (catalog no. F3165; Sigma) and anti-HA (Babco) are monoclonal antibodies directed against the epitope tag. The pan anti-acetyl-lysine is a monoclonal antibody (Ac-K-103) from Cell Signaling. To detect SIRT1, we used the monoclonal antibody 2G1/F7 (catalog no. 05-707; Upstate).

The secondary antibodies were anti-rabbit and anti-mouse immunoglobulins and a horseradish peroxidase-linked whole antibody from Amersham.

Confocal microscopy. Cos-7 cells were cultured on coverslips in 12-well plates and transfected as described above. Twenty-four hours after transfection, they were washed, fixed for 20 min in cold 3% paraformaldehyde, permeabilized in 0.1% Triton X-100 for 5 min, saturated for 30 min in 300 μl of PBS with 10% goat serum, incubated for 30 min with primary antibody diluted 1/500 in PBS with 10% goat serum, and incubated in the dark for 30 min with the secondary antibody diluted 1/200 in PBS with 10% goat serum. Between each stage, they were washed three times for 5 min in PBS. Then, they were placed inverted on a drop of Immuno-Fluore mounting medium on a slide. The slides were stored in the dark at 4°C and visualized with a Leica TCS-NT confocal microscope.

Repression assays. The repression assays were carried out as previously described with minor modifications (16). Two hundred nanograms of Gal4 plasmid and 250 ng of reporter plasmid were cotransfected. The β-OS *lacZ* vector (50 ng) was cotransfected in each assay to correct for variations in transfection efficiency. Forty-eight hours after transfection, cells were rinsed in PBS and lysed with Luc assay buffer (25 mM glycylglycine [pH 7.8], 15 mM $MgSO_4$, 4 mM EGTA, 1% Triton X-100). Luciferase and β-galactosidase activities were measured by using beetle luciferin (Promega) and the Galacto-Light kit (Tropix), respectively, with a chemiluminometer (Berthold).

Docking model of the MKHEP peptide in the catalytic domain of SIRT1. The structures of some low-energy conformers of the MKHEP peptide were obtained using the minimization and random searching tools within the SYBYL software (Tripos, Inc., St. Louis, MO). The Tripos force field was mainly used except for torsional coordinates and electrostatic interactions, for which the procedure described by Meziane-Tani et al. was used (39). In particular, partial atomic charges were calculated using quantum chemical methods based on the density functional theory (B3LYP hybrid functional and the 6-31G* basis set). For that purpose, Jaguar software was used. To derive atomic charges, the molecular electrostatic potential was fitted to a set of point charges located at the atomic centers also reproducing the dipole moment. Among the low-energy structures of MKHEP, the one displaying an extended conformation of the acetylated side

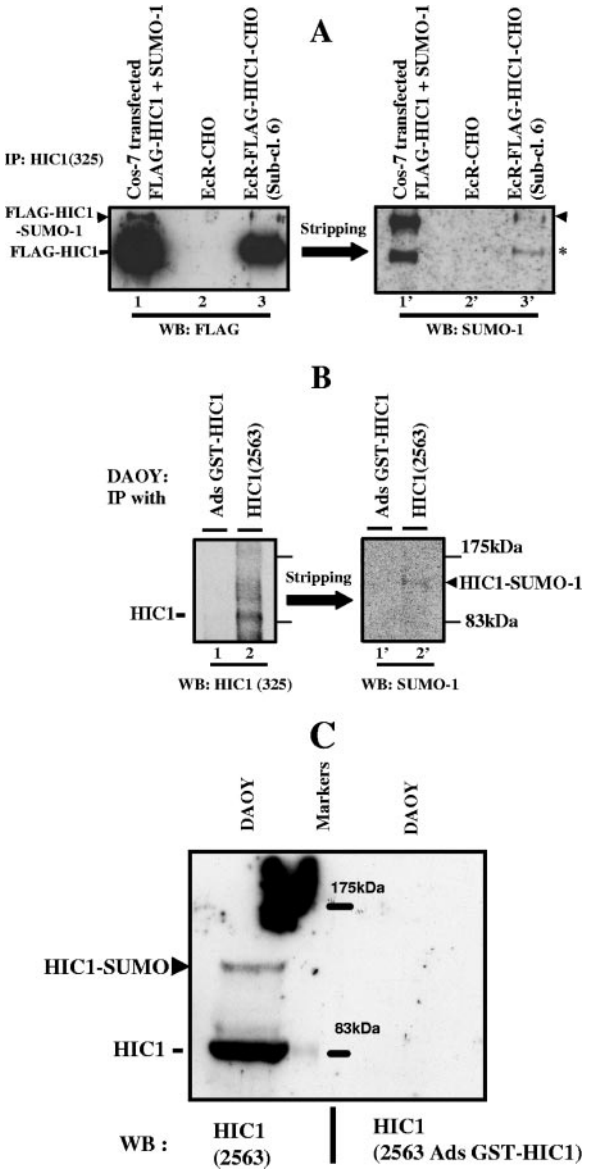

FIG. 2. HIC1 is SUMOylated in vivo. (A) In vivo SUMOylation of HIC1 in a stable inducible cell line. Cos-7 cells were transfected with expression vectors for FLAG-HIC1 and SUMO-1 (lane 1) as a positive control, whereas the parental EcR-CHO cell line (lane 2) and the stable inducible EcR-CHO-pIND-FLAG-HIC1 clone 6 cell line were treated with 10 μM ponasterone A for 48 h (16). Cell lysates were prepared in the presence of NEM and then subjected to immunoprecipitation (IP) with an anti-HIC1 polyclonal serum directed against a C-terminal peptide of HIC1 (325) (16). The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-FLAG M2 monoclonal antibody (left panel). The blot was stripped and probed with an anti-SUMO-1 monoclonal antibody (right panel, lanes 1′, 2′, and 3′). A remnant of the wild-type HIC1 proteins resistant to the stripping procedure yielded a convenient size control (*). (B) In vivo SUMOylation of endogenous HIC1 in the DAOY medulloblastoma cell line. Lysates were immunoprecipitated with antibodies raised against the C-terminal end of HIC1 (amino acids 590 to 714 fused to glutathione *S*-transferase [GST]) (2563) (16) (lane 2) and by the same immune serum adsorbed with an excess of the purified GST-HIC1 fusion protein used to immunize the rabbit (Ads-GST-HIC1, lane 1). The immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blotting (WB) with the anti-HIC1 325 antibody. After stripping, the blot was reprobed with an anti-SUMO-1 monoclonal antibody (right panel, lanes 1′ and 2′). (C) Detection of endogenous SUMOylated HIC1 proteins in the DAOY medulloblastoma cell line. Cells were directly lysed in Laemmli loading buffer. The lysates were immediately boiled for 10 min, and equal amounts were resolved by SDS-PAGE and analyzed by Western blotting using the polyclonal anti-HIC1 antibodies (2563) or the same antibodies but adsorbed with an excess of the purified GST-HIC1 polypeptide used to immunize the rabbit (2563 Ads GST-HIC1).

chain for the lysine residue was chosen according to structural information given in the 1SZC file of the Protein Data Bank (http://www.rcsb.org). Flexible docking was performed for MKHEP and 1SZC using the GOLD 3.1 software (30). The most stable docking model was selected according to the best-scored structure predicted by the Goldscore function (30).

RESULTS

In vivo SUMOylation of HIC1. By sequence comparisons of HIC1 proteins from various species, we have previously identified, in the central repression domain of HIC1, a conserved GLDLSKK motif that allows the recruitment of the CtBP corepressor (16). When cloned zebrafish (2) or in silico-deduced *Fugu rupibres* HIC1 sequences were included in these analyses, a second phylogenetically conserved peptide, YRWM/VKXEP, containing a potential SUMOylation consensus site (ψKXE) became obvious in the central region (Fig. 1).

To demonstrate that endogenous HIC1 proteins are covalently modified by SUMO in vivo, we first used our previously described CHO cell line with inducible human FLAG-HIC1 expression under the control of ecdysone-responsive elements (16). Upon induction by ponasterone A, two bands can be observed in the inducible HIC1 cell line (Fig. 2A, lane 3), but not in the parental EcR-CHO cell line (lane 2), after immunoprecipitation with anti-HIC1 antibodies and Western blot analyses with the monoclonal FLAG antibodies. The major band migrated with an apparent molecular mass of ca. 83 kDa, corresponding to HIC1 proteins, whereas the slower-migrating band showed a ca. 14-kDa increase in molecular mass, consistent with the addition of a single SUMO molecule. Indeed, the same bands were observed in Cos-7 cells cotransfected with FLAG-HIC1 and SUMO-1 expression vectors (Fig. 2A, lane 1). To more directly demonstrate that these slower-migrating HIC1-immunoreactive forms were SUMO-1-modified HIC1 proteins, the same blot was stripped and probed with anti-SUMO-1 monoclonal antibodies. As shown in Fig. 2A (lanes 1′ and 3′), the SUMO-1 antibodies recognized the upper band in the transfected Cos-7 cells and in the induced EcR FLAG-HIC1 CHO cells.

