Interaction between Smad-interacting protein-1 and the corepressor C-terminal binding protein is dispensable for transcriptional repression of E-Cadherin.

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Running title: CtBP independent repression of E-Cadherin by SIP1
Summary

δEF1 and SIP1 (or Zfhx1a and Zfhx1b, respectively) are the only known members of the vertebrate Zfh1 family of homeodomain/zinc finger-containing proteins. Similar to other transcription factors both SIP1 and δEF1 are capable of repressing E-Cadherin transcription through binding to the E2 boxes located in its promoter. In the case of δEF1, this repression has been proposed to occur via interaction with the corepressor C-terminal binding protein (CtBP). In this study, we show by co-immunoprecipitation that SIP1 and CtBP interact in vivo and that an isolated CtBP-binding SIP1 fragment depends on CtBP for transcriptional repression. However, and most importantly, full-length SIP1 and δEF1 proteins do not depend on their interaction with CtBP to repress transcription from the E-Cadherin promoter. Furthermore, in E-Cadherin-positive kidney epithelial cells, the conditional synthesis of mutant SIP1 that cannot bind to CtBP abrogates endogenous E-Cadherin expression in a similar way as wild-type SIP1. Our results indicate that full-length SIP1 can repress E-Cadherin in a CtBP-independent manner.
Introduction

Smad-interacting protein-1 (SIP1) is a transcriptional repressor with structural similarities to the Drosophila Zfh-1 protein (1), and has eight zinc fingers, seven of which are grouped in two clusters, and one homeodomain-like motif (2). SIP1 therefore belongs to the small Zfh1x gene family of transcriptional repressors, with the only other vertebrate member being δEF1 (3). Whereas DNA-binding activity has been determined for the isolated zinc finger clusters (Carboxy-terminal zinc finger cluster; CZF) of SIP1 and δEF1 (2,3) and within the respective full-length proteins (4), the homeodomain-like domain of δEF1 failed to show an interaction with DNA (5). The zinc finger clusters specifically recognize CACCT(G) DNA motifs and SIP1/δEF1 full-size proteins demonstrate the highest DNA-binding efficiency in vitro when both zinc finger clusters are intact, and when two spaced CACCT motifs are present in the target DNA (4). SIP1 (ZEB2; Zfhx1b) has meanwhile been characterized in several species including mouse, human and frog (2,6-8), and a δEF1-like gene (Kheper) has also been reported in fish (9).

Recently, the gene encoding the transmembrane protein E-Cadherin, a major player in homotypic cell adhesion, has been identified as a target for transcriptional repression by δEF1 and SIP1 in vitro (10,11). SIP1-knockout mouse embryos clearly show up-regulation of E-Cadherin in the neuroepithelium and the neural tube where SIP1 is normally expressed (12). Many potential target promoters have been identified for both SIP1 and δEF1 but the precise mechanism of action of these repressors is still unknown. In the case of the E-Cadherin promoter and its regulation by δEF1, the co-repressor C-terminal binding protein (CtBP) was proposed to be necessary for transcriptional repression (10). CtBP was originally identified as a protein that interacts with the C-terminal segment of the adenovirus E1A oncoprotein via a PLDLS sequence in the latter (13), and interacts with a growing list of transcription factors from Drosophila and vertebrates (14,15). Two highly related CtBP proteins, CtBP1 and
CtBP2, have been identified in vertebrates and have overlapping but also unique roles during embryogenesis, as shown by loss-of-function studies in the mouse (16). Although it has recently been shown that CtBP possesses dehydrogenase activity (17), the precise mechanism by which CtBP represses transcription is still subject to controversy as well.

Both SIP1 and δEF1 have 3 PXDLS motifs and interaction between CtBP and those domains has been shown in vitro (6,18). In addition, CtBP significantly enhances the repression mediated by full-length δEF1 of the muscle creatine kinase enhancer (19). Furthermore, using truncated δEF1 proteins, two studies demonstrated that intact CtBP binding motifs are necessary for these δEF1 polypeptides to repress the human E-Cadherin promoter in luciferase reporter assays (10, 20). In this paper we investigate the possible role of CtBP in SIP1-mediated transcriptional repression of E-Cadherin. We show that anti-CtBP antibodies can immunoprecipitate endogenous CtBP: SIP1 complexes from a human breast carcinoma cell line (MDA-MB435S) and from human embryonic kidney (HEK293T) cells. Using the CtBP-interaction domain (CID) of SIP1, we observe a CtBP-dependent repression when recruited to the SV40 early promoter or an E-Cadherin promoter driven luciferase reporter. In contrast, full-length SIP1 and δEF1 proteins do neither need CtBP binding sites to repress the E-Cadherin gene in reporter assays nor is CtBP binding necessary for endogenous transcriptional repression of E-Cadherin by SIP1 in cultures of dog kidney epithelial (MDCK) cells.
Experimental procedures

Plasmids and reagents — pCS3 with inserts of Myc-tagged wild-type or the double zinc-finger mutant of mouse SIP1 cDNA are described in (2,4). The 3xCtBP\textsubscript{mut} and 4xCtBP\textsubscript{mut} SIP1 mutants were generated by PCR and verified by sequencing before exchanging a\textit{SmaI} insert containing these mutations with the corresponding fragment of wild-type SIP1 cDNA in pCS3. Mouse CtBP2 was obtained by PCR from mouse testis mRNA and cloned in-frame with a flag tag in\textit{pCDNA3.1}. Human CtBP1 and CtBP1-VP16 were obtained from V. Kumar (La Jolla, CA,USA). For the UAS-SV40-luciferase assays, the GBD fusion proteins studied here were obtained by cloning the complete open reading frame of mouse SIP1 and the SIP1CtBP\textsubscript{mut} in-frame with the DNA-binding domain of the yeast GAL4 protein (in\textit{pBind}, Promega). A UAS-SV40 promoter-luciferase vector was created by cloning five GAL4 binding sites (5xUAS of\textit{pGL5}; Promega) in front of the SV40 early promoter in\textit{pGL3} (Promega). GBD-SIP1\textsubscript{CID} and GBD-SIP1\textsubscript{CID-mut} were generated by a similar in-frame cloning of a product, generated by PCR, that comprises the wild-type CID or 3xCtBP\textsubscript{mut} CID of mouse SIP1 (CID, as it is used here, encompasses amino acids 737 to 871 of SIP1). The same fragments were used to generate the CZF-CID SIP1 polypeptide and corresponding CID mutant by cloning these cDNAs in-frame with the CZF-encoding cDNA of mouse SIP1 (CZF, as it is used here, encompassed amino acids 982 to 1107 of SIP1). These mutations were first generated by PCR and the fragments were cloned in-frame with 6 Myc tags and the SV40 T-antigen nuclear localization signal (NLS) in\textit{pCS2-NLS-Myc}, thereby generating\textit{pCS2-NLS-Myc-CZF-CID} vectors.

