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# The Human Candidate Tumor Suppressor Gene *HIC1* Recruits CtBP through a Degenerate GLDLSKK Motif

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***HIC1* (hypermethylated in cancer) and its close relative *HRG22* (*HIC1*-related gene on chromosome 22) encode transcriptional repressors with five $C_2H_2$ zinc fingers and an N-terminal BTB/POZ autonomous transcriptional repression domain that is unable to recruit histone deacetylases (HDACs). Alignment of the HIC1 and HRG22 proteins from various species highlighted a perfectly conserved GLDLSKK/R motif highly related to the consensus CtBP interaction motif (PXDLSXK/R), except for the replacement of the virtually invariant proline by a glycine. HIC1 strongly interacts with mCtBP1 both in vivo and in vitro through this conserved GLDLSKK motif, thus extending the CtBP consensus binding site. The BTB/POZ domain does not interact with mCtBP1, but the dimerization of HIC1 through this domain is required for the interaction with mCtBP1. When tethered to DNA by fusion with the Gal4 DNA-binding domain, the HIC1 central region represses transcription through interactions with CtBP in a trichostatin A-sensitive manner. In conclusion, our results demonstrate that HIC1 mediates transcriptional repression by both HDAC-independent and HDAC-dependent mechanisms and show that CtBP is a HIC1 corepressor that is recruited via a variant binding site.**

The recruitment of corepressors has emerged during the past few years as a widely used mechanism of transcriptional repression. Corepressors are non-DNA-binding proteins that interact with a subset of sequence-specific transcription factors to bring about repression. Examples of such corepressors include mSin3A, SMRT/NCoR, Tup1/Ssn6, Groucho, and CtBP. The compositions of these corepressor complexes as well as the mechanisms whereby they repress transcription are being actively investigated (reviewed in references 25 and 56). A common theme is the recruitment of histone deacetylases (HDACs) that can deacetylate specific lysine in nonhistone proteins as well as in histones, thus leading to a condensed, transcriptionally inactive chromatin. Conversely, many transcriptional coactivators are associated with the opposing histone acetyltransferase activity (27).

The structural requirements for the interactions between transcriptional repressors and cognate corepressors need to be deciphered. In some instances, these interactions are mediated by short sequence motifs. Nuclear hormone receptors recruit SMRT and NCoR via an interaction domain, the CoRNR box, which has the consensus sequence I/L-X-X-I/V-I (8), whereas the Groucho corepressor (7) interacts with transcription regulators such as *Drosophila melanogaster* long-range repressors Dorsal and Hairy via a WPRW motif (43, 58). Another well-established example is CtBP (C-terminal binding protein), which was originally identified as a protein that interacts with the C-terminal region of the adenovirus (Ad) 2/5 E1A oncogene product via a PLDLS motif (4, 46, 53). The binding of CtBP to E1A negatively modulates its cooperation with activated Ras in cell transformation, as well as the tumorigenic and metastatic properties of transformed cells (4, 46). Isolation of the *Drosophila* CtBP homologue in yeast two-hybrid screens by using short-range repressors Knirps and Snail as baits provided the first compelling evidence for a role in transcriptional repression (39). Subsequently, CtBP has been shown to interact with another viral protein, EBNA3C (51), and with a still growing list of transcription factors from *Drosophila* and vertebrates that have key roles in differentiation, such as Hairy (44), BKLF (52), δEF1 (16), ZEB (45), HPC2 (47), XTcf-3 (5), FOG and FOG-2 (15, 20), Ikaros (26), Knirps (24), Tramtrack (55), TGIF (31), MEF2 (57), Hairless (37), Sox6 (38), and Evi-1 (23, 42), or in cell proliferation, such as Net (9), Rb/p130 (33), and BCRA1 (28). The nature of the CtBP-containing complexes and the mechanism by which they affect transcription, notably their relationship with HDAC, are still subjects of controversy (26, 43, 49, 50, 53). Nonetheless, some class II HDACs (HDAC-4 and -7), as well as MITR, contain a PXDLR motif in their N-terminal parts and can interact with CtBP (34, 57). Furthermore, CtBP can interact with HDAC-1 (49, 50), HDAC-2 (26), and HDAC-5, which does not contain a recognizable PXDLS motif (53, 57). HDAC-5 contains a variant PVELR motif at the position analogous to that in MITR, but its deletion did not prevent the interaction with CtBP, indicating the presence of an additional CtBP-binding motif (57). Highly related CtBP proteins CtBP1 and CtBP2 have been identified in vertebrates (52, 53), but functional differences between them still remain elusive. CtBP1 and, to a lesser extent, CtBP2, are widely expressed in normal human tissues and cancer cell lines as well as throughout development in mice (16, 47).

In many cancers, DNA hypermethylation changes of the *Not*I restriction sites at the D17S5 locus in 17p13.3 allowed the positional cloning of *HIC1* (hypermethylated in cancer 1) (30). *HIC1* is a candidate tumor suppressor gene since it significantly decreases the clonogenic survival of various cancer cell lines, and its expression is upregulated by p53 through a functional

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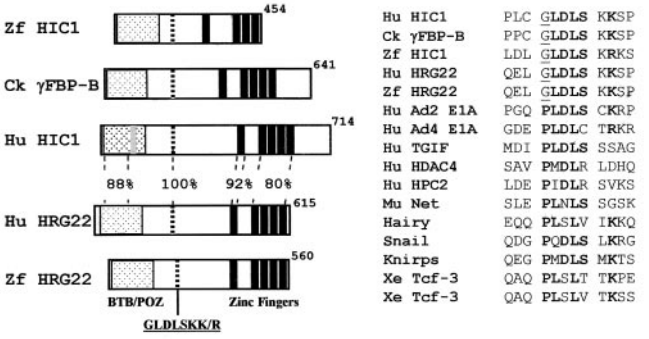


FIG. 1. Identification in the HIC1 and HRG22 proteins of an evolutionary conserved GLDLSKK/R motif related to the CID consensus. (Left) Schematic drawing of the HIC1 and HRG22 proteins from various species. Hu, human; Ck, chicken; Zf, zebra fish. Degrees of conservation (percentages of identity and similarity) between the human HRG22 and HIC1 proteins obtained with the BLAST program are indicated. (Right) The evolutionarily conserved GLDLSKK/R motif can be aligned with CtBP-binding motif PXDLSXK/R, originally found in the Ad E1A proteins and later in a still-growing list of proteins from various species. Notably, the proline residue (see also Fig. 4) is replaced by a glycine (underlined) in HIC1 and HRG22. Mu, murine; Xe, *Xenopus laevis*.

p53 binding site in its 5′ flanking region (18, 30). *HIC1* may also be involved in the Miller-Dieker syndrome (MDS), a severe form of lissencephaly (13). Indeed, together with perinatal death and a reduction in overall size, *HIC1*$^{-/-}$ mouse embryos have other developmental anomalies resembling those found in MDS patients (6), and parts of the *HIC1* expression territories overlap with regions exhibiting abnormalities in MDS patients (19).

*HIC1* encodes a protein with five Krüppel-like $C_2H_2$ zinc fingers in the C terminus and a protein-protein interaction domain called the BTB/POZ domain at the N terminus. Many proteins with BTB/POZ domains and zinc fingers are transcriptional repressors, such as *Drosophila* Tramtrack and human BCL6 and PLZF, which are involved in translocations in some leukemias (2, 3). Previously, we have shown that HIC1 is a transcriptional repressor (10) as is its avian homologue, γFBP-B (γF1 binding protein B) (29). Both the HIC1 and γFBP-B BTB/POZ domains are autonomous transcriptional repression domains but, in sharp contrast with those of BCL-6 and PLZF, they are unable to recruit SMRT/NCoR-mSin3A-HDAC complexes and are insensitive to trichostatin A (TSA), a specific inhibitor of HDACs (10). Recently, we identified by database searches a human homologue of *HIC1* located on human chromosome 22q11.2, which we called *HRG22* for *HIC1*-related *g*ene on chromosome 22 (11). The human and zebra fish *HRG22* genes are similar, in overall gene structure and organization, to the various *HIC1* orthologs. In addition to the zinc fingers and to the BTB/POZ domain that is implicated in TSA-insensitive transcriptional repression (11), there is a short sequence, GLDLSKK/R, that is conserved between the HIC1 and HRG22 proteins from various species (Fig. 1). This motif is highly related to consensus motif PXDLSXK/R of the CtBP-interacting domain (CID) except that the proline residue is replaced by a glycine residue.

