Evi-1 Transforming and Repressor Activities Are Mediated by CtBP Co-repressor Proteins*

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Ectopic production of the EVI1 transcriptional repressor zinc finger protein is seen in 4–6% of human acute myeloid leukemias. Overexpression also transforms Rat1 fibroblasts by an unknown mechanism, which is likely to be related to its role in leukemia and which depends upon its repressor activity. We show here that mutant murine Evi-1 proteins, lacking either the N-terminal zinc finger DNA binding domain or both DNA binding zinc finger clusters, function as dominant negative mutants by reverting the transformed phenotype of Evi-1 transformed Rat1 fibroblasts. The dominant negative activity of the non-DNA binding mutants suggests sequestration of transformation-specific cofactors and that recruitment of these cellular factors might mediate Evi-1 transforming activity. C-terminal binding protein (CtBP) co-repressor family proteins bind PLDLS-like motifs. We show that the murine Evi-1 repressor domain has two such sites, PFDLT (site a, amino acids 553–559) and PLDLS (site b, amino acids 584–590), which independently can bind CtBP family co-repressor proteins, with site b binding with higher affinity than site a. Functional analysis of specific CtBP binding mutants show site b is absolutely required to mediate both transformation of Rat1 fibroblasts and transcriptional repressor activity. This is the first demonstration that the biological activity of a mammalian cellular transcriptional repressor protein is mediated by CtBPs. Furthermore, it suggests that CtBP proteins are involved in the development of some acute leukemias and that blocking their ability to specifically interact with EVI1 might provide a target for the development of pharmacological therapeutic agents.

A small number of transcription factors are frequently targets for de-regulation by recurring chromosome translocations in acute leukemias, and these events play a pivotal role in disease progression (1). The EVI-1 gene encodes one of these transcription factors, which is activated in 4–6% of acute myeloid leukemia (AML)1 patients with various karyotypic abnormalities of chromosome 3q26 (2), which result in the ectopic production of intact or, occasionally, C-terminal-truncated EVI1 proteins (3–6). In addition, novel EVI1 fusion proteins are sometimes produced. For example, patients with karyotypes t(3;21)(q26;q22) or t(13;12(q26;pl3) express AML1/EVI1 (7) and ETV6(TEL)/EVI1 (8) chimeras, respectively, and similar fusions with a naturally occurring MDS1/EVI1 isoform (9).

The precise contribution of ectopic EVI1 and EVI1 fusion protein production in leukemia progression is unknown, but a combination of enforced transgene expression and intervention studies shows a causative role, affecting both cell differentiation and proliferation. Expression of AML1/MDS1/EVI1 induces AML in mice, resulting in the accumulation of myeloblast cells and immature differentiated myelocytic and monocytic lineages (10). EVI1 or AML1/EVI1 expression in either 32Dcl3 cells or murine primary bone marrow cells abrogates granulocyte colony-stimulating factor and erythropoietin-mediated differentiation and survival, respectively (11–13). EVI-1 antisense oligonucleotides inhibit proliferation of leukemic cells expressing the AML1/EVI1 fusion protein (14), and evi-1 gene targeting produces an embryonic lethal phenotype accompanied by widespread hypocellularity (15).

The 145-kDa nuclear EVI1 full-length protein (FL) is a sequence-specific transcriptional repressor protein (16) that is expressed in acute myeloid leukemia (AML)1 patients with various karyotypic abnormalities of chromosome 3q26 (2), which result in the ectopic production of intact or, occasionally, C-terminal-truncated EVI1 proteins (3–6). In addition, novel EVI1 fusion proteins are sometimes produced. For example, patients with karyotypes t(3;21)(q26;q22) or t(13;12(q26;pl3) express AML1/EVI1 (7) and ETV6(TEL)/EVI1 (8) chimeras, respectively, and similar fusions with a naturally occurring MDS1/EVI1 isoform (9).

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1 The abbreviations used are: AML, acute myeloid leukemia; FL, full-length; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; bp, base pair(s); CtBP, C-terminal-binding protein; DBD, DNA binding domain; AD, activation domain.