Using a similar experimental strategy, we next demonstrated that endogenous HIC1 proteins immunoprecipitated from the medulloblastoma cell line DAOY (35) are also SUMOylated, as shown by the presence of SUMO-1-immunoreactive species in the specific HIC1 immunoprecipitates (Fig. 2B, lane 2′). Finally, direct lysis of the DAOY cells in loading buffer allowed the detection of HIC1 and its SUMOylated form (Fig. 2C), as recently described for C/EBPα (47).

Thus, endogenous HIC1 proteins are subject to modification by SUMO-1 in vivo.

The phylogenetically conserved lysine 314 in the HIC1 central region is the target residue for SUMO-1 modification. To

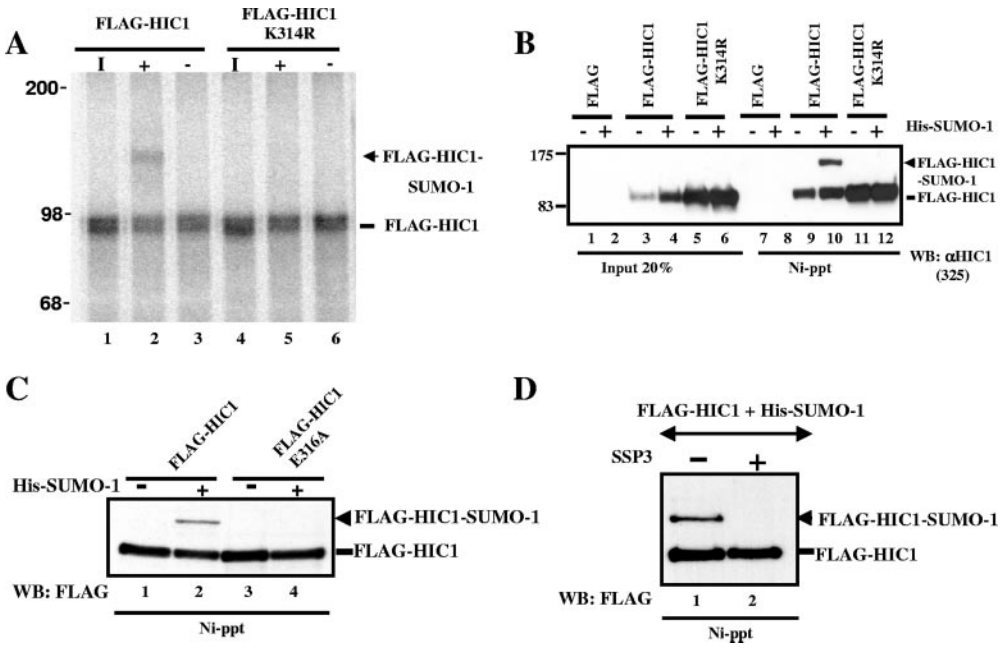

FIG. 3. The evolutionarily conserved lysine 314 is the major SUMO-1 modification site in HIC1 in vitro and in vivo. (A) In vitro-translated, [^{35}S]-labeled, full-length FLAG-HIC1 (lanes 1 to 3) or FLAG-HIC1 K314R (lanes 4 to 6) was analyzed by SDS-PAGE directly (I, Input; lanes 1 and 4) or was subjected to an in vitro modification reaction by incubation with a mix containing a fraction of HeLa cells (as a source of E1 activity), recombinant Ubc9, and ATP in either the presence (+; lanes 2 and 5) or the absence (−; lanes 3 and 6) of recombinant SUMO-1 before SDS-PAGE analysis and autoradiography. (B) Cos-7 cells were cotransfected with the indicated expression vectors and an empty vector (−) or a vector expressing His-tagged SUMO-1 (+). Forty-eight hours after transfection, cell lysates were subjected to nickel-agarose precipitation. Twenty percent of the lysates (lanes 1 to 6) and the proteins retained on nickel-agarose beads (Ni-ppt; lanes 7 to 12) were separated by SDS-PAGE and immunoblotted with the rabbit anti-HIC1 (αHIC1) (325) polyclonal antibody. (C) The SUMOylation of the wt HIC1 protein or the E316A mutant (a point mutation of the other strictly conserved residue in SUMOylation motifs) was analyzed as described for panel B, except that the FLAG monoclonal antibody was used to reveal the HIC1 proteins. (D) Cos-7 cells were cotransfected with expression vectors for wt HIC1 and His-SUMO1 and with an empty vector (−) or a vector expressing the deSUMOylase SSP3 (+). Forty-eight hours after transfection, cell lysates were subjected to nickel-agarose precipitation (lanes 1 and 2), separated by SDS-PAGE, and immunoblotted with the FLAG monoclonal antibody. WB, Western blot.

demonstrate that the K314 residue identified in silico is indeed the target residue that is modified by SUMO, we mutated this residue to arginine in the full-length HIC1 protein. The resulting FLAG-HIC1 K314R point mutant and the wild-type FLAG-HIC1 proteins were in vitro translated and labeled with [^{35}S]methionine in reticulocytes lysates. Then, their ability to undergo SUMO-1 modification was analyzed in an in vitro modification assay in the presence or absence of recombinant SUMO-1 with a fraction of HeLa cells as a source of E1 activity and purified Ubc9 (E2) as previously described (17, 50). A slower-migrating band was obtained with only the wild-type HIC1 protein (Fig. 3A, compare lanes 2 and 5).

To confirm SUMO-1 modification of HIC1 on K314 in vivo, Cos-7 cells were transfected by either the wild-type FLAG-HIC1 or the FLAG-HIC1 K314R point mutant with or without expression vectors for His-tagged SUMO-1 (His-SUMO-1). His-SUMO-1-modified proteins were affinity purified on nickel-agarose beads and analyzed by Western blotting. The transfection of FLAG-HIC1 or FLAG-HIC1 K314R alone resulted in the pull down of a significant amount of HIC1 and HIC1 K314R proteins (Fig. 3B, lanes 9 and 11), most likely due to interactions via the C_2H_2 zinc fingers, as previously described for Tramtrack (36). In the His-SUMO-1-transfected cells, a single SUMOylated band was observed with the wt HIC1 protein (Fig. 3B, lane 10). Consistent with the in vitro results, the FLAG-HIC1 K314R point mutant could no longer be modified by SUMO-1 (Fig. 3B, lane 12). As a further confirmation, a point mutation of the conserved glutamic acid residue in the ψKXE consensus, E316A, also abolishes the SUMOylation of HIC1 at K314 (Fig. 3C, lane 4). Similarly, the overexpression of SENP2/SSP3 deSUMOylase also results in the disappearance of the slower-migrating band, demonstrating that this species corresponds to a SUMOylated form of HIC1 (Fig. 3D, lane 2).

Thus, the phylogenetically conserved lysine 314 in the central repression domain is the target for the in vivo SUMO-1 modification of HIC1.

PIAS family members act as E3 ligases for HIC1. Proteins the PIAS family of have been widely reported to function as E3 ligases in the SUMO-1 conjugation pathway (48). To determine whether PIAS proteins could act as specific E3 ligases for HIC1 in vivo, we used the Cos-7 overexpression system in the presence of a limiting amount (1/10 of the amount used in standard assays) (Fig. 3B) of the His-tagged SUMO-1 expression vector (40). Under these experimental conditions, the SUMOylated form of HIC1 became almost undetectable (Fig. 4A, compare lanes 1 and 2). However, upon the cotransfection of PIAS expression vectors, PIAS1 or PIASXα/ARIP3, but not PIASγ or PIASXβ/MIZ1 (Fig. 4A, lanes 3 to 6), restored HIC1 SUMOylation.