In this report, we demonstrate that HIC1 interacts with CtBP via this phylogenetically conserved GLDLSKK motif, indicating that HIC1 has two functionally distinct transcriptional repression domains. The HIC1 central region containing the GLDLSKK motif interacts with mCtBP1 and represses transcription in a TSA-sensitive manner, whereas the BTB/POZ repression domain, which is TSA insensitive, does not interact with mCtBP1 in yeast or mammalian two-hybrid assays. However, its presence and most likely its dimerization potential are instrumental in the interaction between full-length HIC1 and CtBP. Finally, we show that HIC1 can recruit endogenous HDACs. More generally, our results demonstrate that the consensus binding site for CtBP is more versatile than previously anticipated, with leucine as the sole invariant residue, thus potentially increasing the number of transcription factors that can interact with this corepressor.

## MATERIALS AND METHODS

**Constructs. (i) Yeast two-hybrid vectors.** Bait constructs were obtained by cloning PCR products corresponding to the HIC1 BTB/POZ domain (residues 1 to 140) and the HIC1 central region (residues 135 to 296) into the pLex10A vector containing the LexA DNA-binding domain. The clone containing the complete murine CtBP1 coding sequence fused to the VP16 activation domain in the pASV3 vector has been previously described (9). The HIC1 BTB/POZ domain was also cloned in the prey vector pGAD-GE containing the Gal4 activation domain.

**(ii) Eukaryotic expression vectors.** The eukaryotic expression vectors for glutathione *S*-transferase (GST; pBC) and GST-mCtBP1 (pBC-mCTBP1) and the pTL-mCtBP1 vector have been described (9). The 5′ end of a genomic clone containing the unique HIC1-coding exon (18) was modified by PCR. A *Bam*HI-*Eco*RV fragment was prepared and cloned in a modified pcDNA3 vector (kind gift from D. Monte) containing the coding sequence for a Flag epitope followed by a *Bam*HI cloning site to yield pcDNA3-Flag-HIC1. The ΔGLDLSKK deletion mutant was generated by the two-round PCR mutagenesis strategy.

**(iii) Gal4–DNA-binding domain fusion proteins.** The Gal NC4 containing the CtBP interaction motif from the Net protein, the Gal4-HIC1 (1-140), and the Gal4-HIC1 (1-714) chimeras have been described (9, 10). The other Gal4-HIC1 chimeras were obtained by PCR and/or exchange of restriction fragments from the relevant chimeras. The Gal4-HIC1 (135-296) ΔGLDLSKK mutant was obtained by PCR with convenient flanking oligonucleotides using pcDNA3-Flag-HIC1 ΔGLDLSKK as the template.

For the mammalian two-hybrid assays, the complete mCtBP1 coding sequence from the yeast vector was cloned in frame with the VP16 activation domain in the pACT vector (CheckMate mammalian two-hybrid kit; Promega).

PCR fragments were systematically verified by sequencing on both strands. All clones were checked by appropriate restriction enzyme digestion, and the vector-insert junctions were verified by sequencing.

**(iv) Reporter.** The pG5-Luc (AdML promoter) clone (CheckMate mammalian two-hybrid kit; Promega), which contains five Gal4 binding sites in front of a luciferase gene, was used as the reporter in transient transfection assays.

**Cell culture and transfections.** Cos-1 and RK13 (rabbit kidney) cells were maintained in Dulbecco medium supplemented with 10% fetal calf serum. Cells were transfected in OptiMEM by the polyethyleneimine method as previously described (10) in either 100-mm-diameter dishes (in vivo interaction) with 2.5 μg of DNA or in 12-well plates (immunofluorescence) with 500 ng of DNA for Cos-1 cells or in 6-well plates with 1 μg of DNA for RK13 cells (repression assays). Cells were transfected for 6 h and then were incubated in fresh complete medium. They were rinsed in phosphate-buffered saline (PBS) 48 h after transfection and processed for protein-protein interactions (Cos-1) or luciferase assays (RK13).

**Repression and mammalian two-hybrid assays.** The repression and mammalian two-hybrid assays were carried out as previously described (10). Luciferase (Luc) and β-galactosidase activities were measured by using, respectively, beetle luciferin (Promega) and the Galacto-light kit (Tropix, Bedford, Mass.) with a Berthold (Nashua, N.H.) chemioluminometer. After normalization to β-galactosidase activity, the data were expressed as Luc activity relative to the activity of pG5Luc with empty control vectors, which was given an arbitrary value of 1. Results presented are the mean values and standard deviations from two independent transfections in triplicate.

For experiments using TSA (Sigma), 24 h after transfection, the cells were

either left untreated or were treated with 300 nM TSA for a further 24 h before harvesting.

**Yeast quantitative β-galactosidase assays.** Interactions between mCtBP1 and HIC1 were studied by cotransfecting LexA-DBD-POZ (HIC1 1-140) and LexA-DBD-CR (HIC1 135-296) plasmids with VP16-AD-mCtBP1 (9) or Gal4-AD and Gal4-AD-POZ (1-140) as controls into the L40a strain. Transformants selected on Trp- and Leu-deficient plates were cultured in Trp- and Leu-deficient medium for 24 h. The optical density at 600 nm ($OD_{600}$) of each culture was measured, and the quantitative β-galactosidase assay was performed. The β-galactosidase units were calculated by using the formula activity = $OD_{420} \times (1{,}000/\text{incubation time}) \times (1/OD_{600})$. The results presented are the mean values and standard deviations from two independent experiments, where the quantitative β-galactosidase activity was measured after 2 or 3 min of incubation.

**Western blots and antibodies. (i) Western blots.** Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After 1 h of saturation in PBS–5% milk (PBSM), the membranes were incubated overnight at 4°C with specific primary antibodies in PBS–0.1% Tween 20-5% milk and washed three times for 10 min with PBS–0.1% IGEPAL (PBSN). The membranes were next incubated for 1 h at room temperature with secondary antibodies coupled to peroxidase (diluted 1/10,000) in PBSM, washed two times for 10 min with PBSN, rinsed with PBS, and revealed with the Western blot chemiluminescence reagent kit (NEN).

**(ii) Antibodies.** Anti-mCtBP1 PAb1123 and anti-GST have been described by Criqui-Filipe et al. (9). To detect endogenous CtBP in Western blots, we used the monoclonal antibody (E-12) raised against amino acids 1 to 440 from human CtBP1, which reacts with CtBP1 and CtBP2 from various origins (Sc-17759; Santa Cruz Biotechnology). Anti-Flag M2 is a monoclonal antibody (F3165; Sigma). Anti-HIC1 PAb2563 is a rabbit polyclonal antibody directed again the C-terminal amino acids of HIC1 (590-714) fused to GST (11). Anti-HIC1 PAb325 is a rabbit polyclonal antibody raised against the keyhole limpet hemocyanin-coupled peptide corresponding to amino acids 701 to 714 of human HIC1 (Genosphere, Paris, France). The secondary antibodies were horseradish peroxidase-linked antibodies raised against either rabbit or mouse immunoglobulins (Amersham).

**Eukaryotic GST pull-down and coimmunoprecipitation experiments.** The eukaryotic GST pull-down and coimmunoprecipitation assays were performed as described in detail by Criqui-Filipe et al. (9) with extracts of transfected Cos-1 cells. Proteins recovered on the glutathione-Sepharose or the protein G-Sepharose beads were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting.

**Immunofluorescence.** Cos-1 cells were cultured on coverslips, transfected as described above, and processed at room temperature. 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–10% goat serum, incubated for 30 min with the primary antibody diluted 1/500 in PBS–10% goat serum, and incubated in the dark for 30 min with the secondary antibody diluted 1/200 in PBS–10% goat serum. At each stage, they were washed three times for 5 min in PBS. They were then placed inverted on a drop of Immuno Floure mounting medium (ICN) on a slide. The slides were stored in the dark at 4°C and visualized under fluorescence or confocal microscopes.

**Generation of a stable CHO cell line with inducible Flag-HIC1 expression.** A *Hin*dIII-*Eco*RV fragment encoding a HIC1 protein with an N-terminal Flag epitope was prepared from the pcDNA3-Flag-HIC1 vector and cloned in the pIND vector (Invitrogen), which contains five modified ecdysone response elements upstream from a minimal heat shock promoter, to yield pIND-Flag-HIC1.