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Recruitment of co-repressors is a common theme in transcriptional repression, because several non-DNA binding co-repressors have been identified that become anchored to DNA by interacting with subsets of sequence-specific transcription factors. These co-repressors include pRh (24), SMRT/Ncor (25, 26), Groucho (27), ETO (28), and the CtBP family (29). Recently, it has been shown that the Evi-1 Rp domain binds a CtBP family protein mCtBP2 in a yeast two-hybrid assay (29). There are at least two human CtBP proteins, hCtBP1 and hCtBP2 (30), two murine homologs, mCtBP1 and mCtBP2 (29), and a Drosophila homolog, dCtBP (31), which, based upon a broad range of binding partners, play crucial roles in a number of biological processes. For example, they are essential to Hairy (32), Knirps, Snail (31), and Kruppel (33) activities in early embryonic patterning in Drosophila and mediate the transcriptional repression activities of key regulators of vertebrate differentiation (δEF1, ZEB, FOG, BKL/F, 29, 34–36) and proliferation (Net, Rb, p130, BRC1A1, 37–39).

Although the CtBP proteins have been shown to mediate repressor activity for a number of cellular factors, the role of these interactions in mediating biological activity has not been previously demonstrated in mammalian cells. They are good candidates for mediating aspects of the biological activities of EVI1. CtBP protein binding is mediated by a conserved PLDLS located in the EVI1 Rp domain. In this study, we examined the binding of CtBP and EVI-1 proteins and demonstrate their interaction is necessary for the transformation of Rat1 cells and mediate transcriptional repressor activity.

MATERIALS AND METHODS

Cell Culture—RatFL cells have been described before and Rat1, RatFL, Bosc-23, and 293 cells were all maintained as described previously (31). Procedures for transfections, production of helper free recombinant retrovirus, retroviral infections, growth in soft agar, CAT, and β-galactosidase assays have all been described previously (16). Cells infected with the zeocin resistance marker (neo)-containing retroviral vectors were selected and maintained in 1 mg/ml zeocin (Invitrogen).

Construction of Plasmids—The zeocin gene was initially PCR-amplified from pBS21 using oligonucleotides 12/14 and 15/13 using pBS21 template DNA. The amplified fragments were digested with NotI and EcoRI and inserted as a NotI/EcoRI fragment into the NotI/EcoRI site of pMK20 subclone. pMK20 was digested by simultaneous ligation of NotI/EcoRI fragments from pKSI-BamHI fragment into pBluescript KSII to create pKSI-BamHI fragment and the yeast vector pGBT9 (CLONTECH) was modified by replacing the NotI/EcoRI digested p50MX-neo. The yeast vector pGBT9Rp was created by inserting an NotI fragment from Evi-1 cDNA pBS21 (41) into plasmid pGBT9 (CLONTECH) modified by replacing the NotI/EcoRI site with NotI, utilizing NotI linkers (New England BioLabs), to create pGBT9N. The vector pGBT9Rp was created by inserting an EcoRI/NotI fragment from pGEV514/724 (16) into pGBT9N. pGBT9Rp514–633 was created by inserting EcoRI/NotI fragments, derived by PCR of pBS21 with oligonucleotides 10 and 11 into EcoRI/NotI-digested pGBT9N.

A cassette containing the Rp domain of Evi-1 encompassing amino acids 521–724 was created by PCR of pBS21 using oligonucleotides 12/13 and inserted as an EcoRI/BamHI fragment into pBluescript KSII (Stratagene). This wild type sequence, designated RpWT was then inserted into pGBT9 as an EcoRI/BamHI fragment producing pGBT9RpWT, into pSG424 as an EcoRI/BamHI fragment producing pSG424/RpWT, and as a BglII/BamHI fragment into the BamHI site of p50MFLpΔα to create p50MFLpΔα-neo. Similarly, mutant Rp domain cassettes, created by site-directed mutagenesis, were inserted as EcoRI/BamHI-digested fragments into pBluescript KSII to create pKSI-ICTBpA and pKSIICtBPb. The CtBpa/b double-mutant was created by ligating EcoRI/EcoI019I and EcoI019I/BamHI fragments from pKSI-ICTBpA and pKSIICtBPb, respectively, into EcoRI/BamHI-digested pBluescript KSII. The various mutant Rp domain cassettes were subsequently introduced into expression vectors as described above to create pGBT9RpCtBPa, pGBT9RpCtBPh, pGBT9RpCtBpA/b, pSG424/RpCtBPa, pSG424/RpCtBPh, pSG424/RpCtBpA/b, p50MFLpΔα, p50MFLpΔb, and p50MFLpΔa/b.

