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Oncogenic mechanisms of Evi-1 protein

Abstract Although Evi-1 is thought to promote growth or block differentiation in some cell types, its biological functions have not been elucidated. To explore the mechanisms underlying Evi-1-induced oncogenesis, we investigated whether Evi-1 affects the signaling of transforming growth factor β (TGF-β), which inhibits proliferation of a wide range of cell types and is one of the most studied growth regulatory factors. We demonstrated that Evi-1 represses TGF-β signaling and antagonizes its growth-inhibitory effects. Two separate regions of Evi-1 are responsible for this repression, one of which is the first zinc-finger domain. Through this domain, Evi-1 physically interacts with Smad3, an intracellular mediator of TGF-β signaling, thereby suppressing the transcriptional activity of Smad3. These results define a novel function of Evi-1 as a repressor of signaling components of TGF-β. We also demonstrated that Evi-1 represses Smad-induced transcriptional activation by recruiting CtBP as a corepressor. Evi-1 associates with CtBP1 through one of the CtBP-binding consensus motifs within the region from amino acid 544 to 607, and this association is required for the efficient inhibition of TGF-β signaling. A specific histone deacetylase (HDAc) inhibitor, trichostatin A (TSA), alleviates Evi-1-mediated repression of TGF-β signaling, suggesting that HDAc is involved in transcriptional repression by Evi-1. This identifies a novel function of Evi-1 as a member of corepressor complexes and suggests that aberrant recruitment of corepressors is one of the mechanisms involved in Evi-1-induced leukemogenesis. These results indicate that specific HDAc inhibitors may be useful in the treatment of Evi-1-induced neoplastic tumors, including myeloid leukemias.

Keywords Evi-1 · Transforming growth factor-β · Smad · Corepressor · Histone deacetylase

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Introduction

Evi-1 was first identified as a gene existing in a common locus of retroviral integration in myeloid tumors in AKXD mice [19]. It encodes a 145-kDa nuclear-localized protein [13]. Although Evi-1 expression is barely detectable in healthy murine or human bone marrow and peripheral blood, its frequent transcriptional activation has been documented in a subset of myeloid malignancies [16, 21]. The human Evi-1 gene is located on chromosome 3q26 and rearrangements involving this region, including t(3;21), t(3;12), t(3;3), and inv(3), often activate Evi-1 expression in myeloid leukemias and myelodysplasias [16, 21]. In t(3;21)(q26;q22) found in patients with chronic myelogenous leukemia in blast crisis, we have demonstrated that Evi-1 is fused to the AML1 gene and is transcriptionally activated as the AML1/Evi-1 chimera under the control of the AML1 promoter [12]. Elevated expression of Evi-1 also occurs without cytogenetically evident translocations in some myeloid malignancies [22, 25]. These findings suggest a critical role for Evi-1 in malignant transformation of hematopoietic cells as a dominant oncogene. Furthermore, it has been reported that Evi-1 is also activated in some ovarian cancers, which hints at its potential role in solid tumor formation [2].

Structurally, Evi-1 possesses seven and three repeats of Cys2His2-type zinc-finger motifs separated into two domains (ZF1–7 and ZF8–10) [13]. The transcriptional properties of Evi-1 have not yet been elucidated. There is

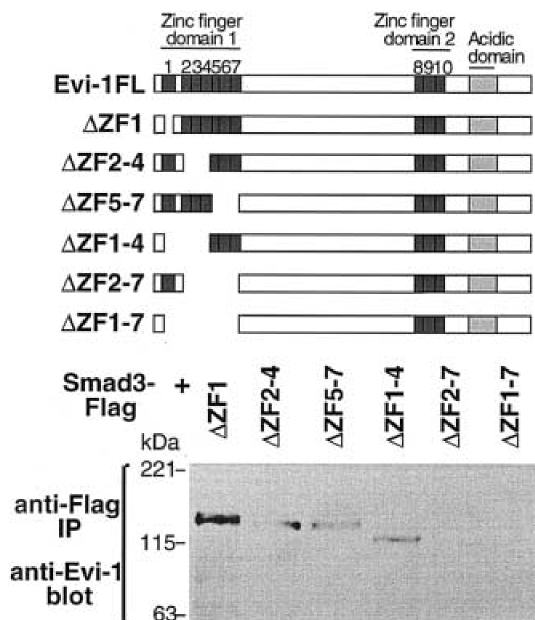
some evidence suggesting that Evi-1 works as a negative regulator of gene expression. It exhibits repressor activities dependent on DNA binding through either the first or the second zinc-finger domain [1, 8]. Alternatively, characteristics of Evi-1 as a transcriptional activator have been described. We have reported that Evi-1 elevates intracellular AP1 activity and stimulates the c-fos promoter with dependence on the second zinc-finger domain [27]. Evi-1 can also activate transcription from the reporter containing the binding sequences for the second zinc-finger domain [18]. However, the authentic target genes that Evi-1 may regulate have not been identified.