The EcR-CHO cell line (Invitrogen), which stably expresses a modified ecdysone receptor from the pVgRXR vector, was used to generate Flag-HIC1-inducible clones by transfection with pIND-Flag–HIC1 followed by a double selection in Ham F-12 medium supplemented with 10% fetal calf serum and containing G-418 (500 μg/ml) and Zeocin (250 μg/ml). Several clonal cell lines were expanded from single foci and subsequently screened by Western blotting and immunofluorescence analyses for inducible expression of Flag-HIC1 in the presence of the ecdysone analogue ponasterone A (Invitrogen) at 10 μM for 48 h. Clone 6 was found to be the best inducible expresser of Flag-HIC1 and was used in further studies.

Nuclear extracts from 10 subconfluent 100-mm-diameter dishes of ponasterone A-treated or untreated EcR-CHO pIND-Flag-HIC1 clone 6 cells were prepared as described by Nielsen et al. (41). Aliquots were immunoprecipitated with the preimmune and immune anti-HIC1 polyclonal rabbit sera. After five washes in NEB buffer (41), the immunoprecipitates were recovered on protein A-Sepharose beads, fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting using the anti-CtBP E12 monoclonal antibody, as described above.

**Immunoprecipitation-HDAC assays.** [$^3$H]acetate-labeled histones were prepared from Jurkat cells essentially as described previously, with minor modifications (14). Flag epitope-tagged proteins were expressed by transient transfection in RK13 cells. The transfected cells were washed with cold PBS, resuspended in 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 120 mM NaCl, 5 mM EDTA, 0.5% IGEPAL) in the presence of protease inhibitors and immunoprecipitated with the anti-Flag M2 monoclonal antibodies. Protein G-Sepharose beads containing the immune complexes were resuspended in 100 μl of HDAC buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% glycerol), and an HDAC assay was performed with 60,000 cpm of acetylated histones as described previously (14). For inhibition studies, the immunoprecipitated complexes were divided into two aliquots and left untreated or preincubated with 300 nM TSA for 30 min at 4°C prior to the assay.

## RESULTS

**Characterization of an evolutionarily conserved GLDLSKK/R motif in HIC1 and the related HRG22 protein.** When HIC1 and HRG22 protein sequences were aligned with the CLUSTAL program, two regions of strong homology (>80%) were identified, the BTB/POZ and the zinc finger domains (Fig. 1) (11). Strikingly, a short GLDLSKK/R peptide located in the central region is perfectly conserved in all the HIC1 and HRG22 proteins (Fig. 1). Such a strong selection pressure on this motif in a region which has otherwise not been conserved suggests that it has an important functional role. Indeed, this motif is highly related to the consensus PXDLSXK/R sequence, which interacts with the CtBP corepressor, with a notable exception: the proline is replaced by a glycine in the HIC1 and HRG22 proteins (Fig. 1). Interestingly, during the in vitro screening of synthetic peptides (14 residues related to the binding site on Ad5 E1A) for their ability to interact with CtBP, replacement of the proline residue by a glycine did not abolish the interaction and only slightly affected the $K_d$ (35). HIC1 and HRG22 are the first examples of naturally occurring proteins containing a GXDLS motif. Thus, a short atypical motif that should predictably interact with CtBP has been phylogenetically conserved in HIC1 and HRG22 proteins.

**HIC1 and mCtBP1 specifically interact in yeast.** To explore a possible interaction between HIC1 and CtBP, targeted yeast two-hybrid experiments were carried out using HIC1 functional domains fused to the LexA DNA-binding domain and the complete coding sequence of mCtBP1 fused to the VP16 activation domain. This clone was isolated from a yeast two-hybrid screen by using the central repression domain of ETS family protein Net as the bait (9). Coexpression of the HIC1 (135-296) bait and mCtBP1 strongly activates the β-galactosidase reporter gene, clearly demonstrating that the central region of HIC1 interacts with mCtBP1 in vivo (Fig. 2, lanes 1 and 2). The HIC1 BTB/POZ domain is an autonomous repression domain whose repression mechanism(s) is still poorly understood (10). Here, we show that the HIC1 BTB/POZ domain did not interact with the mCtBP1 corepressor in the yeast two-hybrid assay (Fig. 2, lanes 3 and 4). As a positive control, we demonstrate that the same HIC1 BTB/POZ bait mediated homodimerization in yeast (Fig. 2, lanes 4 and 5), as previously shown in mammalian cells (10). Thus, the central region of HIC1, but not its BTB/POZ autonomous repression domain, is able to interact with corepressor mCtBP1 in vivo.

**HIC1 and mCtBP1 interact through the GLDLSKK motif in mammalian two-hybrid assays.** To confirm the interaction between HIC1 and mCtBP1 in another experimental system and

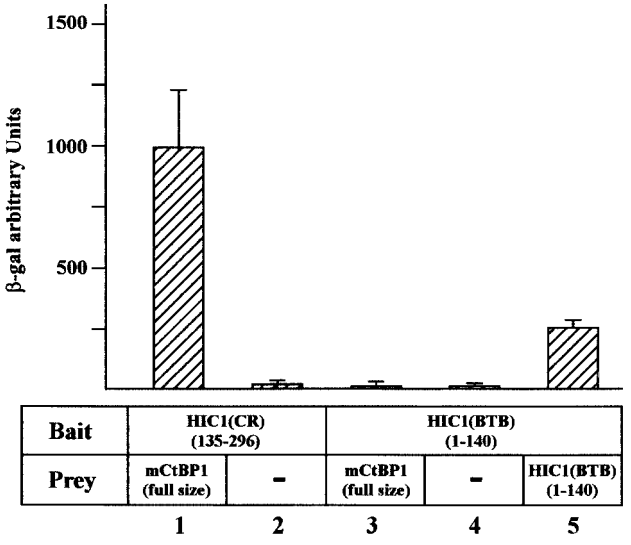


FIG. 2. mCtBP1 specifically interacts with the central region of HIC1 in yeast two-hybrid assays. The baits contain the Gal4 DNA-binding domain fused to the HIC1 central region (CR) or BTB/POZ domain. The preys contain the activation domain of either VP16 (fused to mCtBP1) or Gal4 (fused to HIC1). The baits and preys were transfected in L40a yeast cells. -, pGAD vector. The transcriptional activation levels attained by the bait-prey interactions were estimated by measuring the β-galactosidase (β-gal) activity in liquid cultures of individual yeast colonies. Mean values and standard deviations from two independent experiments are shown.

to demonstrate the importance of the conserved GLDLSKK/R motif, we conducted mammalian two-hybrid assays with rabbit kidney cells (RK13). Chimeras between the Gal4 DNA-binding domain and full-length HIC1 or various subregions of HIC1 containing or lacking this conserved GLDLSKK motif (Fig. 3A) were tested for their ability to interact with mCtBP1. Fully consistent with the yeast two-hybrid experiments (Fig. 2), the HIC1 BTB/POZ domain failed to interact with mCtBP1 (Fig. 3B, lane 2), whereas the central region (residues 135 to 296) showed a robust interaction with mCtBP1 at a level akin to that for the CID motif of Net, used as a positive control (9) (Fig. 3B, lanes 4 and 8). Strikingly, Gal4-HIC1 (1-397), which contains the BTB/POZ domain fused to the central region, and Gal4-HIC1 (full-length) display only a modest (twofold activation) (Fig. 3B, lane 3) or virtually no interaction, respectively, with mCtBP1 (Fig. 3B, lane 6), suggesting that in these fusion proteins the CtBP interaction motif may be masked, as already observed for several proteins (9, 16). Notably, deletion of the BTB/POZ domain in the context of the full-length protein, Gal4-HIC1 (149-714), restored the interaction with mCtBP1 (Fig. 3B, lane 7; see Discussion). Deletion (Δ222-228) of the evolutionarily conserved GLDLSKK motif in the Gal4-HIC1 (135-296) chimera, which strongly interacts with mCtBP1, virtually abolished this interaction (Fig. 3B, compare lanes 4 and 5). These results firmly establish that the GLDLSKK motif is a new CtBP interaction motif (CID) and extend the previously defined consensus-binding site for CtBP (Fig. 4). Indeed, the leucine residue now appears to be the only invariant residue in the five-amino-acid core motif found in all the CtBP-binding proteins known so far.