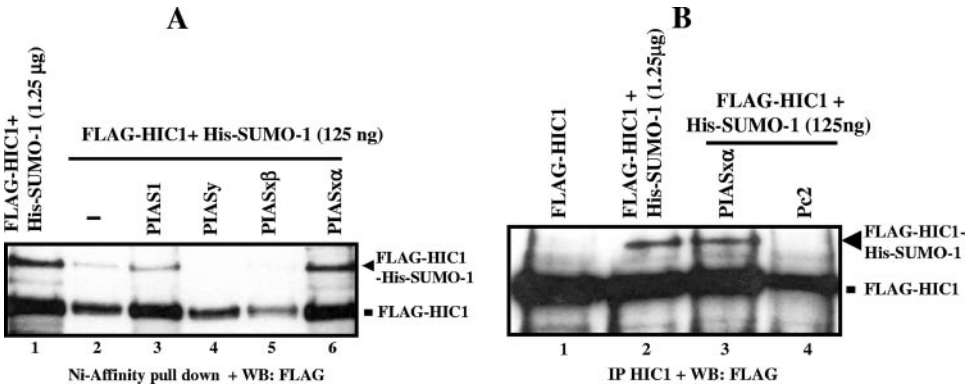

FIG. 4. PIAS family members (but not Pc2) are E3 ligases for HIC1 in vivo. (A) Cos-7 cells were transfected with 1.25 μg of FLAG-HIC1 and 1.25 μg His-SUMO-1 expression vectors (lane 1) or 125 ng of His-SUMO-1 expression vector alone (lane 2) or together with the expression vectors for PIAS1, PIASγ, PIASXβ, and PIASXα (lanes 3 to 6). Forty-eight hours after transfection, cell lysates were subjected to nickel-agarose precipitation, separated by SDS-PAGE, and immunoblotted with the anti-FLAG monoclonal antibody. The lack of HIC1 SUMOylation in the presence of PIASγ and PIASXβ (lanes 4 and 5) is due to the fact that these overexpressed E3 ligases conjugate the limiting amount of SUMO-1 onto their own endogenous substrates, thus precluding the SUMOylation of HIC1. (B) Cos-7 cells were transfected with 1.25 μg of FLAG-HIC1 expression vector alone (lane 1) with 1.25 μg His-SUMO-1 expression vector (lane 2) or 125 ng of His-SUMO-1 expression vector together with the expression vectors for PIAS1Xα or Pc2 (lanes 3 and 4). Forty-eight hours after transfection, cells were lysed in buffer (RIPA) containing 20 mM NEM. Lysates were subjected to immunoprecipitation (IP) with the polyclonal anti-HIC1 antibody, separated by SDS-PAGE, and immunoblotted with the anti-FLAG monoclonal antibody. WB, Western blot.

Since the HIC1 central region contains a CtBP binding site close to the SUMOylation site, we also investigated whether Pc2, the E3 ligase for CtBP (32), could also modulate the SUMOylation of HIC1 in vivo. For this investigation, we used immunoprecipitation instead of Ni affinity pulldown since Pc2 contains a polyhistidine stretch and could thus bind to Ni beads. As shown in Fig. 4B, lane 4, Pc2 does not induce the SUMOylation of HIC1. In addition, the deletion of the GLD LSKK motif of HIC1 responsible for the recruitment of CtBP (16) or the overexpression of CtBP1 or CtBP2 has no effect on the SUMOylation of HIC1 in vivo (data not shown).

These results demonstrate that some PIAS proteins, but not Pc2, stimulate the SUMOylation of HIC1 in vivo.

SUMOylation of lysine 314 is not required for the punctate nuclear localization of HIC1. Since SUMOylation has been implicated in the subnuclear localization of the targeted protein (10), we analyzed HIC1 localization by immunofluorescence confocal microscopy. In transiently transfected Cos-7 cells, wt FLAG-HIC1 and FLAG-HIC1 K314R proteins localized to similar punctate nuclear structures, typical of many overexpressed BTB/POZ proteins (Fig. 5A) (16, 18). Thus, the mutation of the key lysine residue in the SUMOylation site does not significantly alter subcellular localization of HIC1. We next coexpressed FLAG-HIC1 or FLAG HIC1K314R with SUMO-1 and performed double-labeling experiments with the rabbit anti-HIC1 (325) polyclonal antibodies and the mouse anti-SUMO-1 monoclonal antibody. All of the nuclear dots containing the ectopically expressed wild-type FLAG-HIC1 proteins were also labeled by the SUMO-1 antibodies, indicating that they contain SUMOylated HIC1 proteins (33). Consistent with the in vivo labeling assay (Fig. 3B), the expression of the FLAG-HIC1 K314R mutant did not result in the recruitment of SUMO-1 to the HIC1 nuclear dots (Fig. 5B, bottom panels).

Thus, these results confirm that lysine 314 is the target for in vivo SUMOylation of HIC1 and further demonstrate that this modification did not significantly modify the subnuclear localization of HIC1, at least not in transient transfection experiments.

SUMOylation of lysine 314 positively regulates the transcriptional repression activity of HIC1. The SUMOylation site is located in the central region which is an autonomous repression domain (16). To assess the functional consequences of the SUMO-1 modification of HIC1, we next compared the transcriptional repression potentials of wild-type and SUMOylation-deficient K314R HIC1 proteins. As a first approach, we decided to use the Gal-4 fusion assay, which has been widely used to analyze the effects of SUMOylation on various transcription factors. However, preliminary results demonstrated that a chimera containing the HIC1 central region (residues 144 to 422 of the human HIC1 protein) fused downstream of the Gal-4 DNA binding domain (Gal4-CR) was not SUMOylated, although it was nuclear and able to efficiently interact with CtBP (data not shown) (16). These results indicated that a heterologous dimerization domain, namely, the Gal-4 DNA binding domain, could not fully substitute the BTB/POZ domain for SUMOylation of HIC1, whereas it can for the recruitment of CtBP (16).

We thus constructed two other chimeras, BTB-CR-G4 and BTB-CR-G4 K314R, in which the HIC1 BTB/POZ domain and the central region (CR), corresponding to residues 1 to 422 of the human HIC1 protein, are fused to a C-terminal Gal-4 DNA binding domain (DBD), thus mirroring the structure of the wild-type HIC1 protein with its C-terminal *Krüppel*-like C_2H_2 zinc fingers (Fig. 1 and 6A). In vivo SUMOylation assays with Cos-7 cells demonstrated that the wild-type BTB-CR-Gal4 chimera was efficiently SUMOylated in contrast to the K314R point mutant (Fig. 6B, lanes 5 and 6).

RK13 cells were then cotransfected with a reporter plasmid pG5-luc (containing five copies of a Gal4 binding site in front of a luciferase gene driven by a minimal herpes simplex virus thymidine kinase [*tk*] promoter: 5XGal4-tk-luc) and either the empty Gal4 vector or the vectors carrying the wild-type or the mutant K314R BTB-CR-Gal4 chimeras. Both chimeras re-

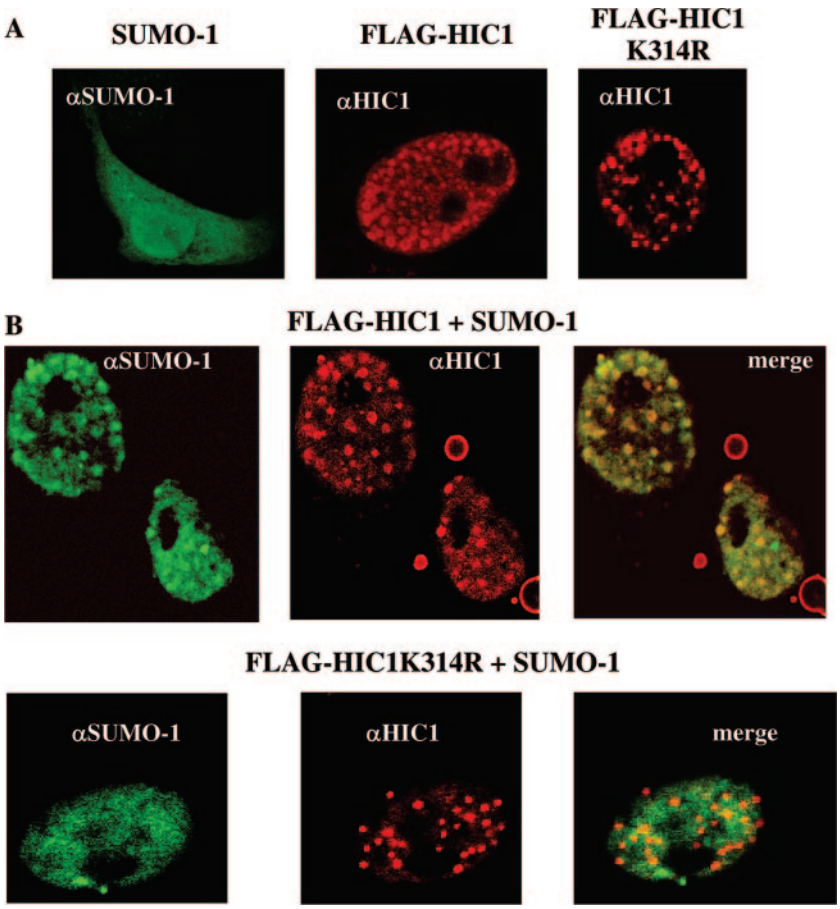

FIG. 5. wt FLAG-HIC1 but not FLAG-HIC1 K314R colocalizes with SUMO-1 on nuclear dots in transfected Cos-7 cells. (A) The mutation of the SUMOylation consensus does not impinge on the subnuclear localization of ectopically expressed HIC1 proteins. Cos-7 cells were transfected with expression vectors for the above-indicated proteins and fixed 24 h after transfection. Cells were labeled with the SUMO-1 monoclonal antibody (αSUMO-1), followed by fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (IgG) antibody, or with the 325 polyclonal antibody (FLAG-HIC1 and FLAG-HIC1 K314R), followed by Texas Red-conjugated anti-rabbit IgG antibody. Confocal images are shown. (B) HIC1 but not HIC1 K314R colocalizes at nuclear dots with SUMO-1. After the coexpression of FLAG-HIC1 (top) or FLAG-HIC1 K314R (bottom) with SUMO-1 in Cos-7 cells, SUMO-1 and FLAG-HIC1 proteins were visualized as described for panel A. Each horizontal lane represents the same cells immunostained with the monoclonal anti-SUMO-1 antibody and then the polyclonal anti-HIC1 325 antibody, and finally, all signals are merged in the last picture.

pressed luciferase gene expression relative to the basal level obtained with the Gal4 DBD alone, G4. However, the K314R chimera repressed transcription almost 1.8-fold less efficiently than did the wild-type HIC1 chimera (Fig. 6C). The same effect was obtained, albeit to a lesser extent, when the repression of the full-length HIC1 proteins, either the wild type or the K314R point mutant, was assessed in U2OS cells on an SV40-luc reporter gene containing five copies of the HIC1 responsive element (5XHiRE) (42) (data not shown). Similarly, a BTB-CR-G4 E316A chimera was not SUMOylated in vivo in Cos-7 cells (data not shown) and was severely impaired in its repression potential (Fig. 6D). Since K314R or E316A point mutations are sufficient to abolish the in vivo SUMOylation of HIC1 (Fig. 3B and C), these results strongly support the idea that this covalent modification in the central region of HIC1 positively regulates its transcriptional repression potential.