Creation of the Myc-tagged EVI1 expression vector will be described elsewhere.2 FLAG-tagged mCtBP2 template created by replacing SalI/PstI in pCMV5B-FLAGSmad2 (gift of Dr. J. Wrana) and inserting a SalI/Smad2 mCtBP2 fragment PCR-amplified from pGAD424 in pBc2 with oligonucleotides 18 and 19.

Site-directed Mutagenesis—Site-directed mutagens were created using a PCR-based method. pGBT9CtBPa, pGBT9CtBPh, pSG424/RpCtBPa, pSG424/RpCtBPh, pSG424/RpCtBpA/b, p50MFLpΔα, p50MFLpΔb, and p50MFLpΔa/b.

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2 A. Gill, unpublished.
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RESULTS

Evi-1 Mutant Proteins, Which Lack the Zinc Finger DNA Binding Domains, Can Revert the Phenotype of Evi-1-transformed Cells—We examined the ability of various murine Evi-1 mutant proteins to revert the transformed phenotype of RatFL cells (21), which express the full-length Evi-1 (FL) protein. Retroviral vectors containing either a zeocin-selectable marker alone (p50MX-zeo) or encoding Evi-1 mutant proteins that lack either the ZF1 (p50M AZF1-zeo) or both ZF1 and ZF2 (p50M AZF1/2-zeo) DNA binding domains were used to infect RatFL cells, and zeocin-resistant cell lines were isolated. The growth of cell lines, selected because the mutant proteins were expressed at either similar or higher levels than the full-length protein (Fig. 1), were examined in soft agar. Both ΔZF1 and ΔZF1/2 proteins behave as dominant negative mutants, reverting the transformed phenotype of RatFL cells as demonstrated by the inhibition of colony formation in soft agar (Fig. 1). RatFL, ΔZF1, ΔZF1/2, whereas expression of zeo alone has no effect (Fig. 1, Zeo).

The Evi-1 Rp Domain Interacts with CtBP Proteins—The ability of the non-DNA binding ΔZF1/2 protein to act in a dominant negative fashion suggests that it can bind to, compete for, and sequester cellular factors necessary for Evi-1 biological activity. Recently, it has been shown that the repressor domain binds CtBP proteins (29). The Rp domain-dependent Evi-1 repressor and cell transformation activities have been shown in human 293 embryo kidney cells and Rat1 fibroblasts, respectively, and Northern blot analysis with an mCtBP2-specific probe confirmed both cell lines express the same proteins have been examined with similar results (not shown).

To determine if CtBP interactions might be required for Evi-1 transforming activity, we compared mCtBP2 binding activity in yeast two-hybrid assays with the Evi-1 repressor domain and a deletion mutant that lacks two CtBP PLDLS-like binding motifs located at amino acids 553–557 (CtBPα) and 584–588 (CtBPβ). The yeast expression vectors pGBT9Rp and pGBT9ΔRp, containing Evi-1 residues 514–724 and 632–724, respectively, fused in-frame with the GAL4 DNA binding domain (DBD), were introduced into yeast strain SFY526 in combination with pGAD424mCtBP2 encoding a GAL4 activation domain (AD) mCtBP2 fusion protein. Yeast cells expressing only ADmCtBP2 and DBD/Rp produce active β-galactosidase (Fig. 2A) clearly demonstrating a functional interaction between these proteins. However, binding activity is lost upon deletion of the repressor domain between amino acids 514 and 631, which includes the two potential CtBP interaction sites (Fig. 2A, ΔRp).