**HIC1 and mCtBP1 interact in vivo mainly through the conserved GLDLSKK motif.** To determine whether the interaction between full-length CtBP1 and HIC1 can also occur in vivo in mammalian cells, we first performed GST pull-down assays with eukaryotic cells. Upon transfection into Cos-1 cells, a full-length HIC1 protein tagged with a small (seven residues) Flag epitope at its N terminus interacted with GST-CtBP1 but not with GST alone (Fig. 5A, lanes 1 and 2). To confirm that the GLDLSKK motif is crucial, we deleted this seven-amino-acid core motif in the context of the full-length protein (Flag-

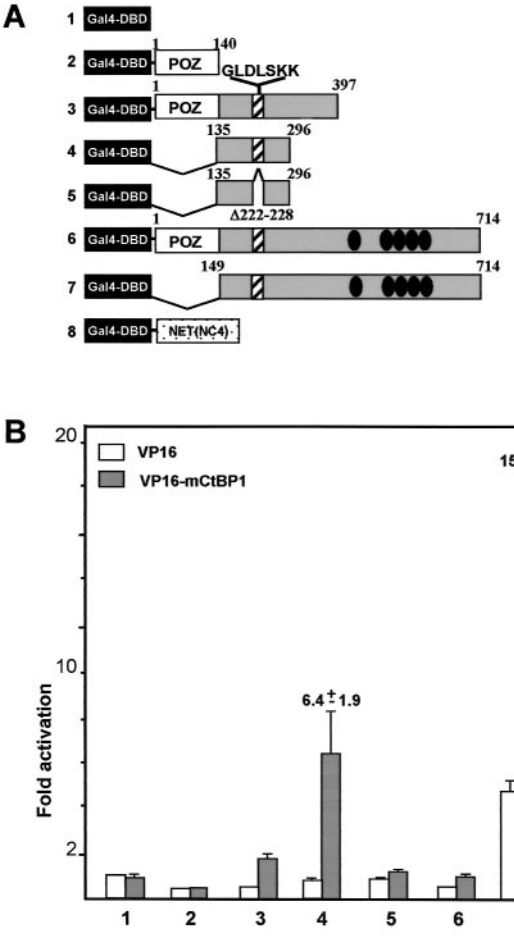


FIG. 3. mCtBP1 specifically interacts with the central region of HIC1 through the conserved GLDLSKK motif in mammalian two-hybrid assays. (A) Schematic structures of the Gal4 DNA-binding domain (construct 1) and of various Gal4-HIC1 chimeras (constructs 2 to 7). Numbering refers to HIC1 residues. Hatched box, GLDLSKK motif; black ovals, five zinc fingers. Gal4-NC4 (construct 8), containing the CID motif of the Net transcription factor, was used as a positive control (9). (B) The GLDLSKK motif is required for the interaction with mCtBP1. Luc and β-galactosidase assays were performed on total extracts from RK13 cells that had been transiently transfected with 750 ng of the pG5Luc vector (Promega), 50 ng of the pSG5 β-galactosidase construct as a control of transfection efficiency, 100 ng of the indicated Gal4 construct, and 100 ng of the VP16 activation domain (white bars) or VP16 activation domain-tagged mCtBP1 (grey bars). After normalization to β-galactosidase activity, the data were expressed as Luc activity relative to the activity of pG5Luc with empty control vectors, which was given an arbitrary value of 1. Results presented are the mean values and standard deviations from two independent transfections in triplicate.

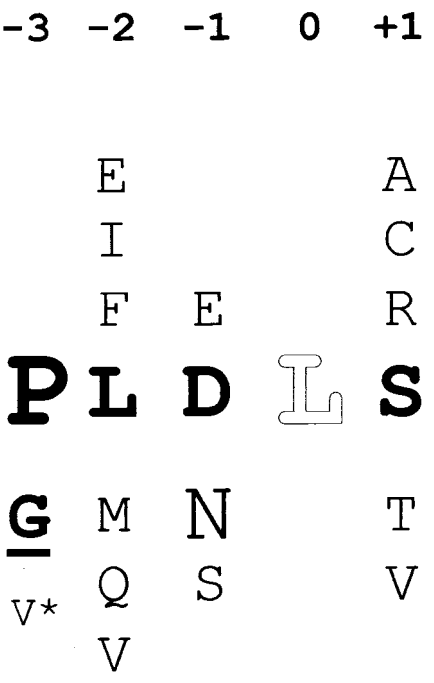


FIG. 4. Definition of a new CID consensus. The new consensus binding site for CtBP is shown according to the nomenclature first proposed by Postigo and Dean (45), who used outlined, boldface, and plain characters of different sizes based on the frequency of the residue. The leucine (outlined) remains the only invariant residue in the core motif. The previously invariant proline can be replaced by a glycine (underlined) as shown in this study for the naturally occurring HIC1 and HRG22 proteins and as previously suggested by in vitro assays with synthetic peptides (35). V*, potential variant residue recently identified. Indeed, 6 out of 41 CtBP-binding partners cloned from a mouse embryonic library in a yeast two-hybrid screen contain a VLDLS motif, but the functionality of this motif still has to be demonstrated (54). Similarly, short-range *Drosophila* repressor Giant interacts with dCtBP but indirectly through an unknown bZIP protein, whereas the Giant VLDLSRR motif could recruit an as yet unidentified corepressor (40).

HIC1 ΔGLDLSKK). In close agreement with the results obtained with the isolated central region (Fig. 3), this mutation abolished the ability of HIC1 to interact with mCtBP1 (Fig. 5A, lanes 3 and 4). The GST fusion proteins (Fig. 5A, bottom) and the Flag-HIC1 wild-type and ΔGLDLSKK mutant proteins (Fig. 5A, middle) were expressed at comparable levels, showing that differences in protein expression levels and/or stability could not account for the lack of interaction.

The interaction between HIC1 and CtBP1 was independently confirmed by coimmunoprecipitation experiments utilizing vectors expressing N-terminal Flag-tagged full-length HIC1 proteins (wild-type or ΔGLDLSKK) and vectors expressing full-length mCtBP1 proteins. As shown in Fig. 5B, our results clearly demonstrate that HIC1 and mCtBP1 can interact in mammalian cells, since the anti-Flag monoclonal antibody coimmunoprecipitated mCtBP1 only from Cos-1 cells expressing both Flag-HIC1 and mCtBP1 (Fig. 5B, lane 6). Notably, a small but significant amount of mCtBP1 was detected in the anti-Flag-immunoprecipitated material when Flag-HIC1 ΔGLDLSKK was expressed instead of wild-type Flag-HIC1 (Fig. 5B, lanes 6 and 7), indicating that the native mCtBP1 protein specifically interacts with the HIC1 ΔGLDLSKK protein, albeit with a much lower affinity than with the full-length HIC1 protein. This slight discrepancy with the GST pull-down experiment, where the HIC1 ΔGLDLSKK mutant protein is totally unable to bind mCtBP1 (Fig. 5A), again highlights the structural constraints underlying this interaction. Furthermore, this coimmunoprecipitation experiment strongly suggests that HIC1, in addition to the GLDLSKK motif, could contain other accessory CtBP weak binding sites, as shown for example for the *Drosophila* Hairy (44) and Tramtrack (55) proteins and for HDAC-5 (57). Taken together, our results indicate that mCtBP1 and HIC1 interact in vivo mainly but perhaps not exclusively via the conserved GLDLSKK motif.

**Dimerization of HIC1 through its BTB/POZ domain is required for the interaction with mCtBP1.** The interaction between CtBP and its partners is highly sensitive to conformational and/or folding constraints, as shown for some constructs used in this study (Fig. 3 and 5) and as previously found by others (9, 16, 26). For HIC1, an obvious candidate for these conformational effects is the correct dimerization of the protein through its BTB/POZ domain since the HIC1 BTB/POZ domain is involved in homo- and heterodimeric interactions (10, 11) (Fig. 2), as are BTB/POZ domains of many proteins (1, 12, 17, 32). In addition, CtBP proteins also homo- or heterodimerize (44, 47, 53). Even though the isolated BTB/POZ domain does not interact with mCtBP1 (Fig. 2 and 3), its removal from the full-length HIC1 protein severely impaired interactions with mCtBP1, in the sensitive coimmunoprecipitation assay (Fig. 5, lanes 6 and 8).

Thus, dimerization of HIC1 through its BTB/POZ domain is required presumably to allow the correct folding of the central region, where the interaction with CtBP takes place, mainly through the GLDLSKK motif.

