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Research Article

Modulation of thyroid hormone receptor transactivation by the early region 1A (E1A)-like inhibitor of differentiation 1 (EID1)

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Abstract

Transcriptional activation (TA) mediated by the effect of thyroid hormones on target genes requires co-activator proteins such as the early region 1A (E1A) associated 300 kDa binding protein (p300) and the cAMP response element binding protein (CREB) binding protein (CBP), known as the p300/CBP complex, which acetylate histones 3 and 4 to allow transcriptional machinery access to the target gene promoter. Little is known on the role of p300 in thyroid hormone receptor (TR) mediated TA but the E1A-like inhibitor of differentiation 1 (EID1), an inhibitor of p300 histone acetyltransferase (HAT), is a functional homolog of E1A and may inhibit myogenic differentiation factor D (MyoD) transcriptional activity and reduces muscle cell differentiation. We evaluated the influence of EID1 on TR-mediated transcriptional activity using transfection and mammalian two-hybrid studies to show that EID1 may partially reduces TA activity of the TR receptor, probably due to p300 blockage since EID1 mutants cannot reduce TR-mediated TA. The EID1 does not affect the function of p160 co-activator proteins (160 kDa proteins of steroid receptor co-activators) and is functionally independent of co-repressor proteins or TR binding. Summarizing, EID1 reduces TR-mediated transcriptional activity by blocking p300 and may play an important role in thyroid receptor activity in muscle and other tissues.

Key words: co-activator, EID1, p300, thyroid receptors, transcription.

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Introduction

The thyroid hormone receptor (TR) is a member of the nuclear hormone receptor (NHR) family of transcription factors, which control growth, development and homeostasis. There are four known TR isotypes (TRα1, TRβ1, TRβ2 and TRβ3) which bind the thyroid hormone 3,5,3'-L-triiodothyronine (T_3) (Williams, 2000). As a hormone-regulated, DNA-binding transcription factor, the TR can either enhance or repress transcription by binding to thyroid hormone response elements (TREs) in the promoters of target genes. The TRs can bind TREs as monomers, homodimers or heterodimers, and there can be variation in the primary nucleotide sequences of TREs as well as their number, spacing and orientation. In particular, TRs can bind to TREs in which half the sites are arranged as palindromes (TREpal), direct repeats (DRs) or inverted palindromes (IPs), the optimal spacing for these half-site arrangements being zero, four (DR4) and six nucleotides respectively. Most of the natural TREs described have DR4 motifs on which TRs can form heterodimers with retinoid X receptors (RXRs), which also are members of the nuclear hormone receptor superfamily (Yen, 2001).

The activating and repressive regulatory properties of TRs are regulated by crucial protein-protein contacts with basal transcription factors and transcriptional co-repressor or co-activator proteins. In the absence of thyroid hormone (TH), TRs typically bind to TREs in the promoters of regulated genes and reduce transcription by binding with the co-repressors nuclear receptor co-repressor (N-CoR) and silencing mediator for retinoid receptors and TRs (SMRT) (Lizcano *et al.*, 2001). The binding of TH induces conformational changes in the TR that promote dissociation of TR and co-repressors and the simultaneous recruitment of several co-activator protein complexes. These co-activators induce changes in nucleosomes, which enable interaction with general transcription factors (Smith and O'Malley, 2004). Some co-activators that interact with TR have histone acetyltransferase (HAT) activity, required for the chromatin unfolding which allows access to transcriptional activator complexes, these co-activators include the 160 kDa proteins of the steroid receptor co-activator (p160), the early region 1A (E1A) associated 300 kDa binding protein (p300), the cAMP response element binding protein (CREB) binding protein (CREBBP, or CBP) and the

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p300/CBP complex associated factor (p/CAF) (Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002). Although the p300/CBP complex may bind directly to TRs, it can be recruited to the promoters through p160 co-activators (Sharma and Fondell, 2002). Furthermore, p300/CBP can bind to switching/sucrose nonfermenting (SWI/SNF) ATP-dependent chromatin remodeling complexes (Becker and Horz, 2002) and the thyroid hormone receptor-associated protein (TRAP) complex (Ito and Roeder, 2002), acting as a bridge between general transcription factors and TR (Huang *et al.*, 2003). This suggests that besides having potent HAT activity, essential for hormone-dependent activation by TR, p300 can also act as an integrator between TR and the general transcriptional machinery (Ogryzko *et al.*, 1996; Korzus *et al.*, 1998).

