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EID3 is a novel EID family member and an inhibitor of CBP-dependent co-activation

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ABSTRACT

EID1 (E1A-like inhibitor of differentiation 1) functions as an inhibitor of nuclear receptor-dependent gene transcription by directly binding to co-regulators. Alternative targets include the co-repressor small heterodimer partner (SHP, NR0B2) and the co-activators CBP/p300, indicating that EID1 utilizes different inhibitory strategies. Recently, EID2 was characterized as an inhibitor of muscle differentiation and as an antagonist of both CBP/p300 and HDACs. Here, we describe a third family member designated EID3 that is highly expressed in testis and shows homology to a region of EID1 implicated in binding to CBP/p300. We demonstrate that EID3 acts as a potent inhibitor of nuclear receptor transcriptional activity by a mechanism that is independent of direct interactions with nuclear receptors, including SHP. Furthermore, EID3 directly binds to and blocks the SRC-1 interacting domain of CBP, which has been implicated to act as the interaction surface for nuclear receptor co-activators. Consistent with this idea, EID3 prevents recruitment of CBP to a natural nuclear receptor-regulated promoter. Our study suggests that EID-family members EID3 and EID1 act as inhibitors of CBP/p300-dependent transcription in a tissue-specific manner.

INTRODUCTION

CBP and p300 are highly related proteins that participate in a variety of cellular functions (1). They serve as transcriptional co-activators for nearly every gene-specific *trans*-activator, participating in multiple regulatory networks, and communicating with other co-activators and multi-protein complexes (2). Therefore, CBP and p300 are also called co-integrators that are involved in multiple biological processes that affect cell growth, transformation and development. The adenoviral transforming protein E1A is one of the most studied viral oncoproteins. It targets both CBP and p300; the latter was originally identified through its interaction with E1A (1). The reprogramming activity mediated by E1A and the host cell response to this reprogramming lead to transformation, growth arrest or apoptosis. E1A mediates the repression of CBP/p300-dependent *trans*-activation by sequestration of CBP/p300, TBP and p/CAF (3).

Recent work has indicated the existence of cellular proteins that share intriguing features with E1A. The first of these proteins, designated EID1 (E1A-like inhibitor of differentiation 1), was cloned on the basis of interactions with the retinoblastoma tumour suppressor protein and was subsequently characterized as inhibitor of CBP/p300-dependent functions on differentiation (4,5). We identified EID1 as cofactor of the orphan nuclear receptor SHP (small heterodimer partner, NR0B2) and demonstrated that EID1 inhibits genes regulated by a variety of nuclear receptors (NRs) (6). EID2 was cloned as the second member of this family and was shown to act as an inhibitor of gene expression. Interestingly, it has been suggested that EID2 inhibition is a combination of two mechanisms, binding to CBP/p300 (7) or recruiting histone deacetylases (8).

Here, we report the identification of a third member of the EID family. EID3 is specifically expressed in testis and, at the sub-cellular level, localized to both nucleus and cytoplasm. We demonstrate that EID3 inhibits gene expression mediated by NRs, most probably by interacting with the SRC-1 interacting domain (SID) of CBP that has been implicated in co-activator assembly, thus preventing recruitment of CBP to a natural NR-regulated promoter. Our study suggests that interference with CBP/p300 function represents a common and crucial feature of at least these two members of the EID family.

MATERIALS AND METHODS

Plasmids

All human EID3 and human EID1 expression plasmids described in this study were generated by PCR-based cloning

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using appropriate primers and verified by sequencing. Full-length cDNAs were originally obtained by PCR from human testis cDNA (EID3) and human breast cancer cells MCF-7 (EID1) and cloned into pGEM-T-vectors (Promega). Primers were as follows: EID3 5′-CCCCGGTAGCGAGTACGCGG-CGAAGTAGGCGGCGGCGG-3′ and 5′-GTGCTTCAGTT-TACATTTTACTCTCCATCTAACTTC-3′; EID1 5′-CGCAG-ATCTGTCTTGCTGGAAGCTTTTTCCTAGAG-3′ and 5′-CCCTCCTCAAGTAGTTTCCTCCTCTTTTAACAGCAT-3′.

pSG5-derivatives were made by cloning EcoRI-fragments from pGEM-T-EID3 or pGEM-T-EID1. pFLAG derivatives were generated by PCR amplification using the 5′ primers 5′-TTTGAATTCATGAAGATGGATGTGTCAGTGAGGGC-CGCGGGCTGC-3′ (EID3) and 5′-TTTGAATTCATGTCG-GAAATGGCT-3′ (EID1) and pGEM-T vector primers, cut with EcoRI and inserted into pcDNA-FLAG vector. The pcDNA-FLAG vector was created using oligos encoding the FLAG tag ligated into pcDNA3 (Invitrogen). pSG5-GAL4-mSF-1-N1 (amino acids 98–462) was cloned by PCR using Steroidogenic Factor-1 (SF-1) cDNA as a template into pSG5-GAL4 using EcoRI. GAL4-mCBP C1 (9), UAS-tk-luc (10), pCMVhGR and MMTV-luc reporter plasmid (11), pSG5-hERa (12), 3xERE-TATA-luc (13), pSG5-mTIF2 (6), pGEX-CBP 2058–2130 (SID) and pGEX-CBP 2058–2130 2103 K→P (14) have been described previously.