The interaction between Evi-1 and mCtBP2 was independently confirmed by co-immunoprecipitation studies utilizing Evi-1myc and FLAGmCtBP2 epitope-tagged expression vectors. Western blot analysis of anti-FLAG M2-immunoprecipitated cell extracts, with anti-myc 9E10, detects the Evi1myc fusion protein only in cell extracts containing both Evi1myc and FLAGmCtBP2 epitope-tagged proteins (Fig. 2B), demonstrating the two proteins can interact in mammalian cells.

To see if CtBP binding correlates with Evi-1 transforming activity, we examined growth in soft agar of various recombinant retrovirus-infected cell populations. Rat1 cells expressing either FL, ΔRp, or ΔRp521–633 Evi-1 proteins (Fig. 2C) were isolated. Macroscopic colonies were observed with populations of cells expressing the entire Evi-1 protein (Fig. 2C, FL). Cells expressing mutants lacking the entire Rp domain, just a partial deletion encompassing both potential CtBP sites, or neo alone produced significantly fewer colonies (Fig. 2C, ΔRp, ΔRp521–633, Neo).

CtBP Mediates Evi-1 Transforming Activity—The deletion mutant studies show a correlation between CtBP binding and Evi-1 biological activity. To directly determine the role of CtBP co-repressors, site-directed mutations were made to destroy the two potential CtBP binding sites in the Evi-1 repressor domain. Previous studies have shown that mutation of the consensus CtBP binding motif PLDLS to PLASS blocks CtBP binding to E1A (40). Similar changes were made in the Evi-1 Rp domain.
CtBP-like binding sites, converting PFDLT (CtBPa) and PLDLS (CtBPb) to PFAST (RpDa) and PLASS (RpDb), respectively. The wild type and mutant Rp sequences were inserted in-frame back into the Evi-1 mutant cDNA of p50Mn-neo (16) to recreate full-length Evi-1 retroviral vectors (Fig. 3). Rat1 cells were infected with the recombinant retroviruses and G418-resistant cell populations isolated. The growth properties of the various Rat1 cell lines were examined in soft agar to determine the impact of these mutations on Evi-1 biological activity. Numerous macroscopic colonies were produced in cells expressing FLRWT (Fig. 3), demonstrating that reconstruction of full-length Evi-1 with wild type RpWT sequences creates a protein with similar properties to the wild type protein. Rat1 cells expressing the FLRpDa mutation still show moderate transforming activity (Fig. 3). However, a dramatic reduction in colony formation is seen with either the FLRpDb or FLRpa/b proteins (Fig. 3), showing that the CtBPa site is partially required and the CtBPb site is essential to biological activity of the Evi-1 protein.

Western blot analysis of cell extracts with Evi-1-specific an-

**Fig. 2.** Protein interaction and transforming activity of Evi-1 Repressor domain deletion mutants. A, histogram showing the relative β-galactosidase activities in yeast SFY526 cells expressing the indicated Gal4DBD and Gal4AD non-fusion and fusion proteins. Results shown are the average assays of five independent colonies. Error bars indicate standard error. Stippled boxes correspond to the yeast Gal4 activation domain (AD), hatched boxes to mCtBP2, light gray to the yeast Gal4 DNA binding domain (DBD), and white to the Evi-1 repressor domain. Vertical thick black lines indicate the potential CtBP binding sites a and b. Rp and ΔRp correspond to Evi-1 amino acids 514–724 and 632–724, respectively. B, Western blot analysis of either whole cell extracts (W.C.E.) or anti-FLAG M2 immunoprecipitated cell extracts with the indicated antibodies. Cell extracts were derived from BOSC-23 cells transfected with the indicated expression vectors, which produce either Evi-1myc or FLAGmCtBP2 epitope-tagged proteins (+ or −). C, histogram showing the production of colonies in soft agar of Rat1 fibroblast cells expressing empty vector control (NEO), full-length Evi-1 (FL), and deletion mutants lacking all (ΔRp) or part (ΔRp521–633) of the repressor domain. Retroviral DNA and Evi-1 Rp domains are as indicated in the legend to Fig. 1 with the exception of the neo gene (stippled box). Inset, Western blot analysis of whole cell extracts derived from the indicated Rat1 cell populations using the Evi-1 N-terminal-specific antibody 34597. The 145-kDa full-length protein is indicated by an arrow.
tibodies (1806) revealed that equal amounts of the proteins were expressed that were indistinguishable in size from the wild type Evi-1 protein (Fig. 3). This showed that we had successfully recreated and expressed full-length wild type and mutant proteins in each case.