Potential roles for Evi-1 in embryogenic development have also been postulated. During ontogeny in mice, Evi-1 shows a temporally and spatially restricted pattern of expression, suggesting a role for the gene in organogenesis. High levels of expression have been reported in limited sites including the urogenital tract, Mullerian duct, bronchial epithelium, focal areas in the nasal cavity, endocardial cushion, and limbs in the developing embryo [24], whereas expression in adult mice is restricted to the kidney and ovary [15]. Several biological effects of Evi-1 have been described. Evi-1 causes cellular transformation when overexpressed in Rat1 fibroblast cells [7]. Overexpressed Evi-1 blocks granulocytic differentiation of a murine myeloid cell line induced by granulocyte colony-stimulating factor [17]. Forced expression of Evi-1 in normal hematopoietic progenitors decreases colony formation in response to erythropoietin [6]. Based on this, Evi-1 is thought to affect growth promotion and differentiation blocking in some types of cells.

Proliferation and differentiation of cells are tightly regulated by a delicate balance of growth factors and growth-inhibitory factors. Transforming growth factor β (TGF-β) is one of the best-characterized growth-inhibitory factors. TGF-β can inhibit proliferation of a wide range of cell types including epithelial, endothelial, and hematopoietic cells [10]. To examine the possibility that Evi-1 may antagonize negative regulators of cell growth, we undertook a study to determine whether Evi-1 can interfere with TGF-β-mediated growth inhibition. We found that Evi-1 blocks TGF-β-induced transactivation of the responsive promoters and abrogates cellular responses to growth-suppressive signaling of TGF-β. At least two regions of Evi-1 are required for this repressor activity, one of which is the first zinc-finger domain. Significantly, Evi-1 physically interacts with the Smad3 protein through the first zinc-finger domain, and this interaction is necessary for its efficient inhibition of TGF-β signaling. Thus Evi-1 acts as an in vivo repressor of Smad3 and provides a paradigm for the regulation of TGF-β signaling by targeting its intracellular signaling component. We further investigated the mechanism of Smad inhibition by Evi-1, and demonstrated that CtBP acts as an essential cofactor in Evi-1-mediated repression of TGF-β signaling.

Evi-1 represses TGF-β signaling by binding to Smad3

To explore the potential roles of Evi-1 in TGF-β signaling, we examined the effects of Evi-1 on the TGF-β-mediated transcriptional responses using cotransfection assays. We first used p3TP-Lux, a TGF-β-responsive reporter plasmid consisting of the plasminogen activator inhibitor 1 (PAI-1) promoter. When p3TP-Lux alone



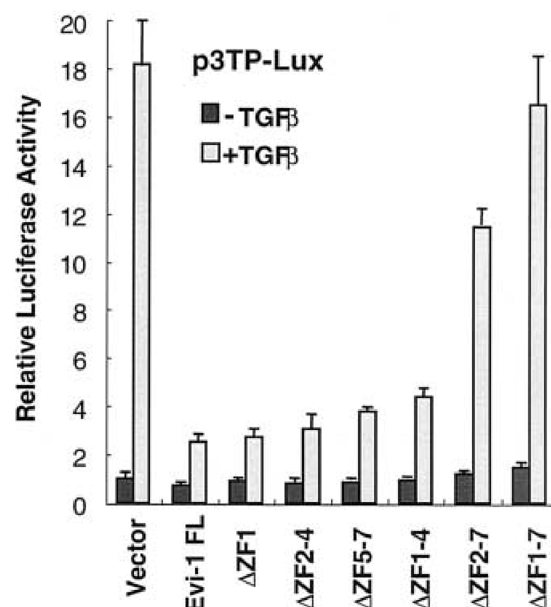


Fig. 1A, B Smad binding ability is required for Evi-1 to repress TGF-β signaling. The indicated forms of Evi-1 mutants were tested for binding with Smad3 (**A**) and for repression of TGF-β signaling (**B**). Evi-1 represses TGF-β signaling by binding to Smad3 through the first zinc-finger domain