Expression analysis of mRNA and protein

Human tissue northern blots [2 μg poly(A)$^+$ RNA per lane] were sequentially hybridized with radioactively labelled cDNA for human EID3 and β-actin (for control) according to the manufacturer's protocol (Clontech). The probes were labelled using Rediprime™^{II} Random Prime Labelling System (Amersham Biosciences), and thereafter purified from unincorporated radiolabelled nucleotides using Microspin™S-200 HR columns (Amersham Pharmacia Biotech). *In vitro* transcribed and translated proteins were produced using [^{35}S]methionine (Amersham) according to the manufacturer's instructions (Promega). The products were then analysed by SDS–PAGE and exposed to an X-ray film.

Confocal microscopy and immunocytochemistry

COS-7 cells were seeded as 250 000 cells/well on cover slips in 6-well plates and transfected as below. Cells were fixed in 3% paraformaldehyde in 5% sucrose/phosphate-buffered saline (PBS), permeabilized with PBS/Tween-20 (0.1%) and blocked with 5% goat serum (Jackson ImmunoResearch). Rabbit polyclonal anti-FLAG (Sigma) were detected with appropriate secondary antibodies conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch). Nuclei were stained using 2 μM 7-aminoactinomycin D (7-AAD) (Molecular Probes). To block nuclear export, 5 nM leptomycin B (LMB) (Sigma) was added 5 h before fixation. Sub-cellular images were determined using a TCS SP multiband confocal imaging system (Leica).

Mammalian cell culture and transfections

HuH7 human hepatoma cells were grown in DMEM (GIBCO, Invitrogen Corporation) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, Invitrogen Corporation) and 5% L-glutamine (GIBCO, Invitrogen Corporation). COS-7 green monkey kidney cells were grown in DMEM (GIBCO, Invitrogen Corporation) supplemented with 10% heat-inactivated FBS (GIBCO, Invitrogen Corporation) and 5% L-glutamine (GIBCO, Invitrogen Corporation). Cells were grown without antibiotics and negatively tested for mycoplasma. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Twenty-four hours before transfection, 30 000 cells/well of HuH7 was seeded in 24-well plates and for whole-cell extracts for western blot, 250 000 cells/well were seeded in 6-well plates. Luciferase assay—cell extracts were analysed for luciferase activity as described previously (6).

Co-immunoprecipitations

HuH7 cells were transfected with indicated plasmids and whole-cell extracts were prepared after 48 h post-transfection in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40 and 5% glycerol containing complete protease inhibitor cocktail tablet (Roche). First, 100 μl extracts were incubated for 2 h with rabbit polyclonal GAL4-DBD antibody (sc-577; Santa Cruz Biotechnology) at 4°C in IP-T150 buffer containing 50 mM Tris–HCl, pH. 8.0, 150 mM NaCl, 0.2% NP-40 and 5% glycerol, then 50 μl of a 50% mixture of protein A/G agarose (Upstate) was added and the incubation continued overnight at +4°C. After three washes in IP-T150 buffer, the precipitates were analysed by SDS–PAGE followed by western blots using mouse monoclonal anti-FLAG M5 antibody (Sigma) at a dilution of 1:10 000.

Glutathione *S*-transferase (GST) pull-down assays

The GST pull-down assays were performed as described previously (15,16). Briefly, [^{35}S]labelled EID1, EID3 and TIF-2 (Redivue, Amersham) were generated by *in vitro* translation using TNT reticulocyte lysate system (Promega). GST fused, CBP amino acids 2058–2130, CBP amino acids 2058–2130 2103 K→P or GST alone were expressed in BL21 (DE3) pLys *Escherichia coli* cells and immobilized on glutathione Sepharose beads (AP Biotech), following a 2 h incubation with the [^{35}S]labelled proteins at 4°C, the complexes were washed five times under stringent condition (150 mN NaCl). Following the washing steps, the complexes were resolved by denaturing PAGE (12% for EID3 and EID1 and 8% for TIF-2) and autoradiographed. Wash buffer: 20 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X and 1× protease inhibitor cocktail (Roche). Binding buffer: wash buffer + 1.5% BSA.

Chromatin immunoprecipitation (ChIP) assays

MCF-7 cells were seeded in 150 mm dishes and grown for 3 days in phenol red-free DMEM supplemented with 5% DCC-FCS. Ligands dissolved in dimethyl sulfoxide (DMSO) were added for the indicated times, and protein–DNA complexes were crosslinked with 1% formaldehyde for 10 min. Crosslinking was quenched by adding 125 mM glycine and cells were washed with PBS, harvested and resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.1% sodium deoxycholate) containing protease inhibitors (Roche,