The E1A-like inhibitor of differentiation 1 (EID1, also known as p/CAF inhibitor 1 or CRI-1) shares functional homology with E1A (Miyake *et al.*, 2000) and is an inhibitor of p300 (De Luca *et al.*, 2003) that has been shown to reduce the activity of other transcription factors (Bavner *et al.*, 2005) and has been described as a functional homolog of the adenovirus E1A protein and may decrease muscle cell differentiation by inhibiting the activation of myogenic differentiation factor D (MyoD) by blocking p300 (MacLellan *et al.*, 2000). Interestingly, while E1A reduces retinoic acid receptor (RAR) transactivation (Chakvarti *et al.*, 1999; Hamamori *et al.*, 1999; Meng *et al.*, 2005), it has also shown activity as a potent TR co-activator in mammalian and yeast cells (Wahlstrom *et al.*, 1999; Meng *et al.*, 2003), probably as a result of direct interaction with TRs.

To investigate the role of p300 in TR activation we evaluated a possible role of the EID1 protein as a functional co-activator of ligand-bound TR transcriptional activity. Our results showed that EID1 reduced TR transactivation, even though it did not bind to TR in mammalian two-hybrid studies. This reduction was probably secondary to blocked p300 activity, because EID1 mutants that could not bind to p300 did not decrease TR-mediated transcriptional activation. We also found that EID1 did not affect the function of p160 co-activator proteins and was functionally independent of binding to co-repressor proteins.

Materials and Methods

Plasmids

Expression vector plasmids containing rat thyroid receptor β1 (TRβ1) and β2 (TRβ2) protein DNA sequences were generated by inserting the *Eco* RI fragment of rat TRβ1 and TRβ2 into the expression vector plasmid pcDNAI/AMP (Invitrogen, USA) as previously described (Koibuchi *et al.*, 1999). An *in vitro* mutagenesis kit (Promega, Madison, WI) was employed to create the TRβ1 mutant (TRAHTm) using a mutagenesis primer that altered codons 223 from A to G, 224 from H to G and 227 from T to A and inserted this into pcDNAI/Amp (Lizcano *et al.*, 2001). These changes in the hinge region of TRβ1 reduce its interaction with the co-repressor complex. Oligonucleotides containing the 5xUAS and DR4 reporter fragments were cloned into the *Bam*HI and *Hin*dIII restriction sites of the pT109 vector, which contains the herpes simplex thymidine kinase (TK) minimum promoter coupled to a luciferase (LUC) coding sequence (Hodin *et al.*, 1989; Koibuchi *et al.*, 1999). The pcDNA3-EID1 and pcDNA3-EID1 mutant plasmids (pcDNA-ΔEID1) have been described previously (Miyake *et al.*, 2000), pcDNA-ΔEID1 (157-Δ53Δ92) lacking the acidic regions created by residues 53-63 and 92-115 that have been reported to be necessary for p300 binding. This mutant also has a deletion of residues 158-187 in the C-terminal region, which contains a retinoblastoma protein (pRB) binding motif (LXCXE, where X equals any amino acid residue) (Miyake *et al.*, 2000). The cMVb-NHAp300 plasmid (Eckner *et al.*, 1996) was a kind donation from the Livingstong's Lababoratory (Dana Farber Institute, Boston, USA) The following plasmids were kindly donated by A. Takeshita (Gunma, Japan): the steroid receptor-1, also referred to as nuclear receptor co-activator 1/NCoA, pcDNA-SRC-1 plasmid (Onate *et al.*, 1995; Kamei *et al.*, 1996); the thyroid hormone receptor activator molecule, also referred to as amplified in breast cancer 1 (AIB1), 1pBK-CMV-TRAM1 plasmid; the activator of transcription of nuclear receptors (ACTR) plasmid; (Takeshita *et al.*, 1997; Anzick *et al.*, 1997; Chen *et al.*, 1997; The thyroid receptor binding protein (also referred as peroxisome proliferator receptor interacting protein (PRIP or PPPAR), receptor associated protein (RAP250/250-kDa), nuclear receptor co-regulator (NRC) or activating signal co-integrator-2 (ASC-2) plasmid pcDNA3-TRBP has been described previously (Ko *et al.*, 2000; Zhu *et al.*, 2000; Caira et., 2000; Mahajan and Samuels, 2000; Lee *et al.*, 2000.