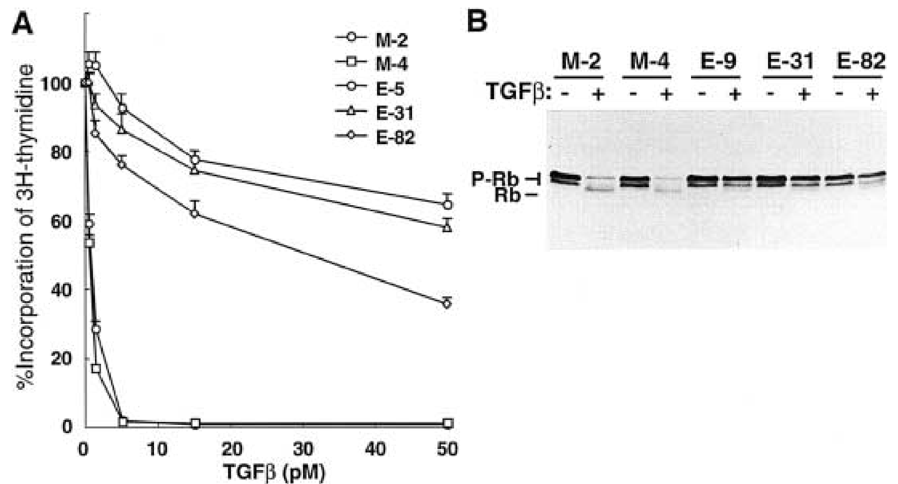


Fig. 2A, B Evi-1 inhibits TGF-β-mediated growth inhibition. Mv1Lu clones stably expressing Evi-1 (*E*) and mock clones (*M*) were subjected to [^{3}H]thymidine incorporation assays in the presence of the indicated concentrations of TGF-β (**A**). Rb proteins in the Evi-1-expressing cells remain hyperphosphorylated even in the presence of TGF-β (**B**)

was transfected, a significant increase in luciferase activity was observed in the presence of TGF-β [9] (Fig. 1). Remarkably, these transactivations were repressed almost to the control level when Evi-1 was cotransfected. Similar repression was observed when another TGF-β-responsive reporter containing the p15 promoter 16 was employed (data not shown). None of the consensus DNA sequences for Evi-1 binding was found in the promoters studied, suggesting that Evi-1 represses TGF-β signaling by interrupting the intracellular signaling pathways rather than binding directly to the target promoters.

To examine whether Evi-1 could affect the antiproliferative effects of TGF-β in vivo, several Mv1Lu cell lines that stably express Evi-1 were established. When cultured in the absence of TGF-β, all the clones exhibited comparable proliferative abilities. In the presence of TGF-β, the growth of the control cell lines M-2 and M-4 was effectively inhibited (data not shown). In contrast, overexpression of Evi-1 prevented growth arrest of the cells even after exposure to TGF-β for 72 h. [^{3}H]Thymidine incorporation assays showed that the growth of M-2 and M-4 was inhibited by 0.3 p*M* TGF-β, whereas overexpression of Evi-1 in E-5, E-31, and E-82 significantly diminished the responsiveness to TGF-β in an expression level-dependent manner (Fig. 2).

We assayed the status of Rb phosphorylation as an in vivo measurement of the effects of Evi-1, and found that the majority of Rb in the Evi-1-expressing cells remained in the hyperphosphorylated state even in the presence of TGF-β, whereas the control cell lines treated with TGF-β showed accumulation of hypophosphorylated Rb, accompanied by a decrease in the overall level of detectable Rb17 (Fig. 2).