Mannheim, Germany) and sonicated 10 times for 10 s. The soluble chromatin was collected by centrifugation, and an aliquot of the chromatin was put aside and represented the input fraction. The supernatants were incubated with 30 µl protein A/G Sepharose (50% slurry; Pharmacia) under gentle agitation for 2 h at 4°C. The supernatant was transferred to a new microcentrifuge tube, and 0.5–1 µg of antibody was added and incubated overnight at 4°C. Protein A/G Sepharose (20 µl of a 50% slurry) was then added and incubated for 1.5 h. The pellets were successively washed for 10 min in 1 ml buffer 1 (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.1% SDS), 1 ml buffer 2 (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.1% SDS), 1 ml LiCl buffer (20 mM Tris–HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% NP-40 and 1% sodium deoxycholate) and 2× 1 ml TE (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA). Protein–DNA complexes were eluted in 120 µl elution buffer (TE and 1% SDS) for 30 min, and the crosslinks were reversed by overnight incubation at 65°C. DNA was purified using a PCR purification kit (Qiagen) and eluted in 50 µl. Antibodies used were rabbit normal IgG (Santa Cruz), anti-oestrogen receptor α (anti-ERα) (H-184; Santa Cruz), anti-FLAG M5 (Sigma), anti-CBP (A-22; Santa Cruz). For real-time PCR, SYBR green qPCR supermix UDG (Invitrogen) was used with primer pairs 5′-GGCCATCTCTCACTATGAATCACT-3′ and 5′-GGATTTGCTGATAGACAGAGACGA-3′ for presenilin 2 (pS2). To calculate the enrichment differences among the receptor and cofactors using real-time PCR, E2 treatments were normalized to the DMSO treatments. The enrichment level for each of the factors during DMSO treatment was set to 1 and the enrichment levels after E2 treatment of the ER and cofactors were compared with it. Since the DMSO treatment was carried out for the same amount of time as the E2 treatment, we defined these as time-matched and solvent-normalized values.

RESULTS

Cloning of a third member of the EID family

NCBI BLAST databases were searched with the EID1 open reading frame (ORF) to identify potential functional homologues and revealed the existence of several unpublished ORFs. In this study, we focused on a predicted human cDNA with an ORF encoding 333 amino acids, which can be accessed through NCBI Protein Database under NCBI accession no. AAH27612. The EID3 gene is located on chromosome 12 (http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html). A mouse homologue with accession number NM_025499 encoding 310 amino acids was also found, located on chromosome 10 (http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html). Both the human and mouse genes were located in the 5′ end of the thioredoxin reductase 1 gene, in the intron between thioredoxin reductase 1 splice variants 2 and 3. The human clone showed similarity in its central/N-terminal part (amino acids 56–92; 73% similarity and amino acids 106–146; 66% similarity) to the C-terminus of EID1 (amino acids 114–147 and amino acids 148–184) (Figure 1). Unlike EID1, EID3 has a conserved C-terminus with unknown function referred to as COG5125 domain (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). We subsequently cloned the human full-length cDNA from testis by PCR and designated it human EID3 as the third putative member of the EID family.

EID3 is expressed in testis and represents a 39 kDa intracellular shuttling protein

In order to determine the tissue distribution of EID3, multiple tissue northern blots containing RNA from 16 different tissues were analysed and revealed an ∼2.0 kb transcript highly expressed in testis (Figure 1B). To characterize the EID3 ORF, we produced [^{35}S]labelled, *in vitro* transcribed and translated protein to ascertain the molecular weight. As shown in Figure 2B, EID3 has a size of 39 kDa consistent with the predicted molecular weight. We next studied the intracellular localization of EID3 in comparison with EID1. FLAG-tagged EID3 was found to be localized in the cytoplasm (66%) and in the nucleus (34%) of transfected COS-7 cells (Figure 2A). However, unlike EID1 (6), the intracellular distribution of EID3 was not affected by the presence of the nuclear export inhibitor LMB, indicating the intracellular distribution of EID3 is regulated by mechanisms independent of CRM1-dependent nuclear export. Further studies also show that increasing amounts of transfected over expressed FLAG-EID3 result in a shift in localization towards a more cytoplasmic and less nuclear distribution, and that it is stable according to western blot (data not shown).

EID3 inhibits nuclear receptor-dependent transcriptional activity

To investigate whether EID3 had inhibitory functions on transcriptional activity comparable with its relatives EID1 (4–6) and EID2 (7,8), we transiently co-transfected EID3, and for comparison EID1, with NRs known to be expressed in testis. SF-1 is expressed in testis and has a role in regulating the steroid hydroxylases (17). The glucocorticoid receptor (GR) is expressed in several cell types in testis and inhibits steroidogenesis (18). The oestrogen receptor α (ERα) is expressed in testis and has a role in the expression of oestrogen-responsive genes (19). The effects of androgens are mediated through the androgen receptor (AR) to regulate genes involved in primarily male sexual differentiation and maturation, and the maintenance of spermatogenesis (20). We observed that EID3 potently inhibited the transcriptional activity of SF-1, GR, ERα (Figure 3) and AR (data not shown). Furthermore, we show by western blot that the expression of the NRs is not altered by the presence of over expressed EID1 and EID3 (Figure 3). In addition, sub-cellular localization studies suggest that FLAG-EID3 preferentially accumulates in the cell nucleus in cells co-transfected with the NRs, which may be due to the formation of a complex between over expressed NR and endogenous CBP/p300 (data not shown).