Transient transfection

The U2OS and CV-1 cell lines were obtained from American Tissue Culture Collection (ATCC) and grown under a 5% (v/v) CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The serum was stripped of thyroid hormones by constant mixing with 5% (w/v) AGI-X8 resin (Mallinckrodt, Raleigh, USA) and subjected to ultra-filtration using a 0.22 μm membrane (Nalgene, Rochester, USA). The cells were seeded at 10^3 cells per well of a twelve multiwell cell culture plates (Falcon, San Jose, USA) 48 h prior to transfection. When cells reached 70% confluence, they were transiently transfected using a calcium-phosphate method (Sambrook *et al.*, 1989). Reporter plasmids (0.85 μg/well) containing DR4 or a constitutive active thymidine kinase-luciferase (TK-Luc) reporter con-

taining five GAL4-binding sites (5x UAS-TK-Luc) were co-transfected with 0.1 μg per well of the Gal4-TRβ1-LBD, VP16-EID-1, Gal4-NCoR, VP16-TRβ1-LBD, EID1, ΔEID1 (1-157Δ53Δ92) plasmids and 0.1 μg per well of a co-activator protein (p300, SRC-1, TRAM1 or TRBP). The total amount of transfected DNA in each well was balanced by adding the pcDNA3.1 plasmid. For both cells types, control wells were transfected with the appropriated amount of empty expression vector. Cells were grown in the presence or absence of 1 x 10^{-6} M T_3, and after 24 h cells were harvested in triton X-100 buffer (USB, Cleveland,USA). Luciferase activity, introduced into the cells using the 5xUAS and DR4 plasmids, was measured with the luminometer (Lumat 9507, Berthold technologies, Bad Wildbad, Germany), beta-galactosidase (β-gal) activity, introduced using the CMV-β-gal plasmid, was measured photospectrometrically at 410 nm (Variant, USA). The fold-change in luciferase or β-gal expression in the cells containing the experimental vectors was calculated relative to expression in the presence of only the control expression vector pcDNA3.1. The data was expressed as the mean ± the standard error of the mean (SEM) and represent a minimum of three independent experiments, with each data point run in triplicate for each experiment.

Mammalian two-hybrid system

The Gal4-blank and Gal4-N-CoR (aa1552-2453) plasmids have been previously described (Miyazaki *et al.*, 2004), while the VP16-EID1 plasmid was generated by recovering EID1 from the pcDNA 3.1 plasmid by double digestion with *Eco* RI and *Hind* III and subcloning into the VP-16 plasmid which was then digested with the same restriction enzymes. The Gal4-TRβ1-LBD plasmid (Miyazaki *et al.*, 2004) was obtained from A. N. Hollenberg (Brigham and Women's Hospital, Boston, USA) and the Gal4-pRB and Gal4-p300 (Miyake *et al.*, 2000) plasmids were obtained from W. Kaelin Jr. (Dana Farber Institute, Boston, USA). The VP16-TRβ-1-ligand binding domain (LBD) was constructed by inserting a PCR-generated in-frame fragment downstream of the VP-16 activation domain in the AASV-VP16 plasmid (Miyazaki *et al.*, 2004). We transiently transfected U2OS cell in twelve-well plates, in duplicate, with 500 ng of CMV-β-Gal plasmid, 1 μg of 5x UAS-TK-Luc reporter plasmid and 1 μg each of the Gal4-Blank, Gal4-pRB, Gal4-TRβ1-LBD, Gal4-NCoR and Gal4-p300 plasmids and 1 μg or 2 μg each of the VP16-blank, VP16-EID1 and VP16-TRβ1 plasmids. The luciferase and β-Gal assays were carried out as described above.

Results and Discussion

We first performed transient transfection studies using the natural (DR-4) reporter plasmid and detected a ligand-independent reduction in the transcriptional activation of the TRβ1 plasmid. After addition of the T_3, there was a significant increase in TRβ1-mediated transcription. Co-transfection of EID1 did not alter the ligand-independent repression, but we observed a 50% reduction in T_3-induced TRβ1 transcriptional transactivation (Figure 1A). In order to evaluate whether a co-repressor complex might be responsible for the reduction in transcriptional activation caused by EID1, transfection studies were performed using TRβ2 and TRAHT mutants, which are thyroid receptors with a lower binding capacity for the co-repressor complex. We found that EID1 was able to significantly reduce the T_3-mediated activation of both TRβ2 and TRAHT receptors (Figure 1B), indicating that perhaps the co-repressor complex was not necessary for the EID1-mediated reduction in thyroid receptor-mediated transcription.