In keeping with these results, the cotransfection of an expression vector for SENP2/SSP3 (24) which is able to deSUMOylate HIC1 (Fig. 3D) has no significant effects on the basal level obtained with the Gal4 DBD alone but induces a decrease of two-thirds in the transcriptional repression mediated by the wt BTB-CR-Gal4 chimera (Fig. 6E). Interestingly, the effect of SSP3 on the wt chimera is more pronounced than that induced by the K314R mutation (Fig. 6C). Furthermore, the overexpression of SENP2/SSP3 also affects the transcriptional repression induced by the non-SUMOylable K314R chimeras (Fig. 6E). An explanation could be that SENP2/SSP3 induces not only the deconjugation of HIC1 itself but also that of other SUMOylated proteins involved in HIC1-mediated repression.

Taken together, these results demonstrate that the SUMOylation of the central region of HIC1 positively regulates its transcriptional repression potential.

Lysine 314 is also subject to reversible acetylation. Several covalent posttranslational modifications, such as ubiquitination, SUMOylation, methylation, and acetylation, can target lysine residues. Recently, the transcriptional activity of some

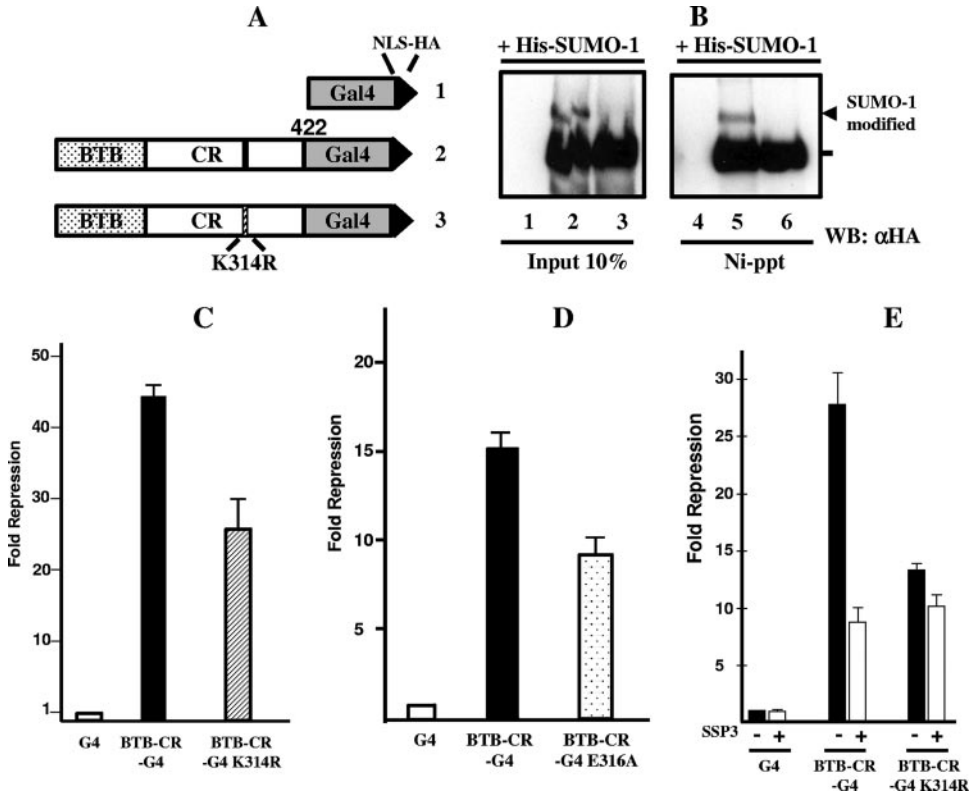

FIG. 6. SUMO-1 modification modulates HIC1-mediated transcriptional repression. (A) Schematic structure of the two HIC1 BTB-CR-Gal4 chimeras. Numbers refer to human HIC1 residues. The BTB/POZ domain, the CR, and the Gal4 DBD domain are represented as dotted, white, and gray boxes, respectively. The SUMOylation consensus site is shown as a black line, and the mutated SUMOylation consensus site is shown as a hatched line. A nuclear localization signal (NLS) and an HA epitope tagged at the C-terminal part of the chimeras are shown as black triangles. (B) The BTB-CR-G4 chimera, but not the mutated BTB-CR-G4 K314R chimera, is SUMOylated in vivo. Whole-cell extracts prepared from Cos-7 cells transfected with vectors expressing His-tagged SUMO-1, and the different Gal4 chimeras were either immunoblotted with anti-HA antibody directly (left panel, lanes 1 to 3) or subjected to Ni affinity chromatography prior to Western blot (WB) analysis (right panel, lanes 4 to 6). The arrowhead indicates the position of the SUMO-1-modified BTB-CR-G4 protein. (C) The mutation of K314 in the SUMOylation site reduces the repression capacity of the BTB-CR-Gal4 chimeras. RK13 cells were transiently transfected in triplicate with 200 ng of the indicated Gal4 chimeras and 250 ng of the pG5-luc reporter (schematically drawn in panel A). The luciferase activity was normalized to the β-galactosidase activity of a cotransfected β-OS-lacZ construct (50 ng). After normalization, the data were expressed as Luc activity relative to the activity of pG5-luc with Gal4-NLS-HA expression vector (G4), which was given an arbitrary value of 1. The results are the mean values and standard deviations (error bars) from one independent transfection performed in triplicate that is representative of three independent experiments. (D) The mutation of the consensus E316 residue in the SUMOylation site also reduces the repression capacity of the BTB-CR-Gal4 chimera. A similar experiment was conducted as described above but with expression vectors for the wt BTB-CR-Gal4 chimera or the E316A point mutant. Error bars indicate standard deviations. (E) Expression of the deSUMOylase SENP2/SSP3 impairs the repression potential of the wt and non-SUMOylable K314R BTB-CR-Gal4 chimeras. RK13 cells were transiently transfected in triplicate with 150 ng of the indicated Gal4 chimeras and 200 ng of the pG5-luc reporter alone (−) or in the presence (+) of 200 ng of the SSP3 expression vector. The luciferase activity was normalized to the β-galactosidase activity of a cotransfected β-OS-lacZ construct (50 ng). After normalization, the data were expressed as Luc activity relative to the activity of pG5-luc with the wt chimera in the absence of the SSP3 expression vector, which was given an arbitrary value of 100%. The results are the mean values and standard deviations (error bars) from two independent transfections performed in triplicate.

transcription factors, such as Sp3, MEF2, and PLAG, has been shown to be regulated by reversible acetylation or SUMOylation targeting the same lysine residue (6). To test whether this holds also true for HIC1 lysine 314, we first asked whether HIC1 could be acetylated by histone acetyltransferases. As shown in Fig. 7A, coexpression in Cos-7 cells of the histone acetyltransferase CBP or p300 can induce HIC1 acetylation (Fig. 7A, lanes 2 to 4), whereas PCAF is unable to do so (lane 5). Relative to wild-type HIC1, the K314R mutant is less acetylated by p300 in the presence of general HDAC inhibitors, TSA (an inhibitor of the class I and II HDACs), and nicotinamide (an inhibitor of the class III NAD^+-dependent Sir2 family deacetylases), either alone (data not shown) or in combination (Fig. 7B, compare lanes 4 and 6). Collectively, these data suggest that HIC1 is acetylated on several lysine residues and, further, that the conserved lysine 314, which is the only SUMO acceptor site, can also be modified by acetylation.