CBP-dependent transcriptional activation is inhibited by EID3

In order to elucidate if EID3 could inhibit CBP-dependent transcriptional activity similar to the EID1 inhibition of p300 (5), HuH7 cells were co-transfected with a plasmid containing the GAL4 DNA-binding domain fused to the C-terminus of

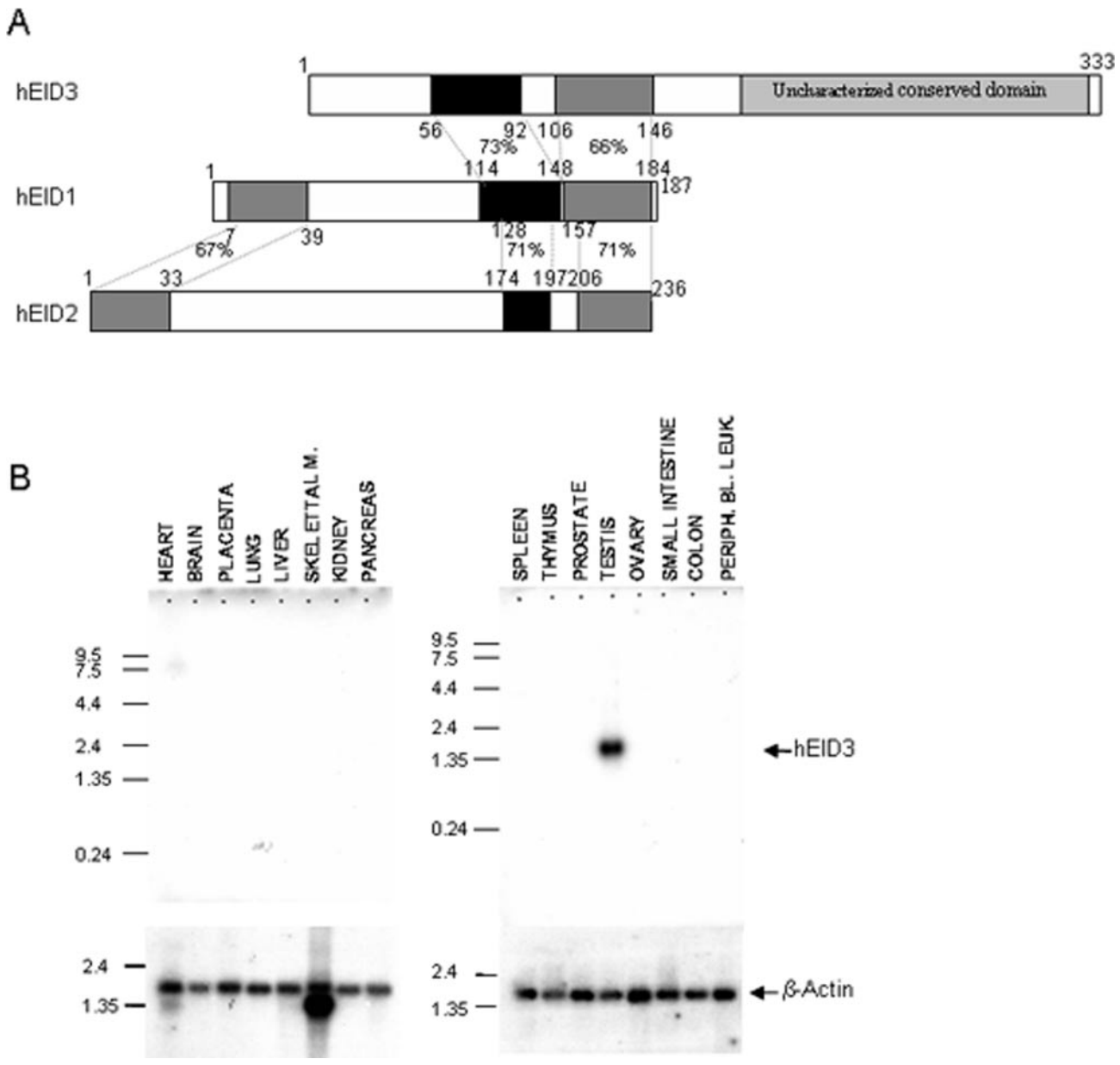

Figure 1. Primary structure and expression analysis of EID3. (**A**) Schematic comparisons of the EID family members 1, 2 and 3, conserved domains are coloured grey and black. (**B**) Tissue distribution of EID3 mRNA. Human multiple tissue northern blots were probed with radiolabelled EID3 or β-actin cDNA.

CBP (amino acids 1678–2441), a reporter plasmid containing GAL4 DNA-binding sites, and a plasmid expressing wild-type EID1 or EID3. As shown in Figure 4A, EID3 had a profound inhibitory effect on the transcriptional activity of CBP, indicating functional similarities to EID1. Furthermore, western blot shows that the expression of GAL4-CBP C is not altered by the presence of over expressed EID1 and EID3 (Figure 4B).