To investigate whether the reduction in TRβ1-mediated transcription caused by EID1 was a consequence of p300 inhibition, CV-1 and U2OS cells were co-transfected with a plasmid containing the Gal4 DNA-binding domain fused to the TRβ1-ligand binding domain (TRβ1-LBD) and a plasmid with the EID1 mutant (ΔEID1-Δ1-157Δ53Δ92), but which lacked the p300 binding sites, together with a reporter plasmid that contained multiple Gal4 biding sites fused to TK-Luc (UAS-TK-Luc). The Gal4-TRβ1-LBD-mediated transcriptional activation following administration of T_3 was not modifying when the ΔEID1 mutant was co-transfected, indicating that the ΔEID1 mutant lacked the necessary sites to cause the reduction in TRβ1-mediated transcription which was observed with EID1 (Figure 2A).

The reduction in transcriptional activation mediated by EID1 was not accompanied by a significant change in the characteristic ligand-independent repression mediated by TRβ1 and was, instead, possibly due to a reduction in p300 activity. This prompted us to study the influence of EID1 on other co-activator proteins with HAT activities, which are recruited to the promoters of genes influenced by TR. It is known that mutual interactions among these co-activator proteins produces complete co-activation due to interactions with general transcription factors and enzymatic changes in the nucleosomes (Ito and Roeder, 2002). Therefore, in order to understand the role of EID1 on co-activators that collaborate with ligand-bound TR, we co-transfected EID1 with plasmid expression vectors containing SRC-1 and TRAM1. In both cases, we observed that the co-transfection of EID1 with p160 proteins did not significantly reduce the strong activation mediated by these co-activators. However, EID1 significantly reduced the remarkable activation mediated by p300 and TRBP co-activator proteins (Figure 2B).

Considering that the EID1-mediated reduction in TRβ1 activation might be a consequence of a direct interaction with the thyroid receptor that obstructs key sites for