To identify the target through which Evi-1 represses TGF-β signaling, we determined whether Evi-1 could interact with the Smad proteins, which are recently identified signaling components of TGF-β [4, 11]. In Smad1–4, Evi-1 was specifically detected in the immune complex of Smad3, as shown in Fig. 1. These results indicate that Evi-1 may antagonize TGF-β signaling by quenching Smad3. To determine the Smad3-binding domain of Evi-1, we evaluated the Smad3-binding abilities of a series of specific-deletion mutants of Evi-1. Evi-1(1–252), which is mainly composed of the first zinc-finger domain, retained the ability to bind Smad3. Detailed internal deletions in the first zinc-finger domain revealed that deletion including the entire first zinc-finger domain (ZF1–7) completely abolished Evi-1-Smad3 binding (Fig. 1). These results indicate that the first zinc-finger domain of Evi-1 is essential for the interaction between Evi-1 and Smad3. We employed another series of region-specific deletion mutants of Evi-1 and determined their repression activity to define the Evi-1 domains that contribute to inhibition of TGF-β signaling. As shown in Fig. 3, both Evi-1D608–732 and Evi-1D544–607 severely impaired the repression activity. Deletion of both regions (Evi-1D544–732) completely abolished the repression activity.

Evi-1 interacts with CtBP1 through its consensus motif

The region between Evi-1 amino acids 544 and 607, which we have shown to participate in efficient inhibition of TGF-β signaling, contains two amino acid sequences, Pro-Phe-Asp-Leu-Thr (PFDLT) and Pro-Leu-Asp-Leu-Ser (PLDLS), which fit the CtBP-binding motif (Fig. 4). These motifs are completely conserved between mouse and human Evi-1 proteins [14]. These findings allow a coimmunoprecipitation assay to determine whether Evi-1 interacts with CtBP in vivo. We introduced T7-tagged CtBP1 with the wild-type or deletion mutants of Evi-1 into COS7 cells. Cell lysates were subjected to immunoprecipitation with anti-T7, followed by immunoblotting with anti-Evi-1. We observed that wild-type Evi-1 was coprecipitated with T7-CtBP1. However, D544–607 or D544–732, which lack putative CtBP-binding motifs, was not coprecipitated. In contrast, D608–732, which retains the domain including the motifs, coprecipitated with T7-CtBP1 (data not shown). From these results, we conclude that Evi-1 interacts with

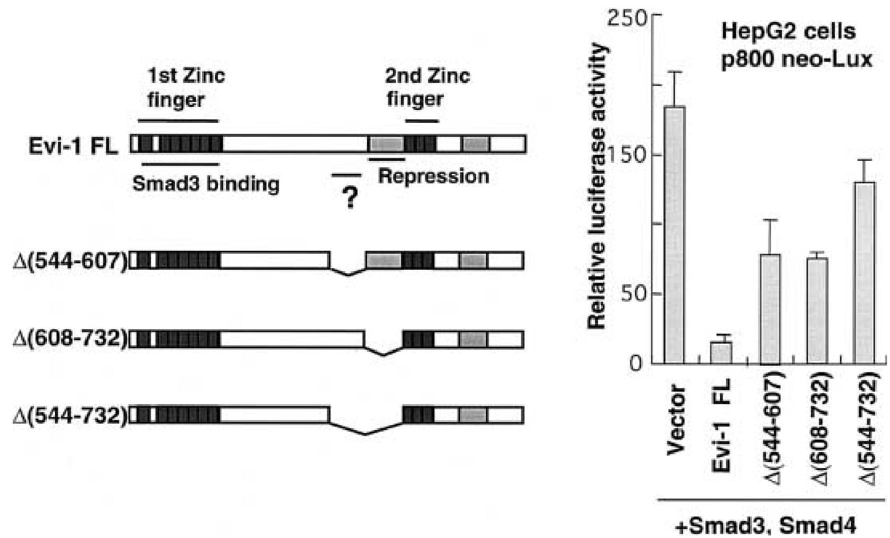


Fig. 3 Amino acids 544–607 of Evi-1 are required for Smad3 inhibition. The indicated series of Evi-1 deletion mutants were examined for repression of TGF-β signaling. Evi-1 mutants lacking amino acids 544–607 and 608–732 showed impaired repressive activity