EID3 binds to the C-terminus of CBP *in vivo* and the CBP SID *in vitro*

Considering that the EID1 C-terminus is homologous to an EID3 domain encompassing amino acids 106–146 and that this domain has been implicated in binding to the C-terminus of CBP/p300 (4–6), we wanted to investigate whether EID3 could interact with the C-terminus of CBP *in vivo*. To test for interactions of these proteins in mammalian cells, HuH7 cells were co-transfected with FLAG-EID3 and FLAG-EID1, and GAL4-CBP C-terminus. CBP complexes were immuno-precipitated using anti-GAL-DBD antibodies, resolved on an SDS–PAGE gel and analysed for the presence of EID3 in the immune-precipitates using anti-FLAG antibodies (Figure 4C). Both EID1 and EID3 were found to be co-immunoprecipitated with the C-terminus of CBP. GST pull-down assays showed that both EID1 and EID3 interacted with the so-called SID of CBP (Figure 4D), indicating a functional conservation of these interactions within the EID family. In order to demonstrate the

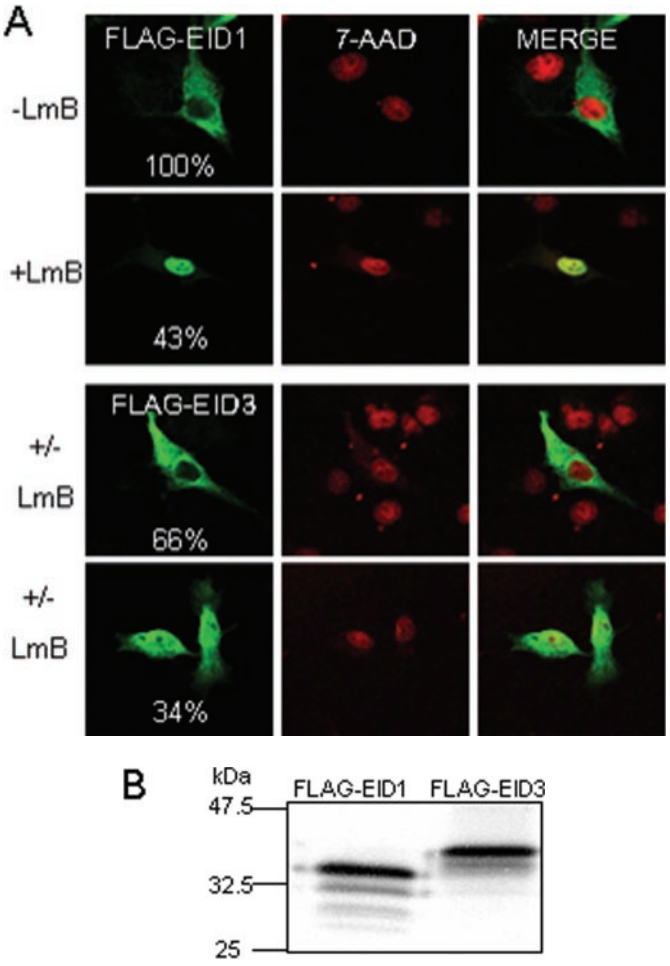

Figure 2. Sub-cellular distribution and protein analysis of EID3. (**A**) Intracellular localization of EID1 and EID3. FLAG-tagged EID1 and EID3 were expressed in COS-7 cells and analysed by indirect immunofluorescence using FLAG antibody (green) in the absence or presence of LMB (5 nM for 5 h). Nuclei were stained with 7-aminoactinomycin D (7-AAD) (red). More than 50 cells were studied and the experiment was independently reproduced at least three times. (**B**) *In vitro* transcribed and translated [^{35}S]methionine-labelled EID1 and EID3 are shown.

competition of EID3 and the p160 co-activator, TIF-2 transient transfection experiments were performed. These results show that EID3 completely abolishes the TIF-2-induced *trans*-activity (Figure 4E).

EID3 and EID1 blocks recruitment of CBP to the endogenous pS2 promoter

The above results suggested that EID3 inhibits NR transcription by means of interference with CBP-dependent co-activation. To further dissect these mechanisms, we performed ChIP assays using the MCF-7 breast cancer cell line, which expresses endogenous ERα and CBP (21). Cells were treated with 10 nM oestradiol (E_2) for 1 h before harvest, and then proteins were crosslinked to chromatin and immunoprecipitated with antibodies against ERα, CBP or FLAG. The ChIP analysis was performed with primers specific for the pS2 promoter, an established oestrogen-responsive gene. The amplified input DNA did not significantly differ among the samples (data not shown). The immunoprecipitated DNA was amplified with primers that recognize the pS2 mRNA located 2000 bp upstream of the transcriptional start site; no amplicon was detected by real-time PCR after 45 cycles, suggesting that the ER and CBP do not bind to that region of the promoter (data not shown).

As shown in Figure 5, EID1 and EID3 inhibit the recruitment of CBP to the pS2 promoter. However, when comparing the occupancy of ERα at the pS2 promoter in the FLAG transfected cells, there is an ~3–4 times higher occupancy than in the FLAG-EID1 and FLAG-EID3 transfected cells. This may be the result of the absence of CBP owing to binding of EID1 and EID3, which might be necessary for the proper assembly of cofactors at the ERα-bound pS2 promoter (21,22). There is no occupancy of EID1 and EID3 at the pS2 promoter, indicating that they might bind to CBP before it becomes recruited to the promoter.

DISCUSSION

In this study, we have identified EID3, a third previously uncharacterized EID family member. The exclusive expression of EID3 in testis contrasts to the more ubiquitous expression pattern of the other two members (4–8), indicating that EID3 might have testis-specific functions. However, unlike EID1, EID3 has an extended C-terminal domain of unknown function.