For the generation of inducible expression plasmids of SIP1, the pTRE plasmid (Clontech) was used to clone a Hygromycin resistance cassette under the control of a thymidine kinase promoter (pTHyg) in the unique\textit{HindIII} site, together with the different Myc-tagged SIP1 open reading frames taken from wild-type SIP1 in pCS3 (see above),
CtBP\textsubscript{mut} (this paper) and the zinc finger mutations (4) downstream of a human cytomegalovirus (CMV)-based enhancer/promoter containing 6 Tet-responsive elements. Wild-type and CtBP\textsubscript{mut} δEF1 cDNAs were a gift from Y. Higashi (Osaka, Japan), and the wild-type and mutant E1A cDNAs were obtained from R. Goodman (Portland, OR, U.S.A).

Antibodies — Rabbit polyclonal α-C\textsubscript{term} SIP1 antiserum raised against a SIP1-specific peptide, the sequence of which is conserved between human and mouse SIP1 (i.e. CSDSEERESMPRDGES), was used at 1:1000 dilution in Western analysis; rabbit polyclonal α-N\textsubscript{term} SIP1 antiserum was raised against another peptide (amino acids 26-129 of mouse SIP1). The rat monoclonal antibody DECMA-1 (Sigma) recognizes both mouse and dog E-Cadherin and was used at 1:100 for immunofluorescence (IF). Mouse anti-Myc-tag antibody (1:200 for IF, 1:3000 for Western analysis), Mouse anti-GBD antibody (1:3000 for Western analysis) and a rabbit anti-CtBP antiserum, which recognizes both CtBP1 and CtBP2 in mouse, human and dog (1:200 for Western analysis), were also used in this study (all from Santa-Cruz).

Transient transfections, and reporter assays — Transient transfections for luciferase assays were done with 25 kDa branched polyethylene-imine (PEI, Aldrich, method described in (21)), using 1.2 μl of a 1 mg PEI/ml stock solution per 300 ng of DNA transfected per well of a 24-well plate. In the case of HEK293T cells, approximately 45000 cells/cm\textsuperscript{2} were seeded, whereas for C90 and C86 fibroblasts 11000 and 16000 cells, respectively, were seeded per cm\textsuperscript{2}. Transfections were carried out 1 day later with 100 ng of E-Cadherin promoter-luciferase (11) or 50 ng of UAS-SV40 promoter-luciferase reporter plasmid, and with the SIP1 and CtBP expression plasmids as indicated. For normalization, 15 ng of a lacZ reporter construct that contains the CMV promoter inserted upstream of Escherichia coli lacZ were
cotransfected. Cell extracts were prepared and assayed for luciferase activity and β-galactosidase activity according to the manufacturers’ protocols (Luciferase assay system (Promega) and Galscreen™ (Tropix), respectively), and only transfections with similar β-galactosidase values were taken into account. The data, obtained in triplicate experiments, were then normalized by calculating the ratio of luciferase and β-galactosidase activities. When the lysates were also used for Western analysis, one fifth of the lysates was used to run on an 8% SDS-PAG followed by Western detection of the produced proteins by means of anti-Myc antibodies and subsequent visualization using the Western Lightning detection system (Perkin-Elmer).

For immunoprecipitation of SIP1:CtBP complexes, HEK293T cells were transfected at 50% confluency with a total of 4 µg DNA and 16 µl of PEI solution, harvested the next day, and immediately frozen down. Immunoprecipitations of overexpressed/endogenous proteins and the Western analysis were carried out as described previously (22). For immunoprecipitation of endogenous CtBP:SIP1 complexes, 20x10^6 cells were used of each cell line, and 5 µg of CtBP antibody were used per immunoprecipitation.

DNA precipitations (DNAP) were carried out as described (23) using 1 µg of biotinylated double-stranded oligonucleotides encoding 1 UAS (GAL4-binding Upstream Activating Sequence; 5’TCTAGACGGGAGT ACTGTCCCTCCGACTCGAG 3’).

Cell culture and generation of MDCK cell lines — Monolayer cultures of HEK293T, HepG2 and MDCK-Tet-off cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, high glucose) supplemented with 10% fetal bovine serum. MDA-MB435S cells and the CtBP+/- and CtBP-/- fibroblasts (the C86 and C90 fibroblasts were a kind gift from P. Soriano, Seattle, WA, USA) were grown in the same medium but supplemented with 2mM L-GLutamine. MCF7 cells were cultured as described (24). For the generation of the MDCK-
Tet-SIP1 cells, 24 hours after seeding, the MDCK-Tet-off cells (24000 cells/cm²) were transfected with 2 µg of the respective linearized pTRE-TKHyg-SIP1 plasmids (wild-type SIP1, SIP1_cTBP-mut and SIP1_ZF-mut) using 6 µl of Fugene (Roche). The next day, these cells were split 1:23 and stable transformants were selected in 0.1 mg Hygromycine B/ml (Roche) for 2 weeks. The SIP1 expression was prevented by adding tetracycline to the cultures during selection for hygromycin resistance (1-2 µg Tet/ml, Sigma). Surviving clones were tested for inducibility and synthesis of the Myc-SIP1 proteins by Western blotting and immunofluorescence. Representative clones were then verified for the incorporation of the different mutant constructs by PCR and sequence analysis. For the analysis of SIP1-mediated transcriptional repression of E-Cadherin, the different cell lines were seeded on glass coverslips at a density of 16000 cells/cm² in the absence or presence of tetracycline, as indicated. After 4 days, cells were washed with phosphate buffered saline, fixed with paraformaldehyde, and analyzed for Myc-SIP1 and E-Cadherin production using standard immunohistochemistry techniques.