Lysine 314 is deacetylated by SIRT1 but not by HDAC4. HIC1 interacts with the deacetylase SIRT1, and this complex binds to and represses the transcription of the *SIRT1* promoter (12). SIRT1 is also known to deacetylate several substrates, including P53 (34) and MEF2D (27). In the latter case, a lysine subject to reversible acetylation catalyzed by CBP and SIRT1 is also SUMOylated. Moreover, this SUMOylation is enhanced by HDAC4 by a still poorly understood mechanism (26, 27, 63, 64). These observations led us first to investigate whether

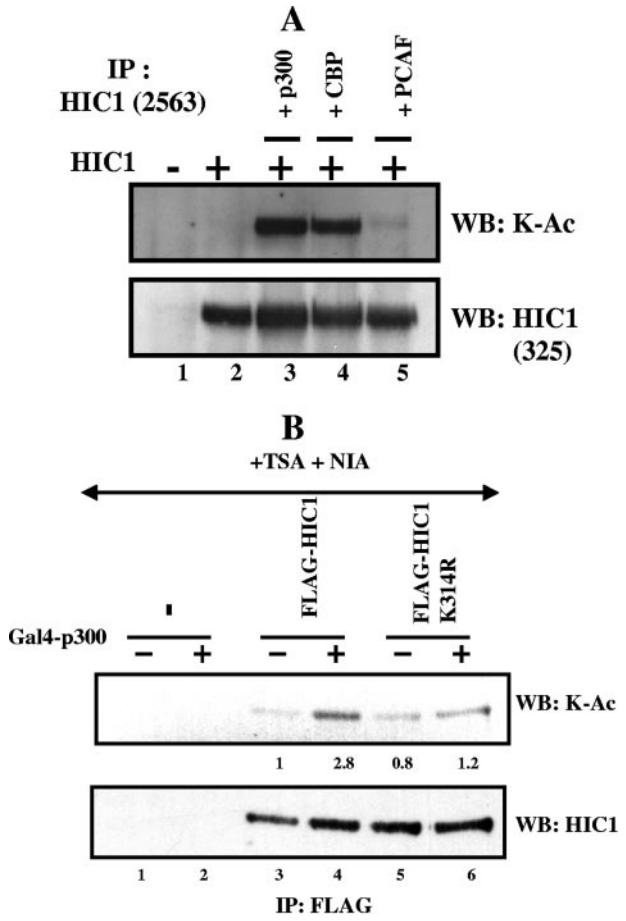

FIG. 7. HIC1 is acetylated on several lysine residues, including K314. (A) HIC1 was expressed alone (lane 2) or with the indicated acetyltransferase (lanes 3 to 5) by transient transfection in Cos-7 cells. Cells were treated with 300 nM TSA for 24 h before lysis and immunoprecipitation (IP). Lane 1 represents untransfected cells used as a control. HIC1 acetylation was detected by immunoprecipitation with the polyclonal anti-HIC1 antibody (2563) and Western blot (WB) analysis with the monoclonal pan acetyl-lysine antibody (K-Ac) from Cell Signaling (top panel). Western blotting with another anti-HIC1 polyclonal antibody (325) was used to ascertain the presence of HIC1 proteins (bottom panel). (B) The acetylation levels of wt FLAG-HIC1 and FLAG-HIC1 K314R were determined (as described above) in the presence of HDAC inhibitors (300 nM TSA and 5 mM NIA) added 24 h before lysis without (−; lanes 3 and 5) or with (+; lanes 4 and 6) expression vectors for P300. Lanes 1 and 2 correspond to controls in the absence of HIC1. Western blots were quantified using Syngene Tools. The ratio between wild-type Ac-HIC1 and total wild-type HIC1 with p300 in the absence of inhibitors was arbitrarily set to 1, and the values obtained are indicated between the two panels.

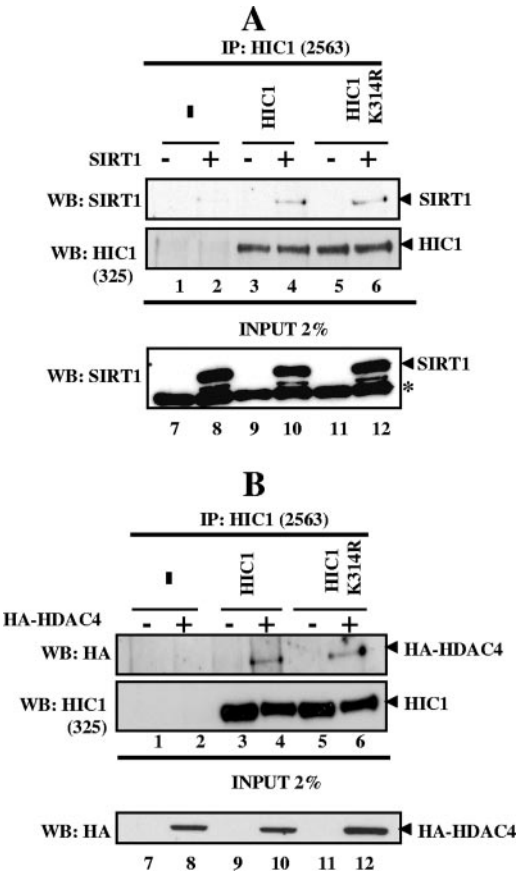

FIG. 8. wt HIC1 and the K314R mutant interact with SIRT1 and HDAC4. (A) Cos-7 cells were mock transfected (lane 1) or transfected with expression vectors for FLAG-tagged HIC1 proteins (1.25 μg; wild type or K314R mutant) and SIRT1 (1.25 μg) (lanes 2 to 6). HIC1 proteins were immunoprecipitated (IP) from cell lysates with anti-HIC1 2563 polyclonal antibodies. The resulting immunoprecipitates were then Western blotted and analyzed with the anti-SIRT1 monoclonal antibody (upper panel). The blot was stripped and probed with the rabbit anti-HIC1 polyclonal antibody (325) to ascertain the presence of HIC1 (middle panel). Two percent of each total cell extract (Input) was resolved by SDS-PAGE and immunoblotted with the anti-SIRT1 antibody (lower panel). The asterisk refers to a nonspecific band as specified by the supplier (Upstate). (B) A similar experiment was conducted as described above but with expression vectors for FLAG-HIC1 proteins (wild type or K314R mutant) and HA-tagged HDAC4. HDAC4 proteins were detected by using an anti-HA monoclonal antibody (Babco). −, absence of; +, presence of.

HIC1 can interact with SIRT1 and/or HDAC4 and whether this interaction can be regulated by SUMOylation. Using transient transfection assays with Cos-7 cells, we first demonstrated that wt HIC1 interacts with both SIRT1 and HDAC4 (Fig. 8A and B, lanes 4), albeit in a SUMO-independent manner, since the wt and the K314R mutant proteins interact equally well with SIRT1 and HDAC4 (Fig. 8A and B, lanes 6).

As to the functional consequence of these interactions, ectopic expression of SIRT1 dramatically reduces the p300-induced acetylation of HIC1 (Fig. 9A, lanes 2 and 3), whereas the overexpression of an inactive SIRT1 enzyme (SIRT1 H363Y) (34) only slightly affects HIC1 acetylation (Fig. 9A, lanes 2 and 4). By contrast, the overexpression of HDAC4 has no effect on the acetylation level of HIC1 (Fig. 9B, lanes 2 and 3).

These experimental data showing that HIC1 is a target of the deacetylase SIRT1 were confirmed by a structural analysis using the Sir2-NAD-H4 complex, *Saccharomyces cerevisiae* (Protein Data Bank code 1SZC), as a template for the catalytic domain of human SIRT1. In that model, the acetylated lysine side chain of the HIC1 MKHEP peptide appears to be close to the H135 catalytic residue and a little bit far from F79. It is also close to the important residues D43, F44, and R45. In particular, the model displays a strong hydrogen bond between the