EID3 shows homology to a region of EID1 (Figure 1A) that is implicated in binding to CBP/p300 (10). As shown in Figure 4A, EID3 had a profound inhibitory effect on the transcriptional activity of CBP, indicating functional similarities to EID1 (4,5). This is most likely due to the binding of EID3 with the C-terminus of CBP (amino acids 1678–2441) observed in co-immunoprecipitation assays (Figure 4C), which indicates a functional conservation of these interactions within the EID family.

The more potent inhibition of NR transcriptional activity mediated by EID3 compared with EID1 (Figure 3A, C and E) could be due to the fact that a larger fraction of EID3 is primarily located in the nucleus (Figure 2A). Furthermore, the inhibition seems to be independent of direct interaction of EID3 with the tested NRs, as judged from mammalian two-hybrid interaction assays and sub-cellular co-localization studies (data not shown). These data suggest that EID3 functions as a co-inhibitor of NRs, and probably other CBP/p300-dependent transcription factors, and thus could be involved in the transcriptional control of testicular tissue homeostasis. Moreover, transcriptional activation by NRs, including SF-1 (17), ER, GR (25) and AR (20), requires the action of CBP/p300.

A sequence within the C-terminus, termed the SID, has been mapped to amino acids 2058–2130 of CBP (26). The SID has also been reported to interact with other nuclear factors in addition to the p160 proteins, including IRF-3, E1A, p53, Tax, Ets-2 and KSHV IRF-1 (27). Proteins that bind to the SID have recently been shown to 'share a sequence motif similar to an amphipathic α-helix in the AD1 domain of the p160s' (14). Interestingly, we have localized putative amphipathic α-helices in EID1 (amino acids 159–167) and EID3 (amino acids 117–125) within the conserved EID regions. Moreover, EID1 and EID3 interacts with the SID