**In Cos-1 cells, HIC1 delocalizes mCtBP1 to nuclear dots.** To study the subcellular localization of mCtBP1 and HIC1 proteins, we performed immunofluorescence confocal microscopy. In transiently transfected Cos-1 cells, Flag-HIC1 and Flag-HIC1 ΔGLDLSKK localized in similar punctate nuclear structures (Fig. 6a and b), as already described for numerous proteins with BTB/POZ domains (12). Thus, deletion of the GLDLSKK motif did not significantly alter subcellular localization. In transiently transfected Cos-1 cells, mCtBP1 gave diffuse nuclear and cytoplasmic distributions (Fig. 6c). Taking advantage of these clearly distinct cellular localizations, we coexpressed Flag-HIC1 or Flag-HIC1 ΔGLDLSKK with mCtBP1 and performed double-labeling experiments with the mouse anti-Flag monoclonal antibody and the rabbit anti-mCtBP1 polyclonal antibody. Strikingly, in the presence of Flag-HIC1, mCtBP1 became organized in discrete HIC1-positive nuclear dots (Fig. 6d to f). Similarly, in U2OS cells, expression of Flag-HIC1 resulted in the relocalization of endogenous CtBP into HIC1-positive nuclear dots (data not shown). By contrast, in Cos-1 cells, the Flag-HIC1 ΔGLDLSKK mutant did not induce relocation of mCtBP1 onto nuclear dots (Fig. 6g to i). Taken together, these results demonstrate that, in Cos-1 cells, HIC1 delocalizes mCtBP1 onto nuclear dots and that this interaction mainly relies on the GLDLSKK motif.

**Endogenous CtBP interacts with HIC1 in a stable CHO cell line with inducible expression of HIC1.** We next tried to detect an interaction between the endogenous HIC1 and CtBP proteins by coimmunoprecipitation and/or immunofluorescence

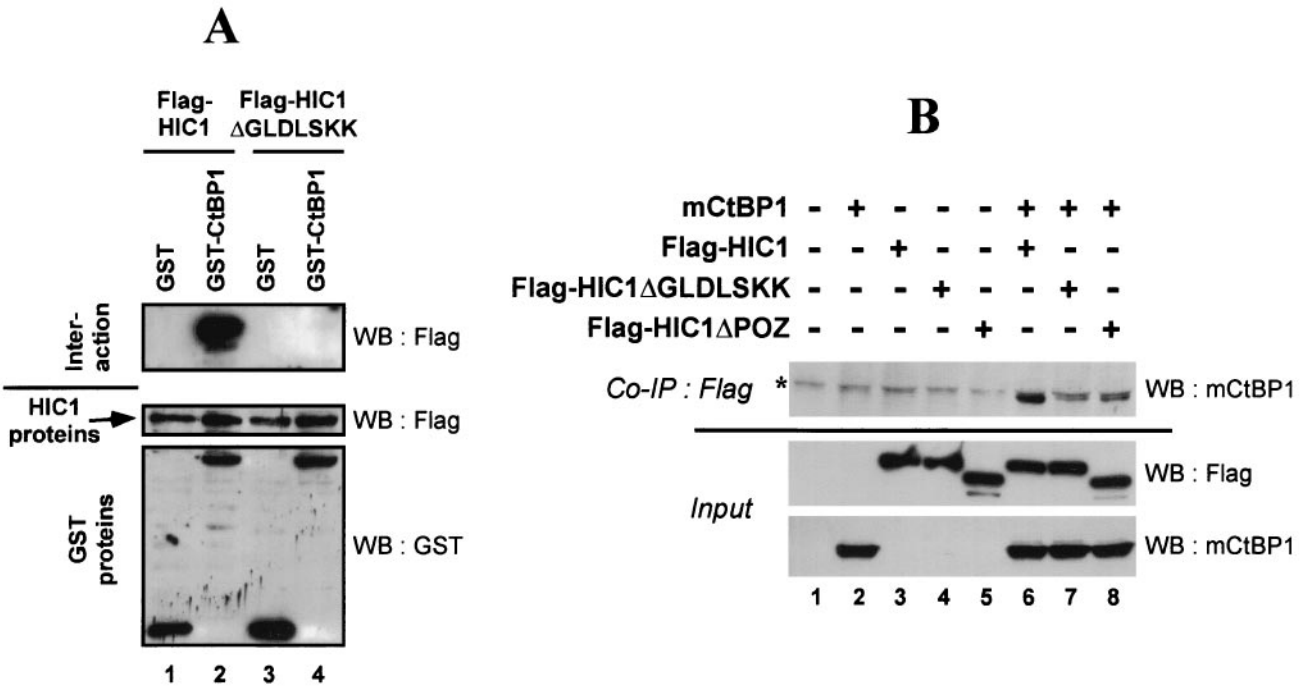


FIG. 5. HIC1 interaction with mCtBP1 in eukaryotic cells requires the GLDLSKK motif. (A) mCtBP1 interacts with Flag-HIC1 but not with Flag-HIC1 ΔGLDLSKK in the eukaryotic GST pull-down assay. Cos-1 cells were cotransfected with the indicated combination of expression vectors. Cell extracts were analyzed by the GST pull-down assay. Immunoblot analysis was used to detect the proteins retained on the beads by protein-protein interaction (top) with the GST proteins (bottom; 5 μl of each total cell extract; input). This blot was stripped and reprobed (middle) with the anti-Flag M2 monoclonal antibody to detect epitope-tagged HIC1 proteins. WB, Western blot. (B) In vivo interaction of Flag-HIC1 and Flag-HIC1 mutants with mCtBP1. Cos-7 cells were mock transfected (lane 1) or transfected with the indicated expression vectors. Flag-tagged proteins were immunoprecipitated (IP) from cell lysates with the anti-Flag M2 monoclonal antibody. The resulting immunoprecipitates were then Western blotted and analyzed with the anti-CtBP1 rabbit polyclonal antibody (top). *, nonspecific band immunoprecipitated by the M2 antibody in each extract under the conditions used. Five microliters of each total cell extract (input) was resolved by SDS-PAGE and immunoblotted with the anti-Flag antibody to control for HIC1 protein expression (middle). This blot was stripped and probed with the rabbit anti-CtBP1 polyclonal antibody to ascertain the presence of the exogenous mCtBP1.

analyses. However, HIC1 is not expressed in transformed cell lines, but only in normal tissues (18, 19, 30), which are not easy to use in this kind of experiment. Weak HIC1 expression has been detected, at least at the RNA level, in normal human fibroblasts (MRC-5 and WI-38) (30) and in human immortalized ovary epithelial cells (HIO) (18). Using several homemade and commercial anti-HIC1 polyclonal antibodies, we failed to detect the endogenous HIC1 and hence its interaction with CtBP in nuclear extracts from MRC-5 or CHO cells, probably due to the low level of HIC1 protein in these cells and/or the insufficient sensitivity of these antibodies.

To circumvent this problem, we generated a stable CHO cell line with inducible human Flag-HIC1 expression using the ecdysone-inducible mammalian expression system (Invitrogen). Upon induction by 10 μM ponasterone A (a synthetic analogue of ecdysone) followed by immunofluorescence analyses with the Flag monoclonal antibody, HIC1 displayed a micropunctate nuclear localization (Fig. 7A).

Nuclear extracts (41) were prepared from both the untreated and treated (induced) cells and used in coimmunoprecipitations with our two different polyclonal anti-HIC1 sera directed against the C-terminal part of HIC1. We were able to detect an interaction between nuclear HIC1 and endogenous CtBP (Fig. 7B, lanes 6 and 8). Notably, the small amount of HIC1 expressed in the uninduced cells due to the leakiness of the minimal hsp promoter used in this inducible system (Fig. 7; compare lanes 1 and 2) was sufficient for the detection of a proportionally lower interaction with endogenous CtBP (Fig. 7B, lanes 5 and 7). Thus, in a stable inducible cell line, HIC1 can associate with endogenous nuclear CtBP.