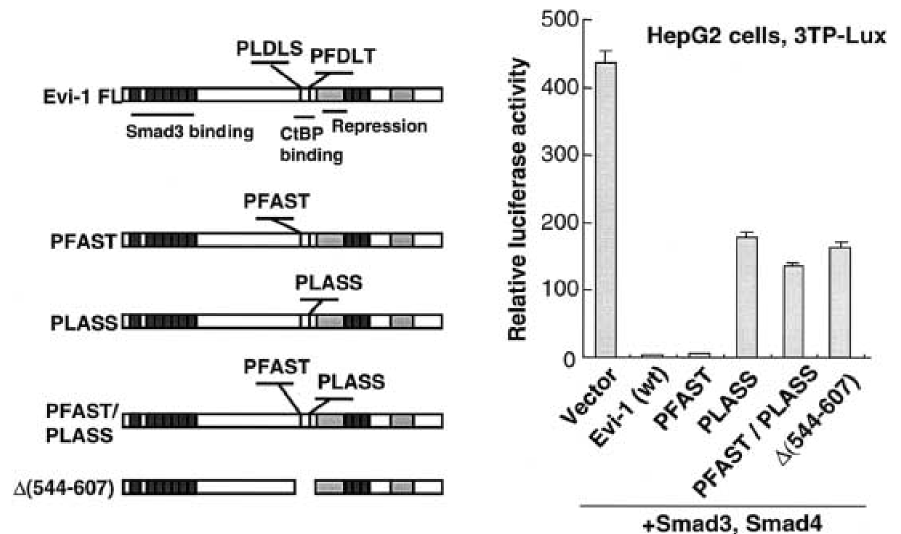


Fig. 4 Disruption of CtBP-binding site abolishes Evi-1 inhibition of Smad3. Mutants for the CtBP-binding motifs were tested for TGF-β-mediated transcriptional activation. Interaction with CtBP is required for efficient repression of TGF-β signaling by Evi-1

CtBP1 in vivo and that the region between amino acids 544 and 607 is responsible for binding to CtBP1.

Next we examined the relative contribution of the two putative CtBP-binding motifs to the interaction with CtBP. For this purpose, we introduced specific amino acid substitutions into Evi-1 at the PFDLT (PFAST) region, the PLDLS (PLASS) region, or both (Fig. 4). We transfected these mutants with T7-CtBP1 into COS7 cells for immunoprecipitation experiments. Each mutant was expressed comparably at the anticipated size. Both wild-type Evi-1 and the mutant for PFDLT (PFAST) interacted with T7-CtBP1. However, neither of the two mutants for PLDLS retained the ability to interact with CtBP (data not shown). Thus, of the two CtBP-binding motifs, PLDLS at amino acid 584 is responsible for the interaction between Evi-1 and CtBP1. To determine whether Evi-1 binds to CtBP directly, we performed an in vitro pull-down assay using GST fusion proteins. Evi-1(544–607) and its mutants for the CtBP motifs were fused to GST and expressed in bacteria. These proteins were immobilized onto glutathione Sepharose 4B beads and incubated with [^{35}S]methionine-labeled, in vitro-translated CtBP1. GST-PLASS failed to bind to CtBP, whereas GST-PFAST interacted with in vitro-translated CtBP1 (data not shown). These findings indicate that Evi-1 interacts directly with CtBP1.

Evi-1 represses Smad-induced transcription by recruiting a corepressor complex

CtBP has been shown to act as a transcriptional corepressor in *Drosophila melanogaster* and in vertebrates [3, 5, 20]. To investigate the role of CtBP in Evi-1-mediated repression, we attempted to determine whether the PLASS mutation, which abrogates the binding of CtBP1 to Evi-1, would impair the ability of Evi-1 to repress TGF-β signaling. p3TP-Lux was transfected into HepG2 cells together with the effector plasmids (Fig. 4). The