Two-hybrid mating assays in yeast — These were performed by the interaction trap cloning method, which is often referred to as the LexA two-hybrid system (25). The inter-zinc finger region of both mouse SIP1 (defined here as from amino acid 315 to 942) and mouse δEF1 (from 330 to 909) were cloned in-frame with the LexA DNA-binding domain (pGilda (Clontech), the bait plasmid) as well as the complete open reading frame of mouse CtBP2. For the LexA-mSIP1_cTBP-mut, the same region (315-942) but containing the SIP1 3xCtBP_mut was used. The open reading frame of mouse CtBP2 as well as the open reading frames of human Smad1, 2, 3 and 4, mouse Smad5, 6 and 7, and rat Smad8 were cloned in-frame with the activation domain of pB42 (Clontech, the prey vector). Transformation of yeast and the two-hybrid mating assays were performed as described (26).
Results

CtBP binds to SIP1 through conserved PXDLS-like motifs

SIP1 (also named Zfhx1b) and δEF1 (Zfhx1a) share similarity not only in the two zinc finger clusters (NZF and CZF) that are necessary for DNA binding but also in the relative position of the CtBP-interaction domain (CID) to these clusters. For SIP1, three PXDLS-like motifs are located in a 78 amino acids-long domain (amino acids 737 to 871) that is situated in-between NZF and CZF (Fig.1A). Unlike δEF1, SIP1 has an additional PXDLS-like motif (PLRLT) in front of the three motifs that they have in common (Fig.1A). Besides the zinc fingers and the CID, SIP1 also has a Smad-binding domain (SBD) that shows only 30% identity with δEF1 (2). This low conservation in sequence is directly reflected by the inability of δEF1 to interact with receptor-regulated Smads (RSmads, Fig.1B). When fused to the DNA-binding domain of LexA, the inter-zinc finger region of both SIP1 (segment 315-942) and δEF1 (330-909) was found to bind to full-length CtBP2 (fused to the activation domain of B42; B42AD) in a yeast two-hybrid mating assay. In contrast, only SIP1 was binding to the B42AD-RSmads 1, 2, 3, 5 and 8 fusion proteins. Moreover weak staining indicates that SIP1 does not show strong direct interaction with Smads 4, 6 and 7 (Fig.1B).

Mutation of PXDLS motifs in other proteins that bind CtBP affects their capacity to interact with CtBP (13). We used yeast mating assays and co-immunoprecipitation experiments to investigate whether combined mutation of the three SIP1 PXDLS motifs was sufficient to abolish the binding with CtBP. In the LexA-SIP1 protein, all three CtBP binding sites (PLNLS, PLDLS and PLNLT) were mutated to AAALS, AAALS and AAALT, respectively, to create LexA-SIP1_{CtBP-mut} (Fig.1A). This SIP1_{CtBP-mut} protein clearly did not interact with mouse CtBP2 when co-expressed in yeast (Fig.1C) while it was still capable of
interacting with Smads, e.g. Smad1 and Smad2. As reported before (27), CtBP has the capacity to form homodimers (Fig.1C).

The additional PXDLS-like motif (PLRLT) sequence however does not seem to be sufficient for binding to CtBP because a LexA-SIP1 fusion which carries mutations in the other three CtBP binding sites did no longer interact with CtBP (Fig.1C).

Although overexpression of an 120 amino acids-long polypeptide of human SIP1 revealed binding to overexpressed CtBP, there is no report describing the interaction of full-length SIP1/ZEB2 with CtBP. Therefore, co-immunoprecipitation experiments were performed to test whether full-length mouse SIP1 interacts with CtBP and whether this interaction is disrupted by the aforementioned PXDLS mutations, but now introduced in full-length SIP1. Expression vectors encoding either wild-type or mutant Myc-SIP1 were cotransfected with a Flag-tagged human CtBP1 or a Flag-tagged mouse CtBP2 construct into HEK 293T cells. Extracts were immunoprecipitated with anti-flag antibodies and immunoblotted with an anti-Myc antibody. Myc-tagged SIP1 was only co-immunoprecipitated when the CtBP binding sites were intact but not when the three PXDLS motifs were mutated (Fig.1D). Therefore, full-length wild-type SIP1 can interact with CtBPs, and this interaction is abolished when the three PXDLS motifs are mutated.

**SIP1 interacts with CtBP in vivo**

In several human carcinoma cell lines, the levels of E-Cadherin RNA and protein are inversely correlated with SIP1 expression (11). The human breast carcinoma cell line MDA-MB435S contains high levels of SIP1 while E-Cadherin is undetectable, which is in contrast with other cell lines (e.g. MCF7 cells), which express no SIP1 and have high levels of E-Cadherin. In addition, in SIP1-knockout mouse embryos, E-Cadherin transcription as well as E-Cadherin protein levels are up-regulated in the neuroepithelium and the neural tube (tissues...
in which SIP1 is expressed in normal embryos) (12). Furthermore, SIP1 can bind to, and transcriptionally repress, the E-Cadherin promoter (11). This prompted us to investigate whether the SIP1: CtBP interaction plays a crucial role in the SIP1-mediated repression of E-Cadherin transcription. To start this, the existence endogenous SIP1: CtBP protein-complexes was verified. First, specificity of raised polyclonal anti-SIP1 antibodies was determined by Western blotting of endogenous SIP1 complexes immunoprecipitated with antibodies directed against the N-terminal part of SIP1 (α-NtermSIP1; this study) or the C-terminal part of SIP1 (α-CtermSIP1 (11)) from MDA-MB435S cells. Figure 2B shows that both antibodies could immunoprecipitate a protein of ± 170 kDa that is recognized by the α-CtermSIP1 antibody. The α-NtermSIP1 antibody is SIP1-specific as illustrated in figure 2A. The α-NtermSIP1 antibody only immunoprecipitated overexpressed Myc-tagged SIP1 and not Myc-tagged δEF1 whereas anti-αMyc antibodies immunoprecipitated both tagged proteins. Subsequently, we used MDA-MB435S and HEK293T cells to determine whether SIP1 and CtBP interact at endogenous levels. To this end, extracts from these cells and from MCF7 and HepG2 cells (these two cell lines do not express SIP1 RNA; data not shown and (11)), were immunoprecipitated with a polyclonal antibody against CtBP1/2 and immunoblotted with a the α-CtermSIP1 antibody. While CtBP is present in every cell line and was immunoprecipitated, Fig.2C clearly shows that the SIP1 protein was co-immunoprecipitated with CtBP only in the cells that express SIP1, i.e. MDA-MB435S and HEK293T. This demonstrates that SIP1 and CtBP interact at endogenous levels.