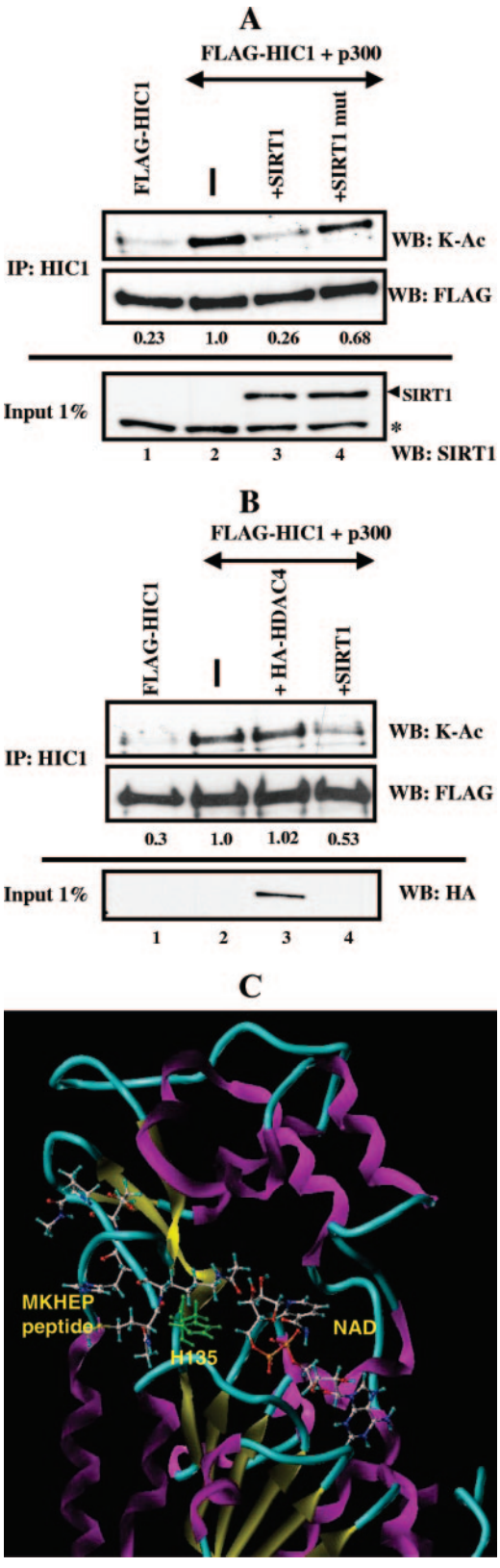

FIG. 9. Class III deacetylase SIRT1 has HIC1 deacetylase activity but not class II deacetylase HDAC4. (A) Cos-7 cells were transfected with expression vectors for FLAG-tagged HIC1 proteins (1.25 μg) in the absence (lane 1) or presence (+; lanes 2 to 4) of expression vectors for the acetyltransferase p300 (1 μg). The class III HDAC SIRT1 or its catalytic dead mutant (H363Y) was cotransfected (1.25 μg; lane 3 or 4, respectively). HIC1 proteins were immunoprecipitated from cell lysates with the anti-HIC1 2563 polyclonal antibodies. The resulting immunoprecipitates (IP) were then Western blotted (WB) and analyzed with the anti-monoacetylated lysine monoclonal antibody (upper panel). The blot was stripped and probed with the FLAG monoclonal antibody to ascertain the presence of HIC1 (middle panel). The ratio between Ac-HIC1 and total HIC1 with p300 (lane 2) was arbitrarily set to 1, and the values obtained are indicated below each lane. One percent of each total cell extract (Input) was resolved by SDS-PAGE and immunoblotted with the anti-SIRT1 antibody (lower panel). The asterisk refers to a nonspecific band as specified by the supplier (Upstate). Note that only SIRT1, not its enzymatic-dead mutant, changes HIC1 acetylation. (B) The acetylation levels of HIC1 in the presence of SIRT1 or HA-HDAC4 were examined as described above. Note that HDAC4 (lane 3) does not change HIC1 acetylation in contrast to SIRT1 (lane 4). (C) A docking model of the MKHEP peptide in the catalytic domain of SIRT1. The K acetylated side chain (C = O group) is strongly hydrogen bonded to the ribose moiety (3′ hydroxyl group) attached to the nicotinamide part of NAD. The catalytic histidine is green.

carbonyl oxygen atom of the acetylated lysine side chain of MKHEP and the 3′ hydroxyl group of the ribose attached to the nicotinamide moiety of NAD (Fig. 9C).

Thus, these results demonstrate that while HIC1 interacts with both HDAC4 and SIRT1, only the latter functions as a HIC1 deacetylase.

HDAC4 upregulates the SUMOylation of HIC1 on lysine 314. In MEF2D, the same lysine residue is a target for reversible acetylation mediated by CBP and SIRT1 and for SUMOylation potentiated by HDAC4 (27). Our results thus prompted us to investigate whether HDAC4 was also able to upregulate the SUMOylation of HIC1 on lysine 314. To that end, we used the Cos-7 overexpression system in the presence of a limiting amount of the His-tagged SUMO-1 (34) in conjunction with a bona fide E3 ligase for HIC1, PIAS1 (Fig. 4A), or HDAC4. Under these conditions, PIAS1 (but not PIASγ, used as a relevant negative control) (Fig. 4A) and, more importantly, HDAC4 restored HIC1 SUMOylation (Fig. 10A, lanes 3 to 5). Thus, HDAC4 is able to promote SUMOylation on HIC1 lysine 314, which is also a target for acetylation by CBP (Fig. 7) and deacetylation by SIRT1 (Fig. 9). To confirm this potential interplay between these two lysine-targeting modifications, we hypothesized that the levels of HIC1 SUMOylation and acetylation would be inversely correlated. Indeed, the SUMOylation of HIC1 K314 is increased when an active SIRT1 enzyme is overexpressed (Fig. 10B, lane 2) and decreased in the presence of its specific inhibitor nicotinamide (NIA) (Fig. 10B, lane 5). Interestingly, although HDAC4 is not able to deacetylate HIC1 and, hence, K314 (Fig. 9B, lane 3), TSA significantly decreased the SUMOylation level of HIC1 on K314 (Fig. 10B, lane 4), indicating that other class I and/or II HDACs can also directly or indirectly regulate the SUMOylation of HIC1. To confirm that SIRT1 directly controls HIC1 SUMOylation, we performed a SUMOylation test with HEK293T cells transfected beforehand with *SIRT1* siRNA or control siRNA. SIRT1 siRNA transfection caused the efficient knockdown of SIRT1 and affected levels of SUMOylated HIC1 proteins (Fig. 10C). This effect is specific since the SUMOylation of

