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Cells Degrade a Novel Inhibitor of Differentiation with E1A-Like Properties upon Exiting the Cell Cycle

SATOSHI MIYAKE,[1,2] WILLIAM R. SELLERS,[1] MICHAL SAFRAN,[1] XIAOTONG LI,[1] WENQING ZHAO,[3] STEVEN R. GROSSMAN,[3] JIANMIN GAN,[1] JAMES A. DECAPRIO,[1] PETER D. ADAMS,[4] AND WILLIAM G. KAELIN, JR.[1,2]*

Department of Adult Oncology,[1] Department of Cancer Biology,[3] and Howard Hughes Medical Institute,[2] Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115, and Department of Molecular Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111[4]

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Control of proliferation and differentiation by the retinoblastoma tumor suppressor protein (pRB) and related family members depends upon their interactions with key cellular substrates. Efforts to identify such cellular targets led to the isolation of a novel protein, EID-1 (for E1A-like inhibitor of differentiation 1). Here, we show that EID-1 is a potent inhibitor of differentiation and link this activity to its ability to inhibit p300 (and the highly related molecule, CREB-binding protein, or CBP) histone acetylation activity. EID-1 is rapidly degraded by the proteasome as cells exit the cell cycle. Ubiquitination of EID-1 requires an intact C-terminal region that is also necessary for stable binding to p300 and pRB, two proteins that bind to the ubiquitin ligase MDM2. A pRB variant that can bind to EID1, but not MDM2, stabilizes EID-1 in cells. Thus, EID-1 may act at a nodal point that couples cell cycle exit to the transcriptional activation of genes required for differentiation.

Tissue homeostasis requires the coordinate regulation of cell division, differentiation, and apoptosis. These fundamental processes are deregulated during malignant transformation. Cellular proliferation and differentiation are typically inversely related such that the most aggressive malignancies are characterized by a high rate of proliferation and absence of differentiation (anaplasia).

p300 (and the highly related molecule, CREB-binding protein [CBP]) and the retinoblastoma (RB) tumor suppressor protein (pRB) play critical roles in cell cycle control and in the induction or maintenance of differentiation (13, 20, 57, 63, 71). The importance of these molecules is underscored by the observation that biallelic inactivation of either p300, CBP, or pRB produces an embryonic lethal phenotype in mice (12, 34, 43, 75). In mice, haploinsufficiency of either p300 or CBP causes developmental abnormalities (65, 75). In humans, haploinsufficiency of CBP causes Rubinstein-Taybi syndrome, characterized by mental retardation, craniofacial abnormalities, and broad big toes and thumbs (20, 51).

p300 and CBP serve as transcriptional coactivators for a variety of transcription factors, including fate-determining proteins such as MyoD (17, 52, 54, 76). p300 and CBP possess histone acetylase (HAT) activity and can also recruit other HATs, such as PCAF and members of the SRC family of nuclear hormone receptor coactivators, to DNA (2, 7, 40, 48, 64, 74, 75). p300 and CBP respond to a variety of intracellular and extracellular signals and have been postulated to act as molecular switches between diverse signaling pathways (3, 10, 40, 50). Recently, p300 was also shown to serve as an adapter molecule that facilitates the ubiquitination of the p53 tumor suppressor protein by MDM2 (23). MDM2 was shown previously to function as an E3 ubiquitin ligase (30, 31).

Like p300 or CBP, pRB can both inhibit cell cycle progression and promote differentiation (15, 57, 71). The former activity correlates with its ability to repress transcription once bound to members of the E2F cell cycle regulatory transcription factor family (15, 39). The latter activity correlates with its ability to activate transcription in cooperation with transcription factors such as MyoD and C/EBP (9, 24, 47, 59). Several mechanisms for transcriptional repression by pRB have been proposed, including recruitment of histone deacetylase, binding to adjacent transcriptional activation domains, inhibition of TAF250, and alteration in DNA bending (39).

As was true for p300 and CBP, pRB can also bind to MDM2 (32, 73). The functional significance of MDM2 binding to pRB is not clear. When overproduced, MDM2 can block pRB-dependent inhibition of cell growth. On the other hand, overproduction of a C-terminal fragment of pRB that can bind to MDM2, but not to E2F, prevented wild-type pRB from promoting differentiation (72).