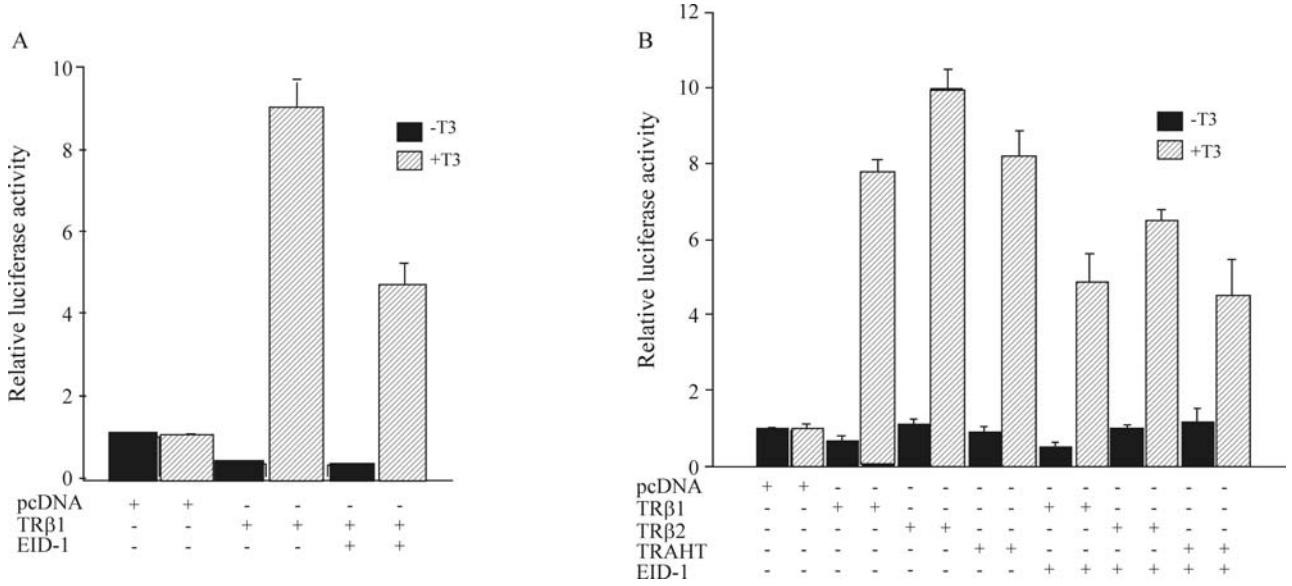

Figure 1 - The early region 1A (E1A)-like inhibitor of differentiation 1 (EID1) reduces ligand-bound thyroid hormone receptor (TR)-mediated transcription of DR-4 reporter plasmids. (A) CV-1 cells were transiently transfected with a luciferase reporter plasmid containing DR-4 natural binding sites for TRβ1 fused to thymidine kinase (DR4-TK-Luc) and plasmid expression vectors containing TRβ1 and EID1 complete cDNAs. After 24 h, thyroid hormone stripped medium was changed and 1 x 10^{-6} M 3,5,3'-L-triiodothyronine (T_3) was added to some wells. Cells were harvested 48 h after transfection and luciferase and β-gal assays were carried out. Luciferase activity was evaluated as relative fold increase relative to the empty vector. The EID1 protein reduced the luciferase activity of TRβ1 by about 50% in the presence of T_3, without significant changes in the absence of ligand. (B) Expression plasmids encoding TRβ1, TRβ2 or TRβ1 mutant (TRAHT), were separately co-transfected with DR4-containing reporters and the CMV-β-Gal control plasmid. Cells were grown in the presence or absence of 1 x 10^{-6} M T_3 for 24 h and analyzed for luciferase activity. When EID1 was co-transfected with each TR there was a significant reduction in T_3 dependent transactivation in all cases.