Site-directed Mutagenesis of Evi-1 CtBP Binding Sites Prevents mCtBP2 Binding—CtBP binding of the various site-directed mutants were tested in yeast. The wild type and mutant Rp domain-encoding fragments were inserted in-frame with the DBD of pGBT9. These constructs were introduced into the yeast strain AH109 with pGAD424mCtBP2 and protein interactions assessed using the β-galactosidase assay. As expected the wild type Rp domain (DBDRpWT) binds ADmCtBP2 as indicated by the production of β-galactosidase activity (Fig. 4A). However, binding of the CtBPa site mutant (DBDRpΔa) is significantly impaired and an even more severe loss of binding is observed with the CtBPb and CtBPa/b site mutations (Fig. 4A, DBDRpΔb, DBDRpΔa/b). Both Rp domain CtBP sites a or b can complement AH109 cell growth on media lacking histidine in the presence of mCtBP2, indicating that these sites can independently bind this protein (Fig. 4B). These results show that mutations of the CtBP sites reduce CtBP binding and that there is a direct correlation of binding activity with transforming activity of Evi-1 proteins containing these mutations.

CtBP Binding Activity Is Required for Evi-1 Repressor Activity—Finally we determined if CtBP binding is necessary for Evi-1 repressor activity. The Rp wild type and mutant fragments were inserted in-frame with the DBD of the mammalian expression vector pSG424. We examined the ability of these constructs to inhibit lexA VP16 induction of the reporter construct pLS85CAT in transiently transfected kidney 293 cells, as we have described previously (16). As expected the wild type Rp sequence represses 80% of lexA VP16 induction of reporter activity, whereas the vector alone has no inhibitory effect (Fig. 5, RpWT, DBD). The CtBPa mutation only partially relieves repressor activity, but the CtBPb and CtBPa/b mutations significantly reduce it to 30% (Fig. 5, RpΔa, RpΔb, RpΔa/b). Western blot analysis of cell extracts with GAL4DBD-specific antibodies revealed that equal amounts of the expected size DBD fusion proteins were expressed in each case. Therefore, the Evi-1 Rp CtBP binding sites mediate transcriptional repressor activity in kidney 293 cells.

**Discussion**

Several invertebrate and vertebrate transcriptional repressors have previously been shown to bind CtBP proteins through evolutionarily highly conserved PLDLS motifs to mediate transcriptional repression. These specific interactions are very likely to be required for some or all of their biological properties, but this has not been previously shown. However, we now show for the first time that CtBP proteins interact with the Evi-1 transcriptional repressor. We demonstrate that this interaction is required for at least one of the known biological activities of the Evi-1 protein, transformation of Rat1 fibroblasts, and in addition transcriptional repressor activity.

CtBP binds Evi-1 site b (PLDLSMG) more efficiently in yeast than site a (PFDLLTK), which probably reflects its closer similarity to the consensus core binding site P-DLS (29). This relative binding activity reflects the situation in mammalian cells, because the greater affinity of site b correlates with the greater impact its mutation has on the efficiency of both Evi-1-mediated cell transformation and transcriptional repressor activity. Thus, mutation of just site b is sufficient to block the vast majority of repressor and transforming activities. However, in each case optimal binding, transcriptional repression, and transformation requires both sites (Figs. 3–5). CtBP proteins can dimerize (32), and the presence of two adjacent Evi-1 binding sites in the Rp domain might enhance dimerization, which could be necessary to either stabilize intermolecular interactions and/or be necessary for function.