The CtBP interaction domain of DNA-bound SIP1 is not required to mediate repression in a full-size context

Many CtBP-interacting transcriptional regulators have been described (for reviews, see (14,15)) and demonstration that these proteins mediate repression in a CtBP-dependent
manner relied on testing the activity of fusions between the CtBP-interaction domain of that protein and the DNA-binding domain of GAL4 (GBD). These fusion proteins are targeted via multiple Gal4 binding sites (UAS) to reporter constructs whose transcription is activated by strong promoters (e.g. the SV40 early promoter). Using this experimental set-up, we transfected wild-type GBD-SIP1 full-length (GBD-SIPFL) or a SIP1-CtBP mutant (GBD-SIPFL-mut) but also wild-type or mutated CID (resp. GBD-SIP1CID and GBD-SIP1CID-mut; (CID is defined as a fragment encompassing from aminoacids 737 to 871) encoding constructs in HEK293T cells, along with a UAS-SV40-luciferase construct. Fig.3A shows that both SIP1FL and SIP1CID repressed the activity of the SV40 early promoter. However, mutation of the CtBP binding sites did not abolish repression by SIP1FL whereas it completely abrogated the repression mediated by SIP1CID. This clearly suggests that in the full-size context of SIP1 the PXDLS domains are not necessary for repression in this assay.

To verify whether this result is not due to the inability of CtBP to bind to full-length SIP1 under these experimental conditions, we used again the UAS-SV40-luciferase reporter assay but, in addition to the GBD-SIP1 constructs, we co-transfected CtBP-VP16, a fusion protein containing the strong transactivation domain of the herpes simplex virus transcriptional regulatory protein VP16. In such two-hybrid type of approach, the interaction between SIP1 and CtBP1 resulted in a strong activation of luciferase synthesis when the CID of SIP1 was assayed (Fig.3B, GBD-SIP1CID + CtBP1-VP16), and in a weak activation when full-length SIP1 was used (from a 40% repression without CtBP-VP16 to a 2-fold activation with CtBP-VP16). Deletion of the CtBP binding sites clearly abolished the interaction of CtBP-VP16 with both GBD-SIP1CID and GBD-SIPFL. This confirms that CtBP can bind to full-length SIP1 when tethered to DNA.

To rule out the possibility that the lower transactivation capacity of CtBP-VP16 in complex with GBD-SIPFL would be due a low affinity of CtBP for DNA-bound full-length...
SIP1, DNA precipitations were carried out. More specifically, the relative amounts of CtBP pulled down with either GBD-SIP1\textsubscript{FL} or GBD-SIP1\textsubscript{CID} polypeptides bound to a double stranded oligonucleotide encompassing 1 UAS was determined. 293T cells were transfected with Flag-tagged CtBP1 and different quantities of GBD-SIP1\textsubscript{CID} or GBD-SIP1\textsubscript{FL} respectively, to obtain similar amounts of GAL4 fusion proteins. While the total protein levels of the two fusion proteins were comparable (Fig. 3C; lysates, lane 3 and 5) the amount of GBD-SIP1\textsubscript{FL}, pulled down by UAS-DNA was lower than the amount of DNA-bound GBD-SIP1\textsubscript{CID} (DNAP; lane 3 and 5). Expression of low levels of GBD-SIP1\textsubscript{CID} (undetectable in lysates, Fig. 3C lane 1 and 2) still resulted in higher levels of pulled-down protein than seen for GBD-SIP1\textsubscript{FL} (Fig. 3C DNAP lane 1 and 5). This very inefficient binding of GBD-SIP1\textsubscript{FL} also resulted in an overall poor recruitment of CtBP to DNA. Most likely, the overall low amount of CtBP1 actually tethered to the DNA by GBD-SIP1\textsubscript{FL} explains the 10 fold less activation by CtBP-VP16 in figure 3B. Nevertheless, the data suggest that CtBP1 is recruited to DNA as efficiently by the entire SIP1 protein as by the SIP1 CID alone since the amount of CtBP pulled down relative to the DNA-bound GBD-SIP1 fusion proteins is comparable (see lane 1 and 5, upper and lower panel Fig3C).

Recently, it has been proposed that CtBP has an ability to detect changes in nuclear NAD\textsuperscript{+}/NADH ratios. Agents like CoCl\textsubscript{2} that are capable of increasing NADH levels stimulate CtBP binding to its partners in cells and potentiate CtBP-mediated repression (20). We used this agent as it may result in an increase of the affinity of CtBP for SIP1 and tested whether under these circumstances a corepressor role of CtBP for full-length SIP1 could be revealed. Transfected cells were treated with 200 µM of CoCl\textsubscript{2} for 18 hours prior to determination of luciferase activities. In accordance with published data the interaction of CtBP with SIP1\textsubscript{CID} was enhanced by this treatment as shown by the increased transcriptional activation of GBD-SIP1\textsubscript{CID} and GBD-SIP1\textsubscript{FL} by CtBP1-VP16. However, even under these conditions, deletion of
the CID did not influence the repressor activity of full-length SIP1 (Fig.3B). All together, these data demonstrate that, although CtBP interacts with DNA-bound SIP1, it does not significantly contribute to the repressor activity of the latter.