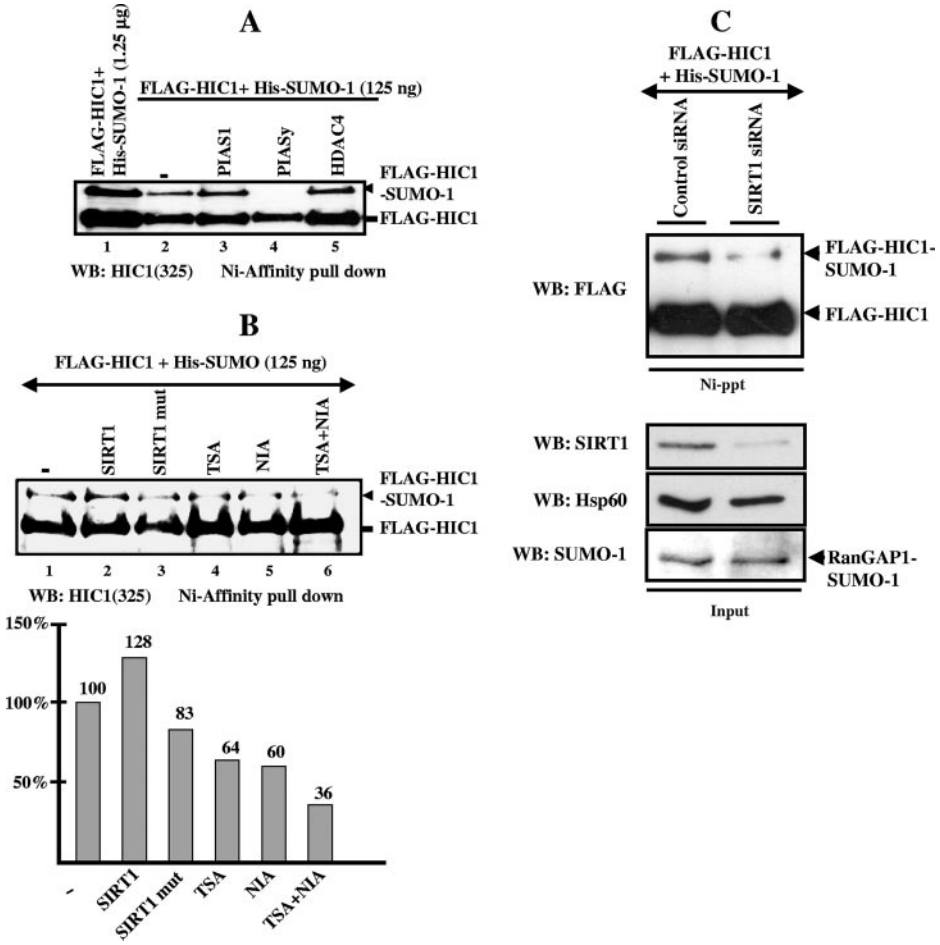

FIG. 10. SUMOylation of HIC1 K314 is enhanced by HDAC4 and SIRT1 but through different mechanisms. (A) HDAC4 stimulates SUMO-1 conjugation to HIC1 lysine 314. Cos-7 cells were transfected with 1.25 μg of FLAG-HIC1 and 1.25 μg His-SUMO-1 expression vectors (lane 1) or 125 ng of His-SUMO-1 expression vector alone (lane 2) or together with the expression vectors for PIAS1 or PIASγ as a positive or negative control, respectively, of canonical SUMO E3 ligases and HDAC4 as indicated (lanes 3 to 5). Forty-eight hours after transfection, cell lysates were subjected to nickel-agarose precipitation, separated by SDS-PAGE, and immunoblotted with the anti-HIC1 (325) polyclonal antibody. (B) SUMOylation of HIC1 on lysine 314 is regulated positively by the deacetylase SIRT1 and negatively by HDAC inhibitors. Cos-7 cells were transfected with 1.25 μg of FLAG-HIC1 and 125 ng of His-SUMO-1 expression vector alone (lane 1) or together with the expression vectors for wt SIRT1 (lane 2) or the catalytically inactive SIRT1 mutant (mut; H363Y) (lane 3), with 300 nM TSA (lane 4), 5 mM nicotinamide (lane 5), or both HDAC inhibitors (lane 6). Forty-eight hours after transfection, cell lysates were treated as described above. The ratio between the SUMOylated and total HIC1 proteins in the absence of SIRT1 or HDAC inhibitors (lane 1) was arbitrarily set to 100%, and the values obtained in the other cases are represented below as a graph. (C) SIRT1 knockdown in HEK293T cells results in decreased SUMOylation of HIC1 on lysine 314. A SUMOylation test has been performed with HEK293T cells transfected 24 h before with negative control siRNA which did not match any sequence in the human genome (Eurogentec) (left) or with SIRT1 siRNA (Ambion) (right). Whole-cell extracts were subjected to Ni affinity (Ni-ppt) (top panel) chromatography prior to Western blot (WB) analysis with the FLAG antibody. As controls, equal amounts of cell extracts were resolved by SDS-PAGE and immunoblotted with the anti-SIRT1 antibody and with the Hsp60 antibody (loading control). In addition, the detection of the SUMOylated form of RanGAP-1, the major SUMO-1 substrate, with an anti-GST-SUMO-1 antibody (J. Seeler, unpublished results), demontrates that SUMOylation is not globally affected. WB, Western blot.

RanGAP1, the most abundant SUMO-1 substrate (4), is unaffected (Fig. 10C, bottom panel).

Thus, as in the case of MEF2D, SIRT1 and HDAC4 are involved in the SUMOylation and deacetylation of the same lysine residue of HIC1.

The ψKXEP motif is a proline-dependent SUMOylation-acetylation switch motif. In striking contrast with MEF2, HIC1 lysine 314 is not embedded in a phosphorylation-dependent SUMOylation motif or SUMOylation-acetylation switch ψKXEXXSP motif but in a ψKXEP motif, with the proline conserved from human to zebrafish (Fig. 1). Since the G/SKXXP motif is a consensus motif for acetylation (44) and since half of the SAS (51) or PDSM (63) motifs contain a proline residue adjacent to the glutamic acid residue, our results emphasize the potential role of this proline residue in the acetylation/SUMOylation switch (Fig. 11). To functionally address this role, proline 317 in the ψKXEP motif of the full-length HIC1 protein was mutated into alanine. The resulting FLAG-HIC1 P317A mutant was compared with wt FLAG-HIC1 for its ability to undergo SUMO-1 modification and acetylation. The SUMOylation of the FLAG-HIC1 P317A was not significantly affected (Fig. 12A, lanes 2 and 4). By contrast, in the presence of P300 and HDAC inhibitors, the acet-

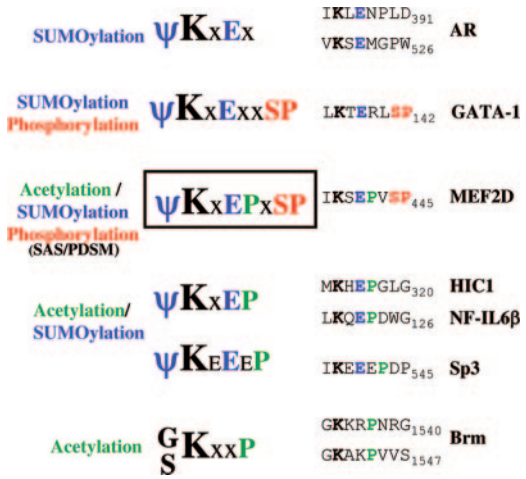

FIG. 11. KXEP: a core motif for coordinated acetylation and SUMOylation? Consensus motifs for which modifications have been fully validated by functional assays (44, 51, 63) are indicated. We noticed that in all known examples of coordinated SUMOylation and acetylation, the glutamic acid residue in the SUMOylation consensus is immediately followed by a proline, except for Sp3, which contains a stretch of three glutamic acid residues.

ylation level of this mutant is significantly decreased (Fig. 12B, lanes 4 and 6).

Thus, our results demonstrate that the ψKXEP motif is an acetylation/SUMOylation switch motif.

DISCUSSION

Here, we show the dynamic modification of HIC1 by SUMO on a single phylogenetically conserved lysine residue, K314, located in the HIC1 central region which, in addition to the BTB/POZ domain, is a second autonomous repression domain. Furthermore, this lysine is also targeted by acetylation by CBP/p300. We have identified the deacetylases SIRT1 and HDAC4 as regulators of the interplay between these two post-transcriptional modifications that affect the transcriptional repression activity of HIC1.

In contrast with the BTB/POZ domain, the zinc fingers and, to a lesser extent, the carboxyl-terminal end, the majority of the HIC1 central region has not been subjected to a strong selection pressure, except for some small peptidic motifs which are perfectly conserved from zebra fish to human. One of these, the GLDLSKK peptide, has been previously identified as a variant of the PXDLS consensus motif required for the interaction with the CtBP corepressor (16). In this study, we have shown that a second motif, M/VKXEP, is a consensus SUMOylation site. Based on these results, we predict that the others also contribute to transcriptional repression in various ways, including the recruitment of HIC1 partners, the stabilization of the protein, and/or the modulation of DNA binding activities. Notably, these motifs, T^{163}PVI, ELY^{200}A, and S^{381}EETGSSE, are centered on residues potentially subject to another regulatory posttranscriptional modification, phosphorylation.

Using the RONN prediction program (http://www.strubi.ox.ac.uk), the HIC1 central region appears to be natively disordered except for the CtBP interaction motif. However, this region is inserted between two highly structured regions which

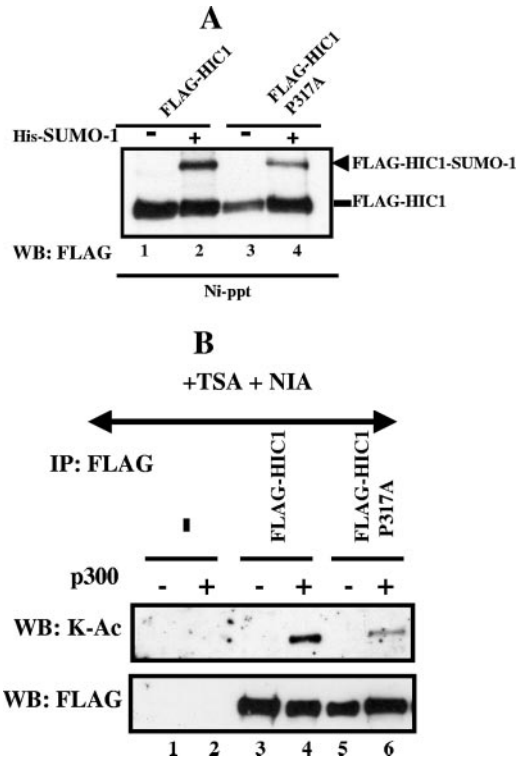

FIG. 12. Mutation of proline 317 in the KXEP motif affects the acetylation but not the SUMOylation of HIC1. (A) The SUMOylation of the wt HIC1 protein or of the P317A mutant (a point mutation of the conserved proline residue in the HIC1 ψKXEP SUMOylation motif) was analyzed exactly as described in the legend for Fig. 3. Lanes 1 and 3 correspond to controls with no His-SUMO-1 transfected. HIC1 acetylation was detected by immunoprecipitation with the monoclonal anti-FLAG antibody and Western blot (WB) analysis with the monoclonal pan acetyl-lysine antibody (K-Ac) from Cell Signaling (top panel). Western blotting with the FLAG monoclonal antibody was used to ascertain the presence of HIC1 proteins (bottom panel). (B) The acetylation levels of wt FLAG-HIC1 and FLAG-HIC1 P317A were determined as described in the legend for Fig. 7 in the presence of HDAC inhibitors (300 nM TSA and 5 mM NIA) added 24 h before lysis without (lanes 3 and 5) or with (lanes 4 and 6) p300. Lanes 1 and 2 correspond to controls in the absence of HIC1.

could impose a strict folding to this otherwise disordered region. Previously, we have shown that the dimerization of the BTB/POZ domain was essential to the creation of an interface for the optimal binding of CtBP though the GLDLS motif but could be replaced by a heterologous dimerization domain, such as the Gal-4 DNA binding domain (16). For the SUMOylation of HIC1, the situation is more complex since a Gal-4-central region chimera is not SUMOylated (data not shown), although it still interacts with CtBP. Whereas the BTB/POZ domain is not directly involved in the recruitment of CtBP, it could be directly involved in the SUMOylation of HIC1. Indeed, yeast two-hybrid screens with the BTB/POZ domain identified the unique E2-SUMO-conjugating enzyme Ubc9 as an HIC1 partner (S. Deltour, unpublished results). In addition, the HIC1 BTB/POZ contains a potential SUMO binding motif, V/I-X-V/I-V/I or V/I-V/I-X-V/I (52). However, crystallographic studies indicate that this motif, which is also found in BCL6, is buried in

its structure, strongly suggesting that it may not be functional (G. Privé, personal communication) (54).