How pRB activates transcription and promotes differentiation is largely unknown. Here, we report the cloning of a putative pRB-binding protein called EID-1 (for E1A-like inhibitor of differentiation 1). Like E1A, this protein contains a canonical pRB-binding motif (LXCXE, where X is any amino acid), can bind to p300, and can inhibit differentiation. Intriguingly, stoichiometric binding to pRB and p300 was not required for EID-1 to block differentiation, suggesting that the observed effects of EID-1 were not due solely to sequestration of pRB and p300. Instead, inhibition of differentiation by EID-1 correlated with its ability to inhibit p300 or CBP HAT activity. EID-1 was rapidly degraded upon cell cycle exit in a ubiquitin-dependent manner. Ubiquitination of EID-1 required an intact pRB- and/or p300-binding unit, and EID-1 was stabilized by a dominant-negative pRB mutant. These studies support a role of pRB and/or p300 in the degradation of EID-1 upon cell cycle exit and suggest that neutralization of EID-1 might be one mechanism by which pRB promotes differentiation.

* Corresponding author. Mailing address: Howard Hughes Medical Institute, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA 02115. Phone: (617) 632-3975. Fax: (617) 632-4760. E-mail: william_kaelin@dfci.harvard.edu.

MATERIALS AND METHODS

Cell culture and transfection. SAOS-2 osteosarcoma cells and 293T cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2.0 mM L-glutamine (PSG). U-2OS osteosarcoma cells were grown in DMEM supplemented with 10% heat-inactivated fetal clone and PSG. U937 leukemia cells were grown in RPMI 1640 medium supplemented with 10% FBS and PSG. To induce differentiation, these cells were suspended at a density of 2.5×10^5 cells/ml and treated with 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma Chemical Co.) for 48 h. C2C12 murine myoblasts were grown in DMEM supplemented with 20% FBS and PSG. WI38 human primary fibroblasts were grown in DMEM supplemented with 10% FBS and PSG. To induce cell cycle exit, these cells were grown with DMEM with 0.1% FBS and PSG for 72 h. Transfection was done by the calcium-phosphate method as modified by Chen and Okayama (6). Where indicated, transfected cells were recovered with anti-CD19 magnetic beads (23).

Yeast two-hybrid assay. To make pGBT9L, the oligonucleotides 5′-AAT TAG GAT CCC GGG AAT TCG AGC TCG TCG AC-3′ and 5′-GAT CGT CGA CGA GCT CGA ATT CCC GGG ATC CT-3′ were annealed in vitro and ligated to pGBT9 (Clontech) that had been cut with *Eco*RI and *Bam*HI. An RB cDNA encoding amino acid residues 379 to 864 was generated by PCR, digested with *Bam*HI and *Eco*RI, and ligated to pGBT9L that had been cut with these two enzymes to make pGBT9L-RB(379-864). This bait plasmid, along with a human fetal brain cDNA library subcloned into the pGAD10 "prey" plasmid (Clontech), was transformed into *Saccharomyces cerevisiae* Y190 (a generous gift of Steven Elledge, Baylor College, Houston, Tex.) which contains integrated reporters with Gal4p DNA-binding sites upstream of *HIS3* and *lacZ*. Approximately 4.0×10^7 transformants were screened. Prey plasmids that supported the growth of yeast in the presence of 20 mM 3-amino-1,2,4-triazole (3-AT) and which induced β-galactosidase expression were rescued by standard techniques.

Plasmids. pRS-hGRα (19), pCD19 (67), pCMVneo (1), pGEX-2TK (37), pCMV-RB and RBΔexon22 (53), pUHC13-3 (21), pCMV-VP16-p300 (16), pSG5-TETr-RB and pTETr-RBΔexon22 (56), pRBG4-Myc-ubiquitin (70), pCMV-MDM2 (8), pCMV-MyoD (47), pCMV-p300-HA (16), pGEX-2TK-p300 (CH3) (17), pGEX-2TK-p300(CH1) (3), 3×GAL4-luciferase (3), pCMV-GAL4-p300 (76), and pSG5L-HA-RB, pSG5L-HA-pRBΔexon22, pSG5L-HA-E2F1, and pMMTV-GRE-luciferase (59) have been described previously. pMCK-luciferase was the generous gift of Bennett Novitch and Andrew Lassar (Harvard Medical School, Boston, Mass.). pCMX-VP16N was the generous gift of Ronald Evans (Salk Institute, San Diego, Calif.).

pSG5L-HA-RB(1-792) was generated introducing a stop codon followed by an *Xho*I site in pSG5L-HA-RB by site-directed mutagenesis using a Muta-gene kit (Bio-Rad) according to the manufacturer's instructions. The RB cDNA 3′ to the stop codon was removed by restriction with *Xho*I followed by religation of the backbone plasmid.