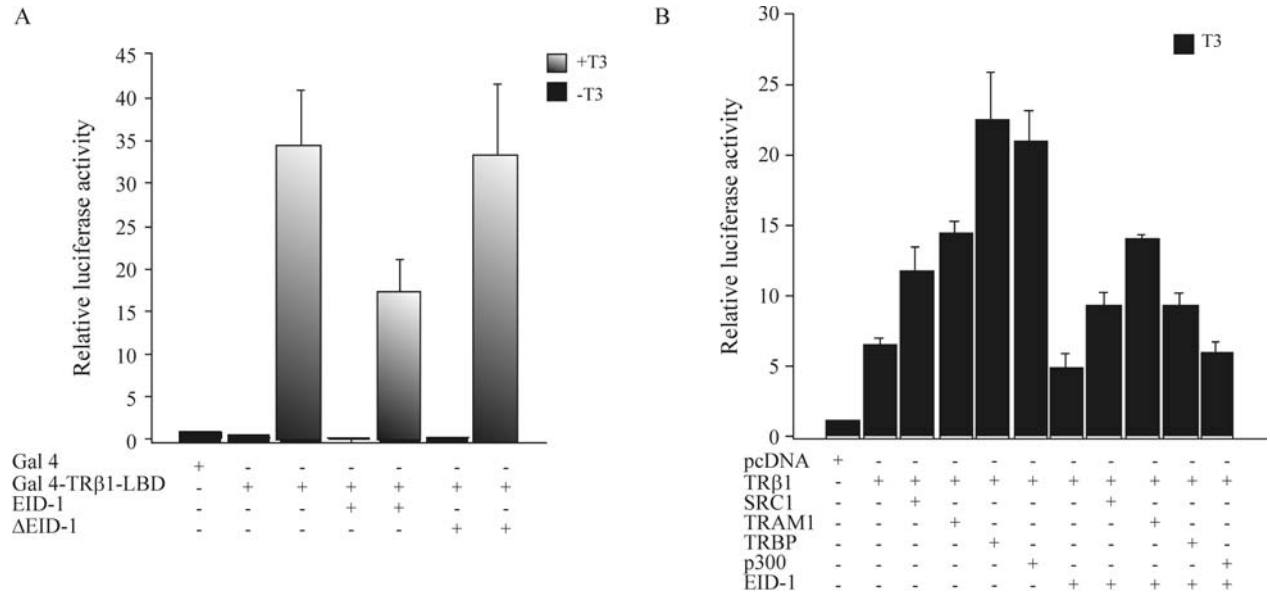

Figure 2 - The early region 1A (E1A)-like inhibitor of differentiation 1 (EID1) reduces transcriptional activity through inhibition of p300 activity. (A) U2OS cells were transiently transfected with a β-gal control reporter plasmid, a luciferase reporter plasmid containing 5x UAS-TK containing multiple Gal4 biding sites fused to thymidine kinase and plasmids encoding the thyroid receptor ligand biding domain (plasmid Gal4-TRβ1-LBD), EID1 or the EID1 mutant (1-157Δ53Δ92) which cannot bind to p300. After 24 h, thyroid hormone stripped medium was changed and 1 x 10^{-6} M 3,5,3'-L-triiodothyronine (T_3) was added to some wells. After 48 h transfection, cell extracts were prepared and luciferase activity, corrected for β-gal activity, was expressed as fold activity relative to the cells producing Gal4 alone. (B) Reporter plasmids encoding DR4 (natural thyroid hormone response elements (TREs) for TRβ1 biding sites) fused to thymidine kinase-luciferase (TK-Luc) reporters were transiently transfected into CV-1 and U2OS cells. Different expression vectors fused to cDNAs encoding p300, SRC-1, TRAM1 and TRBP1 were co-transfected with or without EID1. After 24 h, stripped medium was changed, and 1 x 10^{-6} M T_3 was added to all wells. Cells extracts were prepared after 48 h and luciferase activity, corrected for β-gal activity, was expressed as fold activity relative to cells producing pcDNA as a control plasmid.

co-activator interaction, we evaluated a possible interaction using mammalian two-hybrid studies. There was a significant increase in Luciferase activity when Gal4-N-CoR was co-transfected with VP16-TRβ1-LBD in the absence of T_3 and also when Gal4-p300 and Gal-4-pRB were co-transfected with VP16-EID1 (Figure 3) but there was not increase in luciferase activity when Gal4-TR TRβ1-LBD was co-transfected with VP16-EID1, indicating that these proteins probably do not have a direct interaction.

In this study, we have established the effect of EID1 protein on TRβ1-mediated transcriptional activation. Our results show that EID1 reduces T_3 activation of thyroid hormone receptors without affecting TRβ1-mediated ligand-independent transcriptional repression. An important question in transcriptional regulation by transcription factors, which are regulated by a ligand, is how they can either activate or silence the promoters of target genes. Binding of T_3 results in a conformational change in the TR (Glass and Rosenfeld, 2000), which facilitates the dissociation of TR/co-repressor complexes such as N-CoR and SMRT and the recruitment of co-activating proteins with intrinsic HAT activity such as p160 co-activators and p300/CBP.

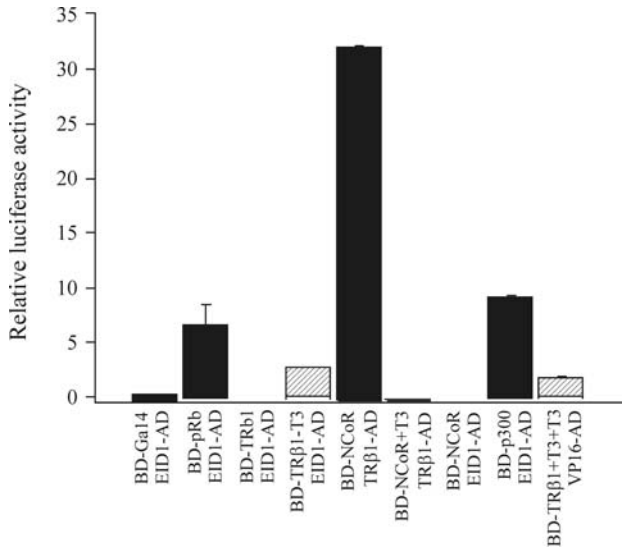

Figure 3 - The early region 1A (E1A)-like inhibitor of differentiation 1 (EID1) cannot bind to TRβ1. Mammalian two-hybrid assays were performed using U2OS cells co-transfected with β-Gal control reporter plasmid (a luciferase reporter plasmid containing thymidine kinase-luciferase (UAS-TK-Luc) and five Gal4 DNA-binding sites). Column 1 = Gal4-BD co-transfected with VP16-EID1; column 2 = Gal4-pRB co-transfected with VP16-EID1; column 3 = Gal4-TRβ1-LBD co-transfected with VP16-EID1; column 4 = Gal4-TRβ1-LBD co-transfected with VP16-EID1 in the presence of 1 x 10^{-6} M 3,5,3'-L-triiodothyronine (T_3); column 5 = Gal4-NCoR co-transfected with VP16-TRβ1; column 6 = Gal4-NCoR co-transfected with VP16-TRβ1 in the presence of 1 x 10^{-6} T_3; column 7 = Gal4-NCoR co-transfected with VP16-EID1; column 8 = Gal4-p300 co-transfected with VP16-EID1; column 9 = Gal4-TRβ1-LBD co-transfected with VP16 only. Cell extracts were prepared after 48 h, and luciferase activity, corrected for β-gal activity, was expressed as fold activity relative to cells producing Gal4-empty as a control plasmid.