SUMOylation has been shown to regulate protein function in different manners. First, it can affect subcellular localization of several proteins, such as, for example, PML in the so-called nuclear bodies. In transient transfection assays, HIC1, like many BTB/POZ proteins, displays a punctate nuclear localization and the endogenous HIC1 proteins have been recently associated with "HIC1 bodies" in medulloblastoma DAOY cells or in WI38 primary human fibroblasts (55). Mutant K314R also displays a punctate nuclear localization, indicating that SUMOylation might not be essential for this localization. Second, SUMOylation may regulate protein-protein interactions. For example, the SUMOylation of two *Drosophila* BTB/POZ proteins, Mod(mdg4)2.2 and CP190, two components of the *gypsy* chromatin insulator, does not affect their ability to bind to chromatin per se but instead inhibits long-range interactions of insulator proteins and hence the establishment of chromatin domains (8). The majority of known SUMO substrates are transcription factors or cofactors, and in most cases, this modification is associated with transcriptional repression (22, 57). For example, SUMOylation negatively regulates the activity of transcriptional activators, such as Sp3, p300, c-jun, c-myb, or Elk1. In that last case, SUMOylation results in the recruitment of HDAC2 to promoters and, hence, transcriptional repression of Elk1 target genes (62). The SUMOylation of transcriptional repressors is also required for their optimal silencing activity as shown for BKLF (40). In the case of HIC1, SUMOylation likely plays both direct and indirect roles in repression. A direct role is inferred from the mutation of the HIC1 SUMOylation site which reduces transcriptional repression, whereas an indirect role is supported by the similar effects exhibited by the deSUMOylase SENP2/SSP3 on the repression mediated by wt HIC1 and the non-SUMOylable mutant, suggesting that SUMOylation affects HIC1 partners involved in repression (Fig. 6E). SUMOylation could thus play a transient role in the formation of multiprotein complexes also involved in HIC1-mediated repression, as recently shown also for human p66α and p66β, which are components of the Mi-2/NuRD complex associated with HDACs (25). Notably, the HIC1 central region containing the SUMOylation site is an HDAC-dependent repression domain (16, 53).

Finally, lysine can be targeted by multiple modifications in a competitive and regulated way. The first example has been reported for the NF-κB inhibitor IκBα, whose SUMOylation blocks polyubiquitination on the same residue and, thus, subsequent ubiquitin-dependent proteasomal degradation (17). Recently, several studies have demonstrated that the transcriptional activity of MEF2D is regulated by interplay between acetylation and SUMOylation on the same residue (27). This regulation by different lysine modifications is mediated by two distinct deacetylases belonging to different classes, namely, the class II, TSA-sensitive HDAC4 and the class III, NAD^+-dependent deacetylase SIRT1. These two deacetylases interact and form a dual-deacetylase complex with restricted and complementary properties since SIRT1, but not HDAC4, can deacetylate MEF2 and in which HDAC4 enhances MEF2 SUMOylation by a still poorly understood mechanism relying on an N-terminal coiled-coil domain (64). Here, we have shown that the transcriptional repressor HIC1 is also a target for this dual-deacetylase complex which can thus regulate, via similar mechanisms, two different families of transcription factors endowed with clearly different functional properties, activation and repression. In MEF2, this acetylation/SUMOylation motif is an extended version of the canonical ψKXE motif, ψKXEXXSP. Bioinformatics analysis has shown this motif to be conserved in numerous proteins, with most involved in transcriptional regulation (29, 63). Furthermore, functional assays with MEF2, GATA-1, and heat shock factors have demonstrated that phosphorylation of the adjacent serine residue regulates the SUMOylation of the lysine, hence the names phosphorylation-dependent SUMOylation motif and SUMOylation-acetylation switch (26). Within PDSM motifs, the spacing between the SUMO target and phosphorylation site appears to be crucial since searches using alternate spacing did not reveal SUMO substrates or transcriptional regulators (29). HIC1, which is also SUMOylated and acetylated on the same residue, lacks the adjacent SP motif found in MEF2 factors (Fig. 1). Strikingly, however, HIC1 shares with MEF2 a conserved proline residue adjacent to the glutamic acid residue (KXEP versus KXEPXSP) (Fig. 11). A G/SKXXP motif has been shown to be a consensus site for the acetylation of the Brm proteins by CBP/p300 (44) (5). These data suggest that the spectrum of modifications potentially affecting a given lysine residue could be defined by the presence/absence of several adjacent residues, including a glutamic acid, a proline, and/or a serine-proline motif (Fig. 11). This would give rise to (i) a classical SUMOylation-only ψKXE motif (ii) a ψKXEXXSP motif whose lysine could be targeted by a phosphorylation-dependent SUMOylation, as shown for GATA-1 (29, 63), (iii) a ψKXEP motif whose lysine could be targeted by SUMOylation or acetylation as shown for NF-IL-6β, also known as C/EBPδ (60) and HIC1 (this study), and (iv) a ψKXEPXSP motif whose lysine could be targeted by acetylation and phosphorylation-dependent SUMOylation (26). Interestingly, a ψKXEP motif is also found in Evi-1 and conserved in the C/EPBα, -β, and -γ proteins (57), but in these cases, the acetylation of this particular lysine has not been demonstrated. Sp3 is another well-documented example for SUMOylation/acetylation on the same residue, but here the motif contains three glutamic acid residues, KEEEP (6, 43, 46). Interestingly, half of the described SAS motifs contain an adjacent proline residue (51).

Recently, it has been shown that HIC1 forms a transcriptional repression complex with SIRT1 and that this complex directly binds the *SIRT1* promoter to repress its transcription (12). The HIC1 BTB/POZ domain interacts with SIRT1 (12), but the repression mediated by this isolated domain in the context of a Gal4 chimera is inhibited neither by TSA, a specific inhibitor of class I and class II HDACs (14, 15), nor by NIA, an inhibitor of NAD^+-dependent class III deacetylases, such as SIRT1 (our unpublished results). Our results thus provide the first mechanistic clues to this HIC1/SIRT1 interaction. SIRT1 is not involved in the repression mediated by the isolated BTB/POZ domain, but by deacetylating HIC1, SIRT1 favors its SUMOylation and, thus, the establishment of optimal transcriptional repression. The acetyl-peptide binding specificity among sirtuins remains unclear (44) and SIRT1 was reported to have no substrate specificity in vitro (3). In vivo, SIRT1 can deacetylate a still-growing list of proteins with a

great versatility in the amino acid sequence surrounding the acetylated lysine, such as RHKK(Ac)^{382}L for p53 (56, 37), RK(AC)^{360}LKK for androgen receptor (20), KYKK(Ac)^{379} for the BTB/POZ transcriptional repressor BCL6 (1), and YWMK(Ac)^{314}HEP for HIC1. BCL6 acetylation decreases its transcriptional repression activity (1) by impairing its physical association with MTA-3, a cell-type-specific subunit of the Mi-2/NURD corepressor complex (21). The interaction between CtBP and some corepressors can also be negatively regulated by lysine acetylation of the PXDLSXK motif, as shown for E1A and RIP140 (58). The HIC1 central region also interacts with CtBP through a conserved GLDLSKK motif which could be targeted by acetylation. In that case, acetylation and deacetylation by SIRT1 and/or TSA-sensitive HDACs would be global regulators of the transcriptional repression mediated by the central region by modulating its SUMOylation and the recruitment of CtBP.

Deciphering the complex interplay between different modifications and their influence on the recruitment of repression complexes will be necessary to better understand the repression mechanisms brought about by HIC1 on its target genes and hence its tumor-suppressive properties.

ACKNOWLEDGMENTS

We thank M. Crossley, R. Hay, T. Kouzarides, S. H. Lin and D. Wotton for their generous gifts of reagents. We are indebted to Bénédicte Grasland and Aurélie Bauduin for the construction and the initial analyses of the Gal4-HIC1 and HIC1-Gal4 chimeras. We thank Brian Rood and Alexis Verger for comments and critical reading of the manuscript and Gil Privé for helpful discussions on the BTB/POZ structure.

This work was supported by funds from the CNRS, the Pasteur Institute, the Ligue Nationale contre le Cancer (Comité Interrégional du Septentrion), the EEC "Rubicon," and the Association pour la Recherche contre le Cancer (to J.S. and D.L.). N. Stankovic-Valentin was supported by a fellowship from the Ministère de la Recherche et de la Technologie and by the Association pour la Recherche contre le Cancer.

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