**The HIC1 central region is a new autonomous repression domain interacting with CtBP.** Having established that the GLDLSKK motif is required for the binding to mCtBP1 and that both proteins interact in the nucleus, we set out to determine if the central region of HIC1 harboring this new CtBP interaction motif has autonomous repressive capacity and whether deacetylase activity is required for this repression. Upon transient transfection into RK13 cells, Gal4-HIC1 (135-296) efficiently repressed (10-fold ± 1.3-fold) the expression of the pG5 reporter gene containing Gal4 binding sites upstream of the Luc gene, compared to that by the Gal4 DNA-binding domain alone (Fig. 8, lanes 1 and 3). Deletion of the GLDLSKK motif in the central region, a mutation that prevents its interaction with mCtBP1 (Fig. 3, lane 5), significantly reduced (4.6-fold ± 0.4-fold) but did not totally abolish its ability to repress transcription (Fig. 8, lanes 3 and 4). These results thus identify a new repression function in the HIC1 central region, depending largely on CtBP binding and mapping mainly to the conserved GLDLSKK motif. Attempts to further delineate a second repression domain in the Gal4-

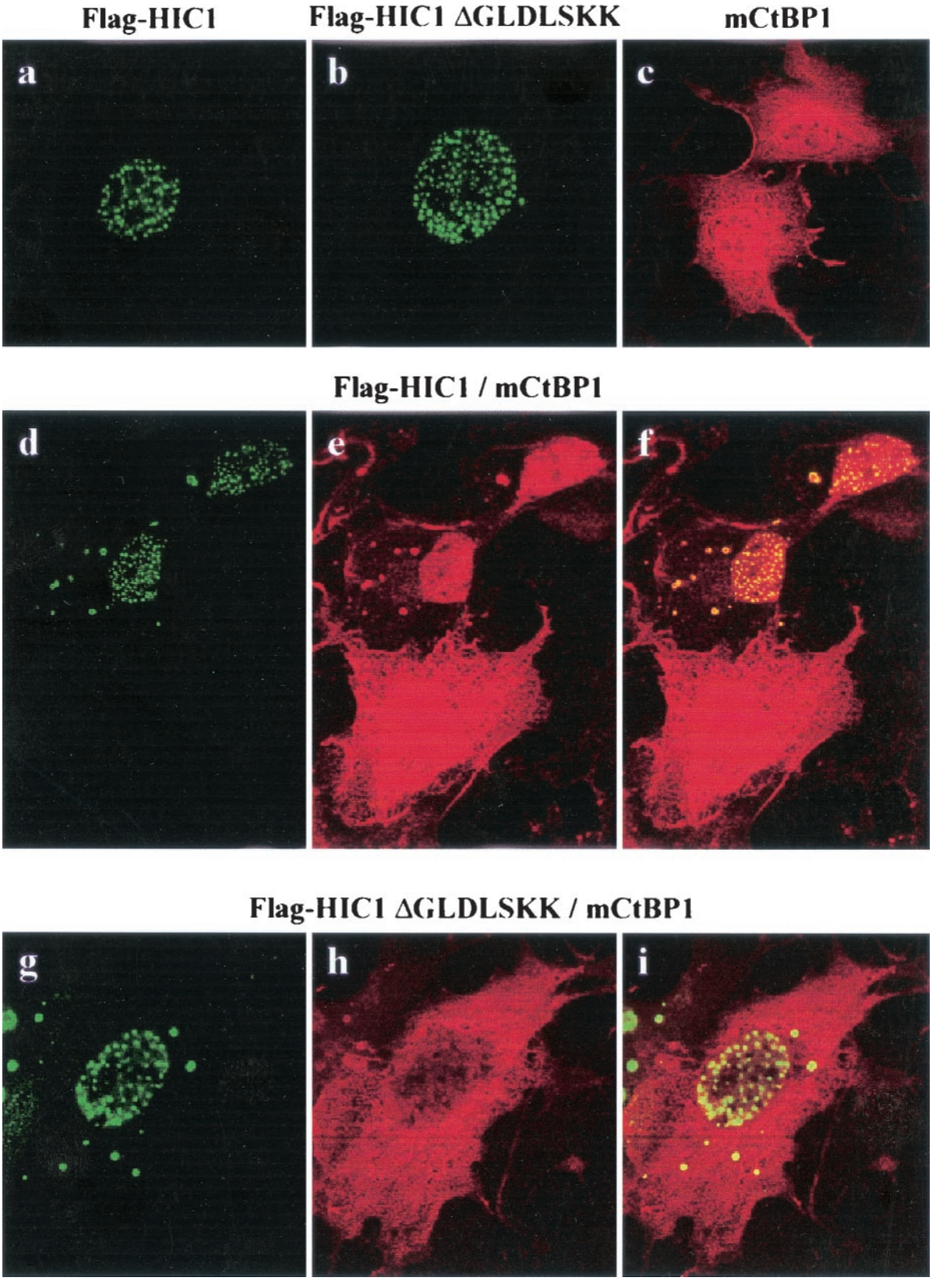


FIG. 6. HIC1 and mCtBP1 colocalize at nuclear dots in transfected Cos-1 cells. (a to c) Transfected Cos-1 cells were labeled with the anti-Flag M2 monoclonal antibody (a and b) or a rabbit anti-CtBP1 polyclonal antibody (c). Flag-HIC1 (a) and the Flag-HIC1 ΔGLDLSKK mutant (b) have punctate nuclear localizations, whereas mCtBP1 (c) has a diffuse nuclear and cytoplasmic localization. (d to f) When HIC1 and CtBP1 were cotransfected, HIC1 (d) recruited CtBP (e) onto nuclear dots (as shown in panel f by the merge). Note the presence in this section of a cell not transfected by Flag-HIC1, where the ectopically expressed mCtBP1 exhibits its typical nuclear and cytoplasmic diffuse pattern (e and f, bottom). (g to i) Flag-HIC1 ΔGLDLSKK mutant exhibits a punctate nuclear localization (g) but is unable to recruit mCtBP1 (h) onto these nuclear dots (i, merge).