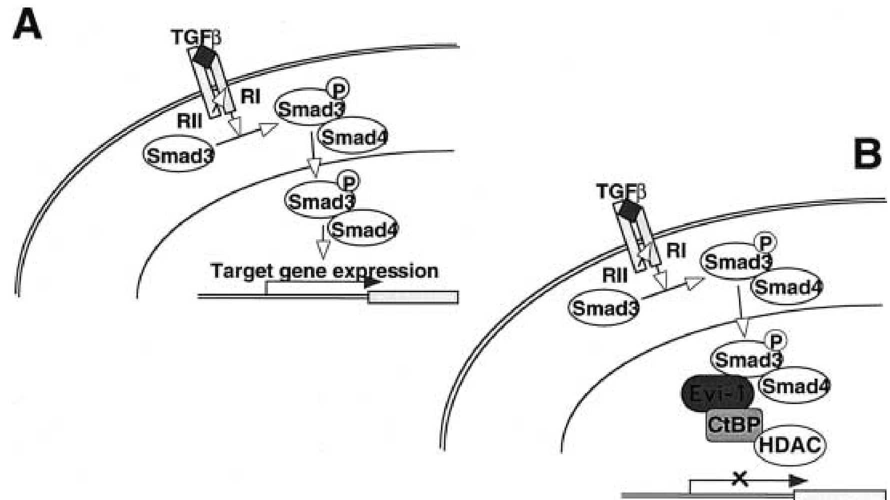


Fig. 5 Schematic mechanism of the repression of TGF-β signaling by Evi-1. Evi-1 binds to CtBP through the second CtBP binding motif, recruits a corepressor complex of CtBP and HDAc, thereby repressing TGF-β signaling and releasing the cells from TGF-β-mediated growth inhibition

PFAST mutant, which does not affect the interaction between Evi-1 and CtBP1, repressed TGF-β-mediated transactivation as efficiently as wild-type Evi-1 (Fig. 4). In contrast, the PLASS mutant, in which the interaction with CtBP1 is specifically abrogated, showed markedly reduced repression activity. We also performed these experiments using overexpression of Smad3 and Smad4 instead of exposure to TGF-β, and similar results were obtained (Fig. 4). These data indicate that recruitment of CtBP plays an important role in full repression of TGF-β signaling by Evi-1.

A histone deacetylase (HDAc) is known to mediate transcriptional repression by rendering the nearby chromatin inaccessible to transcriptional activators through deacetylation of histone proteins [23, 28]. A recent study has demonstrated that CtBP interacts with HDAc both in vitro and in vivo [26], suggesting that CtBP may play a role as a corepressor by recruiting HDAcs. To investigate whether Evi-1-mediated repression involves histone deacetylation, we performed a luciferase assay using the specific HDAc inhibitor trichostatin A (TSA) [29]. We cultured HepG2 cells with TSA 100 ng/ml for 8 h before harvesting. The culture had no effect on the basal or Smad-induced activity of p3TP-Lux (data not shown). In contrast, repression of TGF-β signaling elicited by Evi-1 was significantly attenuated. These results suggest that HDAc activity is involved in repression of TGF-β signaling by Evi-1 (Fig. 5).

To investigate the role of CtBP in the Evi-1-mediated blocking of the antiproliferative effects of TGF-β, we established Mv1Lu clones that stably express either wild-type Evi-1 or the PLASS mutant. The Mv1Lu clones that overexpress Evi-1 showed reduced responsiveness to TGF-β compared with mock-transfected clones. In contrast, the growth of clones overexpressing the PLASS mutant was inhibited in response to TGF-β as efficiently as that of mock-transfected clones. These results suggest that recruitment of CtBP is required for Evi-1 to release cells from the growth-inhibitory effects of TGF-β in vivo.

Conclusions

We found that Evi-1 functions as a negative regulator of TGF-β signaling through binding to Smad3. Identification of the interaction between the two distinct transcriptional regulators has broad implications for the repertoire and complexity of the regulatory mechanisms of TGF-β and Smad signaling, and provides insight into the physiologic and oncogenic activities of the Evi-1 oncoprotein. We further found that the CtBP protein acts as a corepressor of Evi-1. One of the mechanisms that underlie Smad3 inhibition by Evi-1 could be the recruitment of an HDAc complex through the CtB that interacts with DNA-binding transcription factors. Evi-1 may, in addition to binding to DNA directly, regulate transcription by interacting with another DNA-binding protein, as in the case of Smad3 and by recruiting corepressor complexes including CtBP. Our observations also provide insight into potential therapeutic approaches, and indicate that specific HDAc inhibitors might be useful in the treatment of Evi-1-induced leukemias.

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