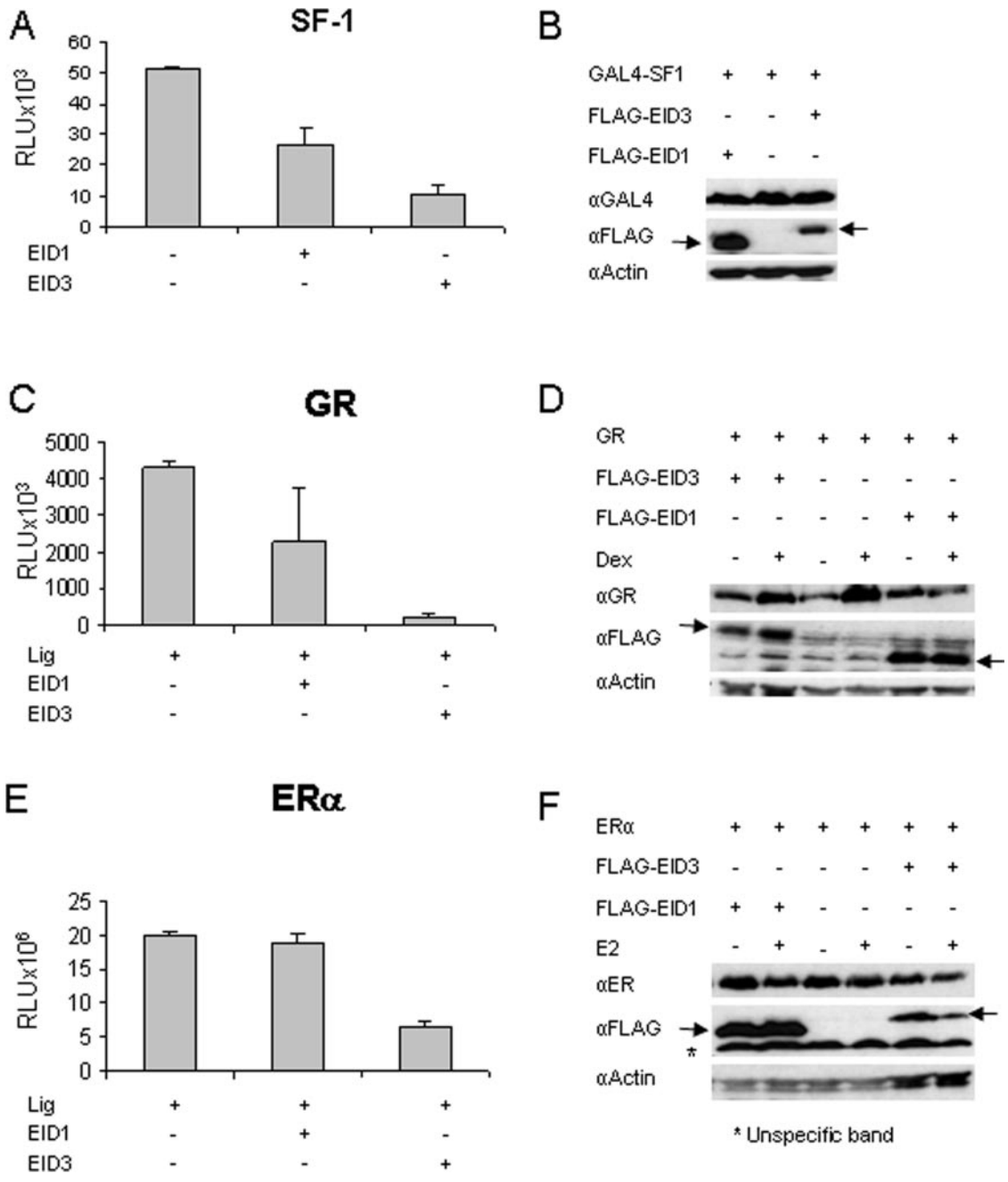

Figure 3. Analysis of NR inhibition. HuH7 cells were grown in media containing phenol red and untreated serum. The cells were transiently transfected with plasmids encoding EID1 or EID3 and luciferase reporter assays were performed. Western blots were performed to see whether the NR expression is not altered by over expressed EID1 and EID3 (indicated with arrows). (**A**) A luciferase reporter plasmid containing GAL4-DBD binding sites, and a plasmid expressing GAL4-SF-1. (**B**) Western blot using GAL4-DBD antibody to detect GAL4-SF-1 and a FLAG antibody to detect FLAG-EID1 and FLAG-EID3. (**C**) A luciferase reporter plasmid containing the MMTV promoter, and a plasmid encoding GR, and ligand (10 nM dexamethasone) as indicated. (**D**) Western blot using GR antibody to detect GR and a FLAG antibody to detect FLAG-EID1 and FLAG-EID3. (**E**) A luciferase reporter plasmid containing the ERE, and a plasmid encoding ERα and ligand (10 nM 17-β oestradiol) as indicated. (**F**) Western blot using ERα antibody to detect ERα and a FLAG antibody to detect FLAG-EID1 and FLAG-EID3.

of CBP (Figure 4D) possibly via these amphipathic α-helices, thereby competing with the p160s of the same surface. This might disrupt an NR/co-activator complex at the promoter, leading to a decrease in NR target gene expression as shown in the model (Figure 6). Consistent with this idea, we have demonstrated that EID1 and EID3 prevent recruitment of CBP to a natural NR-regulated promoter using ChIP assays (Figure 5). These results suggest that EID1 and EID3 interfere with the recruitment of CBP to the promoter-bound ERα/co-activator complex and demonstrate a functional interaction between EID1 and EID3 and CBP *in vivo*.

We propose a model where EID1 and EID3 act as inhibitory 'anti co-activators' that could be critically involved in the fine-tuning of transcription, by targeting the promiscuous co-activators CBP/p300 without interfering with the present transcription factor.