**The CID of SIP1 does not contribute to transcriptional repression of the E-Cadherin promoter**

SIP1, as well as δEF1, can repress transcription from the E-Cadherin promoter and the full-length proteins most likely need two E2 boxes separated by 44 basepairs that are present in the human, mouse and dog E-Cadherin promoter, to do this efficiently (4,10,11). Nevertheless, their isolated CZF is also capable of binding to these E2 boxes (4). It has been suggested that δEF1 represses E-Cadherin promoter activity in a CtBP-dependent manner, i.e. mutation of all three CtBP-binding sites of the CID of δEF1 (tested as segment 700-776), and fused to a CZF-containing segment of δEF1, abolished repression of an E-Cadherin promoter-luciferase construct (10). We wanted to test whether the CID of SIP1 has a similar activity as δEF1 in this assay. Therefore we created similar constructs that consist of fusions between the CID of SIP1 and its CZF segment (and provided with an NLS and a Myc tag, see Experimental Procedures) creating CZF-CID (Fig.4A). Since this is an artificial construct in which the fourth potential PXDLS motif might also function, we did not only generate mutants that had the three PXDLS motifs mutated (3xmut; as shown in Fig.1) but another mutant in which this fourth motif (PLRLT) was also mutated (4xmut in Fig.4A). These SIP1-encoding constructs were transfected together with luciferase reporter plasmids driven by the E-Cadherin core promoter (11) in HEK293T and MCF7 cells, respectively. Fig.4A shows the schematic representation of the CZF fusion proteins and the normalized luciferase assays (done two days after transfection). When the CID of SIP1 is recruited to the DNA by the
DNA-binding domain of SIP1, repression of the E-Cadherin promoter was observed. Moreover, when the CtBP binding sites are mutated, the CZF-CID\textsubscript{mut} fusions, like the GAL4-SIP1\textsubscript{CID-mut}, could not repress the E-Cadherin promoter (similar results were obtained using MCF7 cells, data not shown). Thus, as found previously for δEF1, the E-box binding domain CZF, fused to the CID of SIP1, can mediate repression of the E-Cadherin promoter in a CtBP-dependent manner.

To verify whether the full-length SIP protein also displays a CtBP-dependent E-Cadherin repression, the experiment was also carried out using full-length Myc-tagged SIP1, either as wild-type SIP1 or as SIP1 in which the three PXDLS motifs were mutated (SIP1\textsubscript{CtBP-mut}; Fig.4B). In order to allow a comparison between full-length SIP1 and CZF-CID fusion proteins, different amounts of DNA were transfected to obtain comparable protein quantities (see insert of anti-Myc probed Western blots of the same lysates that were also used for luciferase activity measurements in Fig.4A, B). Unlike for CZF-CID of SIP1, the mutation of the PXDLS motifs did not lead to abrogation of E-Cadherin repression by SIP1 full-length proteins (Fig.4B). Similar results were obtained using the MCF7 cells that do not endogenously express SIP1 (data not shown). These data suggest that, when placed in a full-length SIP1 context, the PXDLS motifs are not key for SIP1 to repress the E-Cadherin promoter.

δEF1 and CtBP-dependent repression of E-Cadherin

The results obtained with full-length SIP1 proteins prompted us to test whether also full-length δEF1 does not need CtBP to repress E-Cadherin transcription. To this end we transfected the δEF1 wild-type and CtBP\textsubscript{mut} expression plasmids used by Furasawa and colleagues (19) and compared their capacity to repress E-Cadherin-driven luciferase transcription. As control we transfected wild-type and CtBP\textsubscript{mut} SIP1 constructs. Fig.5A shows
that full-length SIP1 and δEF1, as well as their CtBP-binding deficient mutants, were capable of repressing the E-Cadherin promoter to similar extents. This result suggests that both SIP1 and δEF1 do not require CtBP to repress E-Cadherin.

It has been shown that adenovirus protein E1A can relieve repression of a similar E-Cadherin promoter construct in a CtBP-dependent manner, presumably by binding to - and outtitration of - CtBP. This has led to the suggestion that CtBP would be necessary for E-Cadherin repression, at least in the cell lines tested in that study (10). We therefore examined if, in our cell lines, CtBP is necessary for E-Cadherin repression. For this, we used plasmids encoding wild-type E1A or a mutant that does not bind to CtBP (28) in our E-Cadherin promoter luciferase assay. As shown in Fig.5B, the wild-type E1A protein indeed caused activated transcription from the E-Cadherin promoter whereas the E1A CtBP mutant did not, which is in agreement with the results of Grooteclaes et al. (2000) (10). Thus CtBP contributes to repression of E-Cadherin in these cells but our data suggest that this corepressor would not act through SIP1 or δEF1. Following a similar rationale as the one used for E1A we decided to see whether an overexpressed mutant SIP1 protein could outtitrate a protein necessary for E-Cadherin regulation. We generated a construct that expresses a SIP1 protein carrying two point mutations, which result in a loss of DNA-binding of the full-length protein (SIP1 ZFmut H300S; H1073, (4)) and also lacks the three PXDLS motifs (SIP1 ZF/CtBPmut). This mutant will not be capable to compete for CtBP like wild-type E1A does, but is also incapable of repressing E-Cadherin repression because it cannot bind to DNA (11). As a control, we used the wild-type SIP1 construct. When cotransfected with an E-Cadherin luciferase construct in HEK293T cells, the SIP1 ZF/CtBPmut protein indeed did not repress the promoter but rather appeared to activate transcription, in a concentration-dependent manner (Fig.5C). This suggests that an endogenous protein that is
capable of binding to SIP1, and differs from CtBP, is also necessary for E-Cadherin repression in general.

**Repression of endogenous E-Cadherin transcription by SIP1**

The precise mechanism by which CtBP mediates transcriptional repression remains unclear. It appears that the mammalian CtBPs may mediate repression through Histone Deacetylase (HDAC) recruitment as well as through Polycomb Group (PcG) proteins. HDACs as well as PcGs can induce modifications of the nucleosomal organization (14,15).