The most probable effect of EID1 on TRβ1 is a reduction in the activity of co-activators, since we did not observe any effect of EID1 in the absence of T_3. This is consistent with our results and those of previous studies (Bavner *et al.*, 2005). To determine if the EID1-mediated reduction in TRβ1 activation might be due to inhibition caused by the co-repressor complex, we performed studies with thyroid receptors that could not interact with the co-repressor complex. The TRβ2 protein is a thyroid hormone receptor isoform derived from the same gene as TRβ1, but with an alternative splicing pattern that produces additional N-terminal amino acids (Yen, 2001). These receptors are expressed primarily in the hypothalamus and pituitary gland, and the additional amino terminus of TRβ2 may block the recruitment of components of the co-repressor complex (Yang *et al.*, 1999). The TRβ1 mutant (TRAHT) contains three amino acid substitutions in the hinge region of TRβ1 and has a decreased affinity for the co-repressor complex (Lizcano *et al.*, 2001). However, EID1 still reduced ligand-dependent TR activity when co-transfection studies were performed with TRβ2 and TRAHT receptors (Fig. 1B). These results preclude the possibility of a dual inhibitory mechanism, which would include the recruitment of co-repressor complex and might explain the effect of EID1 on TRs.

The results of transfection studies with EID1 showed only a partial reduction in TRβ1 activation, which was probably a consequence of interference with a specific co-activator. This effect was not seen after co-transfection with the ΔEID1 mutant, which cannot interact with p300, suggesting that EID1 might block the activity of this co-activator. The p300 protein has been characterized as a binding partner of E1A viral protein, and subsequently, as a histone acetylase (Chan and La Thangue, 2001). Functionally, p300 may also act as a bridge between the TR transcriptional complex and general transcription factors (Vo and Goodman, 2001). The EID1 protein has been recognized as a functional homolog of the viral protein E1A, with both proteins having the capacity to block p300 co-activation activity (Chakvarti *et al.*, 1999). However, while E1A is a potent co-activator of TR-mediated transcription (Wahlstrom *et al.*, 1999), we observed that EID1 acted as an inhibitor of T_3-dependent transactivation. The co-activation mediated by E1A requires a direct interaction with the nuclear receptor but in our mammalian two-hybrid studies we did not observe an interaction between EID1 and TRβ1 (Figure 3).

Our data shows that EID1 reduces the activity of p300 and TRBP but has practically no effect on the function of p160 co-activator proteins (Figure 2B), which can bind directly to TR and have HAT activity. Given that the TRBP co-activator shows no HAT activity, its activation requires the presence of p300 as an integrator protein (Mahajan and Samuels, 2005). Therefore, we suggest that EID1 not only

acts as an inhibitor of HAT activity but also blocks the presence of p300 in the activation complex. Still, it is possible that histone acetylation is necessary for TRBP-mediated contributions to transcription (Huang *et al.*, 2003). Some studies have shown that EID1 may bind to the small heterodimer partner (SHP), an atypical orphan nuclear receptor that lacks a DNA-binding domain and is responsible for reducing the activation of some nuclear receptors (Bavner *et al.*, 2002; Park *et al.*, 2004). The SHP protein binds to the C-terminal regions that harbor activation function 2 (AF2) in ligand-bound TRs and blocks the recruitment of co-activator proteins in the presence of ligand. Because we observed that the reduction in TR function occurred mainly through inhibition of CBP/p300 activity, without a major effect on p160 co-activator proteins, SHP probably did not play a major role in EID1-mediated inhibition of ligand-bound TR transactivation activity.

In conclusion, EID1 may reduce the activity of ligand-bound thyroid hormone receptors through inhibition of p300 activity. It is possible that EID1 block p300 co-activators from binding to the promoter regions of target genes and may play an important role in thyroid receptor activity in muscle an other tissues. EID1 might have and important role in the differentiation of tissues associate with energy expenditure and a target for overweight and obesity drugs.

Acknowledgments

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