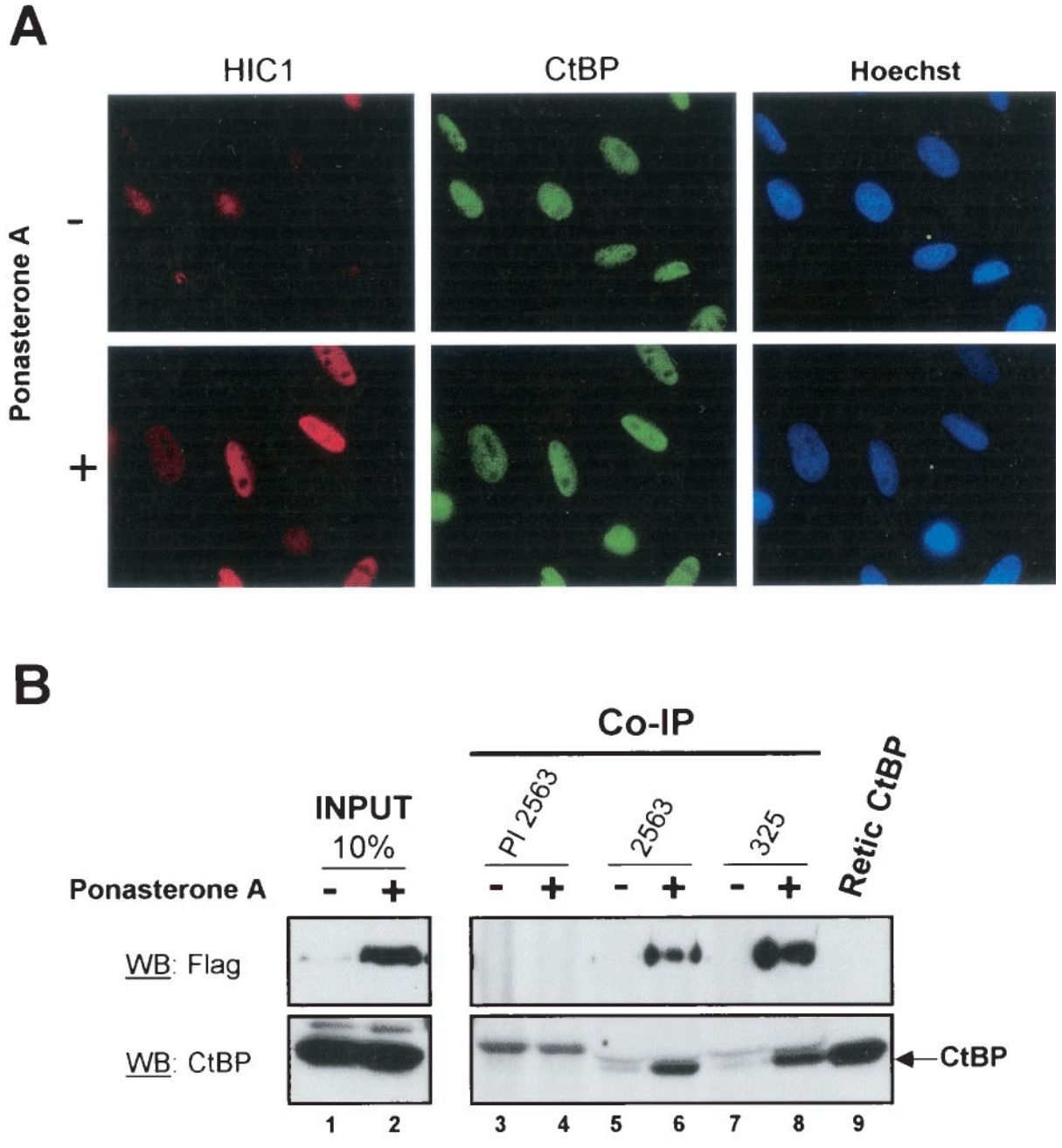


FIG. 7. Inducible HIC1 can recruit endogenous CtBP. (A) Characterization of the stable inducible EcRCHO-pINDFlag-HIC1 clone 6 cell line. EcR-CHO pIND-Flag-HIC1 clone 6 cells were untreated (−) or treated with 10 μM ponasterone A for 48 h (+) and analyzed by conventional immunofluorescence microscopy with the anti-HIC1 PAb325 polyclonal antibody (left) or the anti-CtBP E12 monoclonal antibody (middle). Hoechst staining of the same field is shown on the right. Note the weak expression of HIC1 in a few uninduced cells, probably due to the leakiness of the promoter. (B) Endogenous nuclear CtBP proteins can interact with HIC1 in nuclear extracts prepared from the stable inducible EcRCHO-pIND-Flag-HIC1 clone 6 cell line. Nuclear extracts were prepared as described previously (41) from the untreated EcR-CHO pIND-Flag-HIC1 inducible cell line (−; lanes 1, 3, 5, and 7) or from cells treated with 10 μM ponasterone A for 48 h (+; lanes 2, 4, 6, and 8). Aliquots were immunoprecipitated with the indicated rabbit preimmune serum (lanes 3 and 4) or with two distinct anti-HIC1 immune sera directed against the HIC1 C-terminal region (lanes 5 to 8). The immunoprecipitates, 10% of each nuclear extract (input; lanes 1 and 2) and 3 μl of a mCtBP1-programmed reticulocyte lysate as a control (lane 9) were resolved by SDS-PAGE and immunoblotted with the anti-Flag M2 monoclonal antibody (top). Notably, a small amount of HIC1 can be detected in the uninduced cells (lanes 1, 5, and 7), presumably due to the leakiness of the hsp promoter. The blot was stripped and probed with the anti-CtBP monoclonal antibody (bottom) to detect the interaction with endogenous CtBP (lanes 6 and 8). The band detected in lanes 3 and 4 by the anti-CtBP monoclonal antibody is not endogenous CtBP, as clearly shown by its distinct migration in SDS-PAGE (compare with lanes 6, 8, and 9). It rather corresponds to a nonspecific band brought down by the polyclonal anti-HIC1 preimmune rabbit serum under these experimental conditions. Interestingly, the small amount of HIC1 protein present in the uninduced cells is able to coimmunoprecipitate a proportionally smaller amount of endogenous CtBP (lanes 5 and 7).

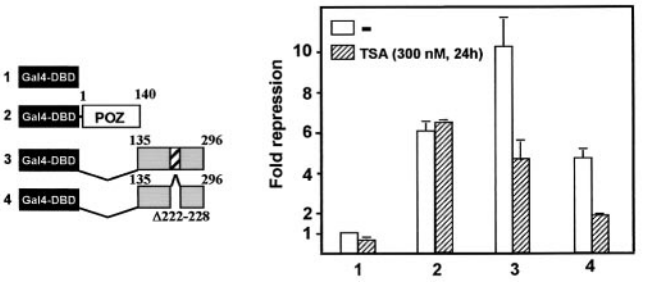


FIG. 8. The HIC1 central region contains two TSA-sensitive repression domains. (Left) The Gal4 DNA-binding domain (construct 1) and the various Gal4-HIC1 chimeras (constructs 2 to 4) used in this assay are shown. Numbering refers to HIC1 residues. Hatched box, GLDLSKK motif. (Right) RK13 cells were transiently transfected in triplicate with 200 ng of the indicated constructs and 750 ng of the pG5Luc reporter. The cells were treated 24 h later with 300 nM TSA (dissolved in dimethyl sulfoxide [DMSO]) (hatched bars) or mock treated with an equal volume of DMSO (-, white bars) for a further 24 h before harvesting. The Luc activity was normalized to the β-galactosidase activity of a cotransfected pSG5 β-galactosidase construct (50 ng). After normalization to β-galactosidase, the data were expressed as Luc activity relative to the activity of pG5Luc with empty control vectors, which was given an arbitrary value of 1. The results are the mean values and standard deviations from two independent transfections in triplicate.

HIC1 (135-296) ΔGLDLSKK chimera have been so far unsuccessful (data not shown), suggesting that the whole region could be required for this function.

To explore the possible involvement of deacetylase activities, we used specific HDAC inhibitor TSA. As shown in Fig. 8, addition of 300 nM TSA substantially alleviated repression by Gal4-HIC1 (135-296) (lane 3), strongly suggesting that the CtBP-dependent repression mechanism exhibited by the HIC1 central region is TSA sensitive. TSA also inhibited Gal4-HIC1 (Δ222-228), lacking the major CtBP interaction motif (Fig. 8, lane 4), showing that this mutated region can also interact with HDACs, albeit weakly. By contrast, the repression mediated by the HIC1 BTB/POZ domain is insensitive to TSA (Fig. 8, lane 2), as previously described (10, 11). Thus, HIC1 contains two functionally distinct repression domains, its BTB/POZ domain, unable to recruit HDAC activities, and its central region, which interacts with CtBP and which can recruit HDAC.

**HIC1 interacts with endogenous HDACs.** To confirm that HDACs are involved in the repression mediated by HIC1, we tested whether HIC1 interacts with endogenous HDACs. Flag epitope-tagged proteins were expressed by transient transfection of Cos-1 cells and immunoprecipitated with Flag monoclonal antibodies. Immunoprecipitates were incubated with [$^3$H]acetate-labeled histones and quantified by scintillation counting of released [$^3$H]acetate. These experiments indicated that HIC1 can associate with endogenous deacetylase enzymatic activities in vivo (Fig. 9A). The levels of HDAC immunoprecipitated by Flag-HIC1 were similar to those immunoprecipitated by Flag-BCL6, which in contrast to HIC1 (10) is able to recruit SMRT/NCoR/mSin3A/HDAC1-2 complexes (12). In this assay, Flag-HIC1 ΔGLDLSKK, which interacts weakly with mCtBP1 in coimmunoprecipitation experiments (Fig. 5B), exhibits a significant deacetylation activity, which is reduced compared to that of wild-type Flag-HIC1 but which is still 2.5-fold above the background levels (Fig. 9A). In all cases, addition of 300 nM TSA greatly reduced the amount of [$^3$H]acetate released, demonstrating the presence of bona fide HDACs in the Flag-HIC1 and Flag-HIC1 ΔGLDLSKK immunoprecipitates (Fig. 9B).

## DISCUSSION

In this report, we demonstrated that the transcriptional repressor HIC1 contains two functionally distinct repression activities and identified CtBP as a HIC1 corepressor which is recruited via a variant consensus binding site.

Using various protein-protein interaction assays, we have shown that the GLDLSKK/R motif, first identified in silico through multiple alignments of HIC1 and HRG22 proteins from various species, is a new CtBP interaction motif. The HIC1 central region containing this motif is a new autonomous repression domain that is sensitive to TSA, an HDAC inhibitor (Fig. 8). The BTB/POZ repression domain is functionally unrelated to the central region, as supported by two lines of evidences. First, the repression activity of the HIC1 (10) and related HRG22 (11) BTB/POZ domains is not alleviated by TSA, in contrast to what was found for the central region (Fig. 8). Second, the isolated BTB/POZ is unable to interact either directly or indirectly via bridging partners (e.g., CtIP) (28) with mCtBP1 in yeast and mammalian two-hybrid assays (Fig. 2 and 3). This is not due to incorrect folding of the domain since the same construct is able to mediate homodimerization (Fig. 2). The presence of several repression activities in the same transcription factor could broaden its range, both qualitatively and quantitatively, and contribute to the fine-tuning of gene expression. A repressor able to interact with several corepressors could exert qualitatively different repression effects, depending