To make pCMX-VP16L, the oligonucleotides 5′-AAT TGC GGA TCC CTC GAG GAA TTC-3′ and 5′-GAT CGA ATT CCT CGA GGG ATC CGC-3′ were annealed in vitro and ligated to pCMX-VP16N that had been cut with *Eco*RI and *Xho*I. A full-length RB cDNA, digested with *Bam*HI and *Xho*I, was ligated into pCMX-VP16L that had been cut with these two enzymes to make pCMX-VP16L-RB.

EID-1 cDNA fragments were recovered from the pGAD10 prey plasmids by digestion with *Eco*RI and subcloned into the *Eco*RI site of pcDNA3 (Invitrogen). The EID-1 cDNA sequence was deduced by comparing the DNA sequence of multiple overlapping clones. EID-1 cDNAs encoding wild-type EID-1, or mutants thereof, were made by PCR with oligonucleotides that introduced a 5′ *Bam*HI site and a 3′ *Eco*RI site. To make internal deletion mutants, a two-step PCR strategy was used as described previously (29). The PCR products were restricted with *Bam*HI and *Eco*RI and subcloned into pSG5L-HA, pcDNA3-T7, pSG5-TETr, pGEX-2TK, and pTrcHisA that had been linearized with these two enzymes. All of the PCR products were confirmed by direct DNA sequencing.

EID-1 cDNAs encoding 1-187Δ158-167, 1-187Δ168-177, and RRR (in which all three EID-1 lysine residues were changed to arginine) were made with the Transformer Site-Directed Mutagenesis kit (Clontech) according to the manufacturer's instructions and with pcDNA3-T7-EID-1 as a template. The mutant cDNAs were confirmed by direct DNA sequencing.

Monoclonal antibody production. Glutathione *S*-transferase (GST)–EID-1 was produced in *Escherichia coli*, purified by glutathione-Sepharose affinity chromatography, and used to immunize BNR5.12 mice. Two mice whose sera specifically immunoprecipitated EID-1 in vitro translate were sacrificed, and their splenocytes were fused to NS1 cells. Hybridomas producing anti-EID-1 antibodies were identified by enzyme-linked immunosorbent assay using immobilized GST-EID-1 and confirmed by immunoblot analysis of GST–EID-1 versus GST alone. Individual clones were isolated by limiting dilution. Data shown are with clone SH-18.

GST pull-down assay. GST pull-down assays were performed basically as described previously (38). Binding reactions contained 10 μl of ^{35}S-radiolabeled in vitro translate and approximately 1 μg of the indicated GST fusion protein in 1 ml of NETN (38). Following 1 h of incubation at 4°C with rocking, the Sepharose was washed five times with NETN. Bound proteins were eluted by boiling in sodium dodecyl sulfate (SDS)-containing sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. Comparable loading of GST fusion proteins was confirmed by Coomassie brilliant blue staining, and ^{35}S-radiolabeled proteins were detected by fluorography.

Immunoprecipitation and immunoblot analyses. Cells were lysed in EBC buffer as described previously (36). Immunoprecipitation assays of extracts prepared from transfected cells contained 2 mg of cell extract and 1 μg of anti-T7 (Novagen) antibody or 1 μg of anti-Myc (9E10; Santa Cruz) antibody in a final volume of 0.5 ml. Following 1 h of incubation at 4°C with rocking, the Sepharose was washed five times with NETN. Bound proteins were eluted by boiling in SDS-containing sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose filters.

Nitrocellulose filters were blocked in 5% powdered milk–1% goat serum in 10 mM Tris (pH 8), 150 mM NaCl, and 0.05% Tween for 1 h at room temperature prior to incubation in primary antibody. Antibodies were used at the following concentrations or dilutions: anti-RB (G245; PharMingen), anti-HA (12CA5; Boehringer-Mannheim), anti-MDM2 (N-20; Santa Cruz), and anti-p53 (DO-1; Santa Cruz), 1.0 μg/ml each; anti-EID-1 (SH-18), 1:5 dilution (vol/vol); anti-TetR (Clontech), 1:500 dilution (vol/vol); anti-MHC (MY-32; Sigma), 1:400 dilution (vol/vol); anti-troponin T (JLT-12; Sigma), 1:200 (vol/vol), anti-tubulin (B-5