We considered the possibility that our inability to show a CtBP-dependent repression of the E-Cadherin promoter by SIP1 could be due to the use of reporter plasmids. Repression of endogenous E-Cadherin transcription by SIP1 could therefore still be CtBP-dependent. To probe this, we used E-Cadherin-positive MDCK-Tet-off cells. Conditional expression of wild-type SIP1 in these cells leads to down-regulation of E-Cadherin, thereby abrogating E-Cadherin-mediated intercellular adhesion and inducing invasion *in vitro* (11). MDCK-Tet-off cells were stably transfected with tetracyclin responsive expression constructs encoding full-length Myc-tagged SIP1 wild-type, -CtBP mutant (3xmut) and zinc finger mutant proteins. Using semi-endogenous immunoprecipitations we found that the wild-type SIP1 protein as well as the zinc finger mutant counterpart could indeed bind to (dog) CtBPs, whereas the CtBP mutant of SIP1 could not (data not shown). Four days after induction of SIP1 expression by removal of tetracycline from the medium, the cells were fixed and analyzed for SIP1 (shown in green) and E-Cadherin expression (shown in red) by immunofluorescence using an anti-Myc and an anti- E-Cadherin antibody, respectively (Fig.6). As described previously (11), the expression of wild-type SIP1 clearly down-regulated endogenous E-Cadherin levels in these MDCK cells (Fig.6, wild-type; note that tetracycline did not influence the typical E-Cadherin honeycomb-like staining appearance in these cells (control
MDCK)). When SIP1 cannot bind to DNA, it was not capable of repressing E-Cadherin transcription (SIP1 ZFmut), which also demonstrates that the repression of E-Cadherin by wild-type SIP1 is direct \textit{i.e.} requires DNA binding, and is not caused by outtitration of proteins necessary for E-Cadherin transcription. However, when the CtBP-binding sites of SIP1 are mutated, this protein was still capable of repressing endogenous E-Cadherin transcription (Fig.6, SIP1CtBPmut). Even cells that do not express high amounts of either wild-type SIP1 or SIP1CtBPmut show E-Cadherin down-regulation, indicating that the repression is not caused by elevated expression of SIP1. Furthermore, it was already shown that the induced SIP1 expression levels in MDCK-Tet-off cells are comparable to SIP1 levels in the E-Cadherin-negative MDA-MB435S cells (11). These data suggest that CtBP is dispensable for SIP1 to repress endogenous E-Cadherin transcription.
Discussion

Transcriptional down-regulation of E-Cadherin is important for invasion and subsequent metastasis of epithelial-derived tumor cells (29) and is also essential throughout gastrulation and other cell migratory processes during embryogenesis. SIP1 has been identified as a transcriptional repressor of E-Cadherin (11). Recently, increased SIP1 expression has been detected in 50% of intestinal-type gastric cancer samples that all showed reduced E-Cadherin expression (30). In addition, SIP1 was identified in two independent large-scale screens to identify genes relevant to cancer (31,32). Moreover, many mutations in SIP1, the majority of which lead to truncation of the protein, have recently been reported to cause congenital defects sometimes associated with Hirschsprung disease (33-38). Therefore, in addition to the study of SIP1’s function in vivo in multiple biological and pathological processes, the elucidation of the mechanism of action of SIP1, preferably at the level of important target genes such as E-Cadherin, remains crucial.

It has previously been reported elsewhere that ectopic expression of the polypeptide composed of amino acids 700 to 871 of human SIP1 can interact with ectopically expressed CtBP (6). We show here that endogenous SIP1 indeed interacts with endogenous CtBP in MDA-MB435S and HEK293T cells. This interaction depends on the three PXDLS motifs in SIP1 because mutation of these three motifs abrogates the binding between SIP1 and CtBPs. Moreover, we confirm that the CID of SIP1 can repress transcription in a CtBP-dependent manner when recruited to a UAS-SV40 early promoter or to the E2-boxes of the E-Cadherin promoter. However, the CtBP-dependent transcriptional repression cannot be evidenced when E-Cadherin-driven luciferase assays are carried out with full-length SIP1, which was never tested before. In addition, this CtBP-independent repression of E-Cadherin is confirmed in MDCK cells that are stably transformed with tetracycline-regulated SIP1
expression constructs. All these data strongly suggest that CtBP is dispensable for SIP1 to repress E-Cadherin transcription.

To verify this further we have used available CtBP1+/−, CtBP2+/− and CtBP1−/−, CtBP2−/− fibroblast lines (16). We could confirm the CtBP-dependence of the GBD-SIP1CID in CtBP null fibroblast cells but we could not detect a SIP1-mediated repression of the E-Cadherin luciferase construct either of the two cell lines (with or without cotransfection of CtBPs, data not shown).

Another way to investigate whether CtBP plays a role in the repression of E-Cadherin is to outtitrate CtBP using another CtBP-interacting protein. When we used this approach and overexpressed E1A in an E-Cadherin reporter assay, this indeed resulted in transcriptional derepression of the E-Cadherin promoter as reported previously (10). These data indicate that CtBP is involved in E-Cadherin repression but not through a mechanism involving SIP1 or δEF1. In addition, the observation that the SIP1-ZFmut/CtBPmut protein can activate transcription of E-Cadherin also suggests that transcriptional repression of E-Cadherin could involve a protein other than CtBP.

The observation that CtBP is not required for SIP1 to repress E-Cadherin raises two important questions. First, what is the mechanism by which this repression takes place? Second, what is the functional relevance of CtBP binding to SIP1? Certainly, DNA binding is necessary since for repression of endogenous E-Cadherin by SIP1 two intact zinc finger clusters are required (see Fig. 6). Competition with activators of the bHLH family has been put forward as a mechanism for δEF1 to repress transcription of the immunoglobulin heavy-chain enhancer in vitro (39) and of the p53 family member, p73 (40). In contrast, an active repression mechanism has been proposed for δEF1 with respect to regulation of α4-integrin (41), and a repression domain close to the N-terminus was identified to be necessary for repression of the δ-crystallin enhancer (42). Although no complex was identified
biochemically, the HIV-1 Tat interacting protein TIP60 has recently been put forward as a corepressor for δEF1 in repressing CD4-enhancer/promoter activity (43). In overexpression studies, we can show an interaction between TIP60 and δEF1 but not SIP1 (CM and LvG, unpublished results) excluding TIP60 as a potential corepressor for SIP1.

So far, CtBP was a good candidate that could fulfill a co-repressor role but is not acting as such in the case of SIP1 mediated E-Cadherin repression (see Figs.4-6). Several other studies have shown that mutation of the PXDLS motifs within CtBP-interacting proteins results in loss of repression (18,19,44-47), whereas in others, this has little or no effect on repression (48,49). For example, loss of the CtBP-interaction motif in a hybrid GAL4-full-length Epstein-Barr-viral oncoprotein EBNA3C had little effect on transcriptional repression of a UAS-based reporter (49). However, mutation of the PLDLS domain in a truncated version of the protein (containing the C-terminal 412 amino acids) fused to the DNA-binding domain of GAL4 not only leads to a loss of repression activity, but can even activate a UAS-driven reporter. We do not observe this phenomenon for SIP1, neither for the CID SIP1 polypeptide nor for full-length SIP1. This means that we can exclude a possible function for CtBP as an inhibitor of SIP1 transcriptional activator activity. Activator activity for SIP has not been proven yet but cannot be excluded since analysis of the amino acid sequence reveals proline-rich and acidic amino acids-rich domains. In addition, δEF1 can be a transcriptional activator of ovalbumin transcription via binding to a TACCT site and is potentiated by co-(over)expression of upstream stimulatory factor 1 (USF-1) (50,51).

An interesting approach to verify the functional requirement of CtBP has been used recently for FOG-1, a zinc finger protein essential for the development of the erythroid and megakaryocytic lineages and a partner of GATA-1. Amino acid substitutions in FOG-1 that impair interaction with CtBP relieve repression in reporter assays and augment blood formation in both Xenopus and Drosophila assays (52,53). Interestingly, knock-in mice that
express a FOG-1 variant unable to bind CtBP are normal and fertile, and erythropoiesis at all stages of development is normal (48). Thus whereas CtBP is required for FOG-1 function \textit{in vitro}, the interaction is not strictly required for these functions \textit{in vivo}.

Using the CID of human SIP1 (ZEB2), it has been shown by others that this domain is not capable of repressing transcriptional activation mediated by transcription factors like c-myb, TFE3, MEF2C, CTF and MyoD, whereas a larger domain of ZEB2 (337-996) can (6). This suggests that SIP1 can mediate repression independent of CtBP but at the same time does not exclude the fact that CtBP is a corepressor for SIP1 in a cellular- or promoter-specific context. Possibly, conformational changes of SIP1 due to post-translational modifications or binding of other SIP1 binding proteins could reveal a function for CtBP as a SIP1-corepressor. Further experiments are necessary to identify genes that are regulated in a CtBP-dependent manner by SIP1 but also to identify what other factors are associated with SIP1 in order to modulate its activity.

The findings illustrated in our study have to be validated \textit{in vivo} to demonstrate which non-CtBP corepressor may be responsible for the transcriptional down-regulation of E-Cadherin by SIP1 and in which SIP1-mediated process(es) CtBP would still be involved. Finally, the identification of PXDLS motifs in both δEF1 and SIP1 has led to the assumption that CtBP can act as a corepressor for both proteins. Promoter studies using these PXDLS motifs alone indeed identified CtBP as a possible corepressor for SIP1 and δEF1. The data presented in this paper suggest that although a small portion of δEF1 or SIP1 mediates transcriptional repression through CtBP, this dependence cannot automatically be extrapolated to the full-length proteins regulating specifically E-Cadherin transcription.
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Footnotes

The abbreviations used are:

CtBP, C-terminal binding protein; CID, CtBP-interacting domain; CZF, C-terminal zinc finger cluster; GBD, Gal4 protein DNA-binding domain; HEK, human embryonic kidney; HD, homeodomain-like domain; MDCK, Madin-Darby canine kidney; NZF, N-terminal zinc finger cluster; SBD, Smad-binding domain; SIP1, Smad-interacting protein-1; Tet, tetracycline; UAS, Upstream Activating Sequence.
Figure legends

Figure 1. Interaction between SIP1 and CtBP

A. Schematic representation of SIP1 showing the different domains and the LexA fusion used (only the mutant is indicated); NZF: N-terminal zinc finger cluster, CZF: C-terminal zinc finger cluster, SBD: Smad-binding domain, HD: Homeo-domain-like domain; CID: CtBP interaction domain (showing the PXDLS motifs).

B. Yeast mating assay; fragments encoding amino-acids 315-942 of SIP1 and 330-909 of δEF1 were fused to the LexA DNA-binding domain (LexA<sub>DBD</sub>) to create LexA-SIP1 and LexA-δEF1. When co-expressed in the same cell with the different Smads or mCtBP2 (each fused to the activation domain of B42) the β-galactosidase readouts give an indication about direct interactions. White colonies indicate no interaction, blue colonies indicate an interaction between the proteins.

C. Identical set-up as in B but now 3 of the 4 PXDLS domains were mutated (See also panel A). As controls, wild-type (wt) SIP1 and mCtBP2 were used as they both interact with mCtBP2.

D. HEK293T cells were co-transfected with expression vectors encoding Myc-tagged SIP1 wild-type (wt) or SIP1<sub>CtBmut</sub> (mut) and Flag-tagged CtBP1 (lane indicated with 1) or Flag-tagged CtBP2 (indicated with 2). Cells were lysed and proteins were precipitated with α-flag antibodies and separated by SDS-PAGE (8%). Complexes were obtained by immunoprecipitation with Flag-specific antibodies and analyzed by Western blot using anti-Myc and anti-Flag-specific antibodies (Flag-IP). A portion of each lysate was analyzed for expression of transfected proteins by Western blot (Lysates).
Figure 2. SIP1 and CtBP form a complex in vivo

A. Myc-tagged SIP1 and δEF1 constructs were transfected in HEK293T cells, lysed and immunoprecipitated with either α-Nterm-SIP1 or α-Myc antibodies and visualized by Western using α-Myc antibodies. The arrow indicates Myc-SIP1 and the starred arrow indicates Myc-δEF1.

B. Endogenous SIP1 complexes were immunoprecipitated from lysates of MDA-MB435S cells using either the α-Nterm-SIP1 or α-Cterm-SIP1 antibodies and visualized using by Western using α-Cterm-SIP1 antibodies. The arrow indicates the 170 kDa SIP1 band.

C. MDA-MB435S, MCF7/AZ, HEK 293T and HepG2 cells were harvested, and the cells were lysed and proteins were precipitated with anti-CtBP antibodies, and subsequently separated by SDS-PAGE (6%). Immunocomplexes were analyzed by Western blot using SIP1-specific and CtBP-specific antibodies (CtBP-IP). A portion of each lysate was analyzed for synthesis of SIP1 and CtBP proteins by Western blot (Lysates). SIP1 is absent in MCF7 and HepG2; it can be detected as a 170kDa protein in the lysates but is also detectable in the lanes where CtBP complexes from MDA-MB435S and MCF7 cells were loaded.

Figure 3. Transcriptional repression and DNA binding by GBD-SIP_{FL} and GBD-SIP_{CID}

Full-length SIP1 (SIP1_{FL}) as well as the CID domain of SIP1 (SIP1_{CID}) were fused to the GBD, creating GBD-SIP_{FL} and GBD-SIP_{CID}. Constructs in which the CtBP-binding sites are mutated are indicated as GBD-SIP_{FL-mut} and GBD-SIP_{CID-mut}.

A. HEK 293T cells were transfected with 20 ng GBD-SIP1_{CID} or 100 ng GBD-SIP_{FL} or GBD plasmids and a (Gal)_{5}-SV40-luc reporter in which luciferase gene transcription is under the control of 5 Gal4 binding sites and the SV40 early promoter. 48 hours after transfection, cells
were assayed for luciferase activity, which is presented here as the mean ±S.D. of triplicate transfections (in arbitrary units after β-galactosidase correction).

B. Constructs used in panel A, were co-transfected with a CtBP1-VP16 construct and treated for 18 hours with 200 μM CoCl₂ as indicated by the crosses (X) underneath the graph. Note the increase in luciferase activity only when CtBP1-VP16 is transfected along with GBD-SIP1CID and not with GBD-SIP1FL.

C. Plasmids encoding GBD-SIP1CID (0.2, 0.5 and 5 ug) and GBD-SIP1FL (4 ug) were cotransfected with 0.2 ug of Flag-CtBP1 plasmid in HEK 293T cells. 24 Hours after transfection cell extracts were incubated with biotinylated double-stranded oligonucleotides encompassing 1 UAS. DNA-bound complexes were analyzed by Western blot using GBD- and Flag-specific antibodies (DNAP; resp. upper and lower panel). A portion of each lysate (1/500) was analyzed for production of SIP1 and CtBP proteins by Western blotting (Lysates).

**Figure 4. Repression of E-Cadherin-luciferase by CZF-based SIP1 polypeptides and full-length SIP1**

HEK293T were transfected with constructs yielding the indicated SIP1 polypeptides and the E-Cadherin-Luciferase construct that are schematically represented in panel A. Two days after transfection, cells were analysed for luciferase activity, which is presented as the mean ±S.D. of triplicate transfections.

A. The CtBP interaction domain (CID) of SIP1 was C-terminally fused to the C-terminal zinc finger cluster of SIP1 generating CZF-CIDwt or the respective mutants. CZF-CID constructs (20 ng) were transfected to document their transcriptional repression of the E-Cadherin-driven luciferase gene. The vertical stripes in the CID represent the number of intact PXDLS motifs.
B. Identical experimental set-up as in A, but 50 ng of full-length SIP1 constructs were used.

Synthesis of the proteins in the lysates that were used for the luciferase assays was verified by Western analysis (insert, all SIP1 polypeptides contained a 6xMyc epitope).

**Figure 5. CtBP dependence of E-Cadherin repression**

Cells were transfected with the E-Cadherin promoter construct and each of the indicated plasmids and analyzed 48 hours after transfection.

A. δEF1wt and δEF1 CtBP

B. E1A co-transfection upregulated the transcription from the E-Cadherin promoter while E1A CtBP

C. By cloning the mutated PXDLS motifs into the zinc-finger mutant described in (4), SIP1-ZF

**Figure 6. Repression of endogenous E-Cadherin by SIP1 and SIP1CtBPmut**

Immunofluorescence analysis of SIP1 and E-Cadherin expression in MDCK-Tetoff-cells stably transfected with myc-SIP1 wt, -SIP1CtBPmut and -SIP1ZFmut constructs, respectively. Induction of SIP1 proteins by removal of tetracycline (-Tet) results in a nuclear expression of SIP1 wt and SIP1CtBPmut (analyzed with a Myc-antibody) and loss of E-Cadherin expression (DECMA-antibody). Expression of the zinc finger mutant of SIP1 does not repress E-Cadherin transcription in MDCK cells.
Figure 1

A

mSIP1

LexA-mSIP1_{CtBPmut}

B

pGilda

mSIP1

mδEF1

C

mSIP1 wt

mCtBP2

mSIP-CtBP_{mut}

D

Full-length Myc-SIP1

|   | SIP1 | CtBP | SIP1 | CtBP |
|---|------|------|------|------|
|   | wt   | 1    | wt   | 1    |
|   | CtBP_{mut} | 2 | CtBP_{mut} | 2 |

150 kD

100 kD

50 kD

Flag-IP

Lysates
Figure 2

A

WB: α-Myc

IP: α-Nterm SIP1

Myc-SIP1 Myc-δEF1 Myc-SIP1 Myc-δEF1

B

WB: α-Cterm SIP1

IP: α-Nterm SIP1 α-Cterm SIP1

MDA-MB 435S

C

WB: α-Cterm SIP1

CDM: MDA-MB 435S MCF7 HEK 293T HepG2

WB: α-CtBP

CtBP-IP lysates
Figure 3

A

B

C

Lysates

WB: α-GBD

WB: α-Flag

Flag-CtBP1

250 kD

150 kD

75 kD

50 kD

GBD-SIP1_CID

GBD-SIP1_FL

Flag-CtBP1

UAS-DNAP

250 kD

150 kD

75 kD

50 kD

GBD-SIP1_CID

GBD-SIP1_FL

Flag-CtBP1

γ-globin

CtBP VP16

SIP1

UAS-SV40

5 x UAS

Gal4

SV40 promoter

Luciferase

CtBP1-VP16

200 µM CoCl₂

0

0.2

0.4

0.6

0.8

1.0

1.2

GBD

GBD-SIP1_cid

GBD-SIP1_FL

GBD-SIP1_CID_mut

GBD-SIP1_FL_mut

WB: α-Flag

GBD-SIP1_CID

GBD-SIP1_FL

Flag-CtBP1
Figure 5

A

B

C

Relative luciferase activity

control SIP1 wt SIP1 CtBP\textsubscript{mut} $\delta$EF1 wt $\delta$EF1 CtBP\textsubscript{mut}

Relative luciferase activity

colorbar: 10 ng 20 ng

colorbar: 10 ng 30 ng 75 ng

Relative luciferase activity

colorbar: 10 ng 20 ng

colorbar: 10 ng 30 ng 75 ng

control E1A wt E1A CtBP\textsubscript{mut}

colorbar: 10 ng 30 ng 75 ng

colorbar: 10 ng 30 ng 75 ng

colorbar: 10 ng 30 ng 75 ng
Figure 6

Control MDCK  SIP1 wt  SIP1_{CtBPmut}  SIP1_{ZFmut}

+ Tet

- Tet

E-Cadherin/ Myc-SIP1