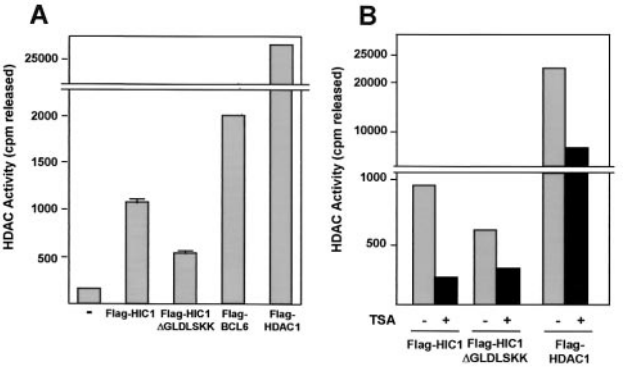


FIG. 9. The full-length HIC1 protein and the ΔGLDLSKK mutant can associate with endogenous HDAC activity. (A) Cos-1 cells were transfected with expression vectors encoding either Flag-HIC1 or Flag-HIC1 ΔGLDLSKK. Flag–BCL-6, which can recruit HDAC-1, and Flag–HDAC-1 itself were used as positive controls, whereas cells transfected with the empty Flag expression vector (−) were used as a negative control to measure background levels. The HDAC activities coimmunoprecipitated with the Flag-HIC1 and Flag-HIC1 ΔGLDLSKK proteins were measured in duplicate from two independently transfected plates, with the standard deviations indicated. (B) To demonstrate the coretention of bona fide deacetylase activity, an experiment similar to that in panel A was performed, except that the immunoprecipitates were divided into two aliquots. One was left untreated (grey bars), and the other was incubated in the presence of 300 nM TSA (black bars) before the enzymatic assay.

on the cellular context and/or the target promoter. Alternatively, several repressing activities could quantitatively increase the global repressive effects on a target promoter, similar to the transcriptional activators, which can engage multiple synergistic contacts with the transcription machinery. For CtBP, however, these additive effects can act negatively as well as synergistically. CtBP-binding proteins Hairy (44, 58), XTcf-3 (5), and E(spl)mδ/C (44) can also interact with the Groucho corepressor. Although Groucho and dCtBP mediate clearly distinct pathways of transcriptional repression, they can act antagonistically when bound to the same repressor, as studied in-depth for the *Drosophila* Hairy protein, where a PLSLVIK CtBP motif is located nine amino acids upstream of a C-terminal WRPW Groucho motif (43, 58). In fact, dCtBP seems to interfere with Groucho-mediated transcriptional repression by impairing the formation of a potent Groucho repression complex (43). For HIC1, it would thus be interesting to define if the repressing activity harbored by the BTB/POZ domain could be modulated by the recruitment of CtBP.

The isolated BTB/POZ domain did not interact with mCtBP1, whereas its removal from full-length HIC1 severely impaired the interaction with mCtBP1 (Fig. 5). Taken together, these data lend further support to the pivotal role of the dimerization (multimerization) potential of the BTB/POZ domain, which creates an interface for optimal binding of the partner. Similar to our results for HIC1, the interaction of CtBP with Tramtrack through a classical C-terminal PLDLS motif and a second uncharacterized binding site requires the presence of a functional BTB/POZ domain (55). Another paradigm for the close interplay among BTB/POZ multimerization, interaction with protein partners, and biological function is the *Drosophila* Mod(mdg4)2.2 protein, which, together with Su(Hw), a zinc finger protein that binds to insulator DNA, is one of the key components of the *gypsy* insulator. The *gypsy* insulator is thought to establish higher chromatin domains by bringing together several individual insulator sites to form rosette-like structures in the interphase nucleus, mainly through the multimerization of the BTB/POZ domains from Mod(mdg4) proteins, which would bring together several individual Mod(mdg4) binding sites (17). In addition, the BTB/POZ domain of Mod(mdg4)2.2 does not directly interact with Su(Hw) but is required in the full-length protein to generate an interface at its C-terminal region through which interaction with Su(Hw) can occur (17). More generally, even when the BTB/POZ domain is directly implicated in interactions with corepressors, e.g., SMRT and BCoR (12, 21, 22), its dimerization also seems to be required. Indeed, point mutations that affect the dimerization of the BTB/POZ domain also affect the interaction between BCL6 and SMRT or BCoR (21, 22) as well as the transcriptional repression properties of PLZF (32).

The strict requirement for an appropriate conformation of HIC1, mediated mainly by the dimerization domain, could explain the lack of interaction between the Gal4-HIC1 (full-length) chimera and mCtBP1 in a mammalian two-hybrid assay (Fig. 3). The lack of accessibility of the CtBP interaction domain to CtBP in chimeras is not unique to HIC1 and has already been observed for Net (9), δEF1 (16), and Ikaros (26). For HIC1, the fusion of large heterologous sequences (i.e., Gal4 DNA-binding domain) to the N terminus could impede dimerization via the BTB/POZ domain. Similarly, in the GST pull-down experiment, the GST-HIC1 chimera also failed to interact with mCtBP1 (data not shown), whereas HIC1 strongly interacts with GST-CtBP1 (Fig. 5A). The interaction between the Gal4-ΔPOZ chimera and mCtBP1, which seems to contradict the strict requirement for BTB/POZ in the full-length protein, in fact strongly suggests that a heterologous dimerization domain, the Gal4 DNA-binding domain, can functionally substitute for the BTB/POZ domain. This observation is highly reminiscent of the Tramtrack-dCtBP interaction, where the *Drosophila* Tramtrack BTB/POZ domain can be replaced by the unrelated human BCL6 BTB/POZ domain, which provides a convenient dimerization interface (55).

Another novel general finding of the present study is the definition and functional characterization of an extended consensus binding site for CtBP, GLDLSKK/R, which is found in two related proteins and which is phylogenetically conserved from zebra fish to humans and in which the virtually invariant proline residue is replaced by a glycine. This proline can eventually also be replaced by a valine (Fig. 4) since a VLDLS motif has recently been found in some CtBP-binding proteins (54), but the functional relevance of this motif has still to be established. An evolutionarily conserved minimal repression domain within the *Drosophila* Giant short-range repressor contains a VLDLS motif (40, 48). However, it is unclear whether this VLDLS sequence participates in Giant-dCtBP interactions, which might be indirect (40). Mutagenesis of the proline or of the Pro-Leu residues in the CtBP-binding sites of various proteins has in most cases severely impaired the interaction with CtBP (9, 16, 46), at least in some assays (38). Moreover, in vitro studies using synthetic peptides mimicking the CtBP-binding site in Ad5 E1A have demonstrated that the proline-to-glycine change found in HIC1 and HRG22 only slightly affects the $K_d$ of the reaction (from 2.5 to 11 μM) whereas alanine substitution for the proline severely increases it (71 μM) (35). Fully consistent with these in vitro studies, in vivo Ikaros binds CtBP via a PLDLS motif, whereas the related Helios, containing an AIDLT, and Daedalus and Helios factors, which contain sequences with weak homology to the CtBP interaction motif, cannot (26). Notably, this is the first protein interaction that distinguishes Ikaros from its family members to be described (26). Conversely, mutations at positions that seem to support high variations in the consensus (Fig. 4) could also have dramatic effects. A point mutation in Smad corepressor TGIF, which changes PLDLS to PLDLC, abolishes the interaction with CtBP and is associated with holoprosencephaly, a prevalent defect of craniofacial development (31). Strikingly, the human Ad4 E1A protein contains a PLDLC motif. Thus, these data demonstrate that the interaction between a transcription factor and CtBP cannot be explained by the simple presence of a PLDLS motif. Global structural effects in the transcription factor, such as a correct dimerization and/or folding (35, 36), together with subtle variation in the motif itself or in the flanking residues, could strongly modulate the interaction with CtBP. Acetylation of a lysine flanking the PXDLS motif has recently emerged as a potent regulatory mechanism for the interaction of CtBP and its partners, notably E1A (59) and RIP140 (54). Disruption of repressor-CtBP interactions by acetylation is likely to be a general mode of gene activation, since numerous CtBP-binding proteins contain PXDLSXK or PXDLSXXK motifs (53, 54). Most HIC1

and HRG22 proteins contain a GLDLSKK motif except for the zebra fish HIC1 protein, which contains a GLDLSKR motif (11, 19) (Fig. 1), suggesting that the HIC1/CtBP interaction can also be regulated by acetylation, at least in some species. Arginine is not a known target of coactivator acetyltransferases and, in the context of E1A, a Lys-to-Arg substitution increases CtBP binding in vivo (59). Interestingly, we have recently characterized a new 737-amino-acid zebra fish HIC1 protein that is highly homologous to the 714-amino-acid human HIC1 protein and that contains a classical GLDLSKK motif (data not shown).

Since CtBP seems to be part of different complexes (43, 53) and seems to be involved in HDAC-dependent and -independent repression mechanisms (26, 50), the next challenges in the future will be to decipher the structural constraints for each CtBP-binding protein and to define how they influence the type of CtBP-containing complex that is recruited. For HIC1, these CtBP complexes could participate in the regulation of target genes that are involved in two mutually nonexclusive pathways: the HIC1 tumor suppressor properties altered in many cancers and the developmental processes impaired in the human MDS and in the HIC1$^{-/-}$ mice.

## ACKNOWLEDGMENTS

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