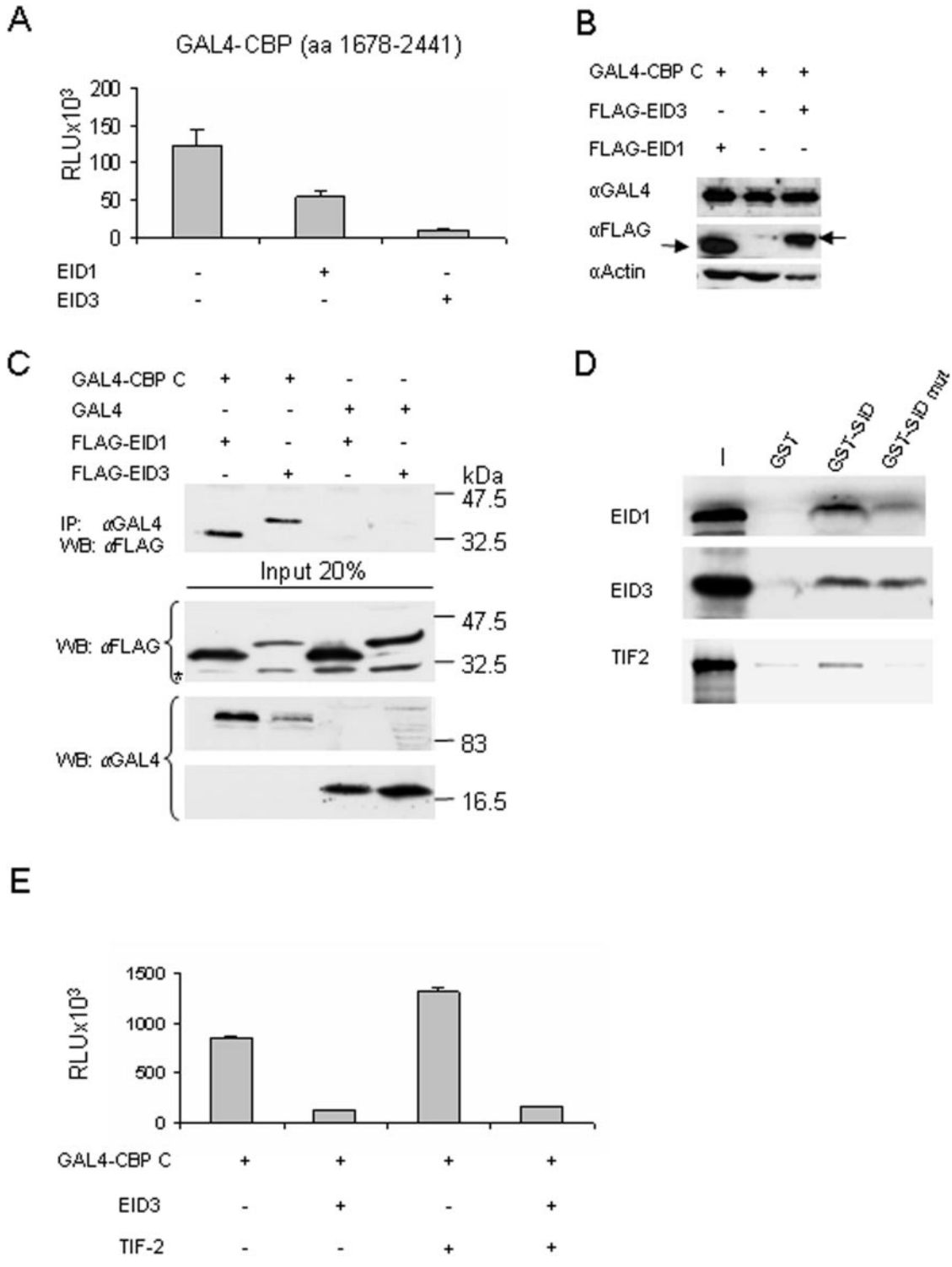

Figure 4. EID3 binds to the CBP SID and shows a functional interaction with the C-terminus of CBP. (**A**) HuH7 cells were transiently transfected with plasmids encoding EID1 or EID3, a luciferase reporter plasmid containing GAL4-DBD binding sites, and a plasmid encoding GAL4-CBP C (amino acids 1678–2441). (**B**) A western blot using GAL4 and FLAG antibodies show that GAL4-CBP C expression is not altered by over expressed EID1 and EID3. (**C**) Co-immunoprecipitation of exogenous CBP (amino acids 1678–2441) and FLAG tagged EID1 or EID3. Immune complexes from HuH7 whole-cell extracts were isolated using a GAL4-DBD antibody. Precipitates were analysed using a FLAG antibody (upper gel). The input corresponds to 20% of the material subjected to immunoprecipitation and was detected using a GAL4-DBD antibody and a FLAG antibody. Asterisk represents unspecific band. (**D**) GST pull-down assay using the CBP SID fused to GST as indicated together with *in vitro* translated EID1, EID3 and TIF-2 as positive control. I (input), GST-SID (CBP amino acids 2058–2130), GST-SID mut (CBP amino acids 2058–2130 K→P). (**E**) HuH7 cells were transiently transfected with plasmids encoding EID3 or TIF-2, a luciferase reporter plasmid containing GAL4-DBD binding sites, and a plasmid encoding GAL4-CBP (amino acids 1678–2441).

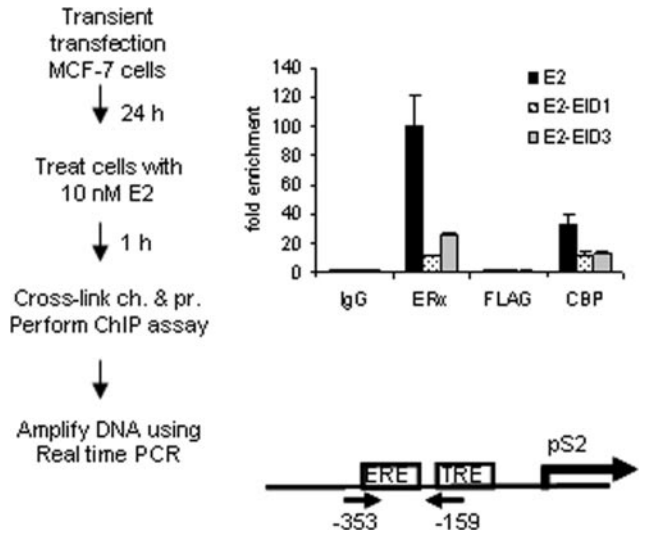

Figure 5. EID1 and EID3 block the recruitment of CBP to the ERα-bound pS2 promoter. MCF-7 cells cultured with oestradiol were transiently transfected with FLAG vector alone for control or with FLAG-EID1 and FLAG-EID3. Soluble chromatin was prepared and immunoprecipitated by using antibodies raised against rabbit IgG as negative control, EPα, CBP and FLAG. Immunoprecipitated DNA was PCR amplified with primers that span the –353 to –159 region of the pS2 promoter. Values were time-matched and normalized for the solvent (DMSO).

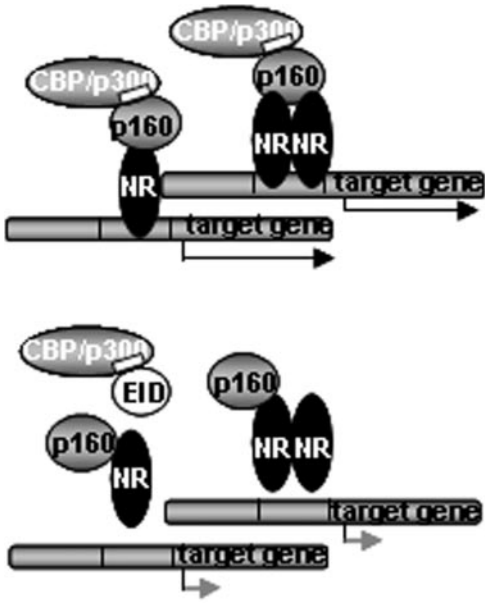

Figure 6. Schematic model of the action of EID3 and EID1 that bind to the CBP SID. The binding leads to a blocking of the interaction surface of the p160 co-activators leading to a reduced target gene transcriptional activity.

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Conflict of interest statement. None declared.

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