In this study we have demonstrated that Evi-1 binds to mCtBP2, which is expressed in 293 and Rat1 cells. There are two murine CtBP genes, mCtBP1 and mCtBP2, which appear to have similar binding specificities (34) and are both likely to bind Evi-1. Therefore, mCtBP1 and mCtBP2 are equally likely to mediate Evi-1 biological activity, but the status of mCtBP1 expression in 293 and Rat1 cells is not known. Interestingly, whole mount in situ hybridization and Northern blot analysis in the mouse show that mCtBP1 is expressed more generally in embryonic and adult tissues, whereas mCtBP2 expression is spatially restricted in the developing embryo (34). Of particular interest is the high level of mCtBP2 expression in the limb buds and dorsal root ganglia of day 10.5 post coitum mouse embryos, which overlaps with the highest levels of Evi-1 expression observed (15). This strongly suggests that mCtBP2 is important to the role of Evi-1 in development, because Evi-1 FL null mouse embryos have multiple defects, including underdeveloped or absent limb buds and no peripheral nervous system (15). However, probably not all Evi-1 functions are mediated by CtBP proteins, because these mutant mice also have severe heart defects where mCtBP1 and mCtBP2 are not expressed (34).

Although it is well established that CtBP proteins are corepressors, their mechanism of action is unknown. There are mixed reports, suggesting CtBP proteins need deacetylase activity to repress transcription. One report shows CtBP-depend-
ent repression is sensitive to histone deacetylase inhibitors (37), whereas another contradicts this (38). It has been reported that hCtBP1 binds histone deacetylase (44). Curiously, a highly homologous and either new member or isoform of the CtBP family designated CtBP3, involved in Golgi structure and function, has intrinsic acyl transferase activity (45). Alternatively, it has been proposed that CtBP proteins might repress transcription by generating specific areas of heterochromatin, possibly by bridging interactions between sequence-specific transcription factors and Polycomb group complex proteins, possibly by bridging interactions between sequence-specific transcription factors and Polycomb group complex proteins, which are involved in silencing homeotic genes (46).

Recently, Evi-1 has been shown to inhibit transforming growth factor-β signaling by the ZF1 domain interacting with Smad3 (47). These studies also show that the Rp domain is necessary for transforming growth factor-β blocking activity. One possibility is that Smad3 binds Evi-1, which then recruits a repressor complex containing CtBP to block signaling. Although the transforming growth factor-β blocking activity is dependent upon amino acids 608–732 of Rp (47), which are outside the CtBP sites, such gross deletions might create changes in protein conformation that prevent binding. This possibility is currently under investigation.

The Evi-1 dominant negative mutants described in this study are likely to act by two distinct mechanisms. Evi-1 mutants, which lack the Rp domain but retain ZF1 and ZF2, also have dominant negative activity (data not shown) supporting the competitive DNA binding mechanism. The dominant negative mutant, which lacks both zinc finger DNA binding domains, most likely sequesters factors that Evi-1 needs to transform cells. Our results suggest CtBP might be such a factor but do not eliminate the possibility that other factors are involved. Therefore, these results show that the expression of partial Evi-1 polypeptides, which either compete for DNA binding activity or co-factors like CtBP, inhibit Evi-1 activity and suggest that smaller peptides designed to block the same targets could be the basis of effective therapeutic agents.

The murine Evi-1 PFDLTTK and PLDLSMG motifs are absolutely conserved in the human EVI1 primary amino acid sequence and, therefore, are very likely to be required for its biological activity as well. The interaction of human EVI1 and CtBP proteins might therefore be necessary in the development of leukemias where either EVI1 or EVI1 fusion proteins are produced. In the case of AML1/EVI1 fusion proteins, EVI1 might be required to recruit a repressor complex comprising CtBP proteins to AML1 DNA binding sites in analogy to ETO recruiting NCoR/mSin3-HDAC1 complexes for the AML1/ETO fusion protein (22).

A possible role for CtBP proteins in the development of leukemias has not been described before. These studies suggest a potential pharmacological use for peptides containing the
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PLDLS motif in blocking the interaction between EVI1 and CtBP proteins in the treatment of some acute myeloid leukemias, chronic myelogenous leukemias in blast crisis (48), and myelodysplasias (49) where EVI-1 is expressed.

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Evi-1 Transforming and Repressor Activities Are Mediated by CtBP Co-repressor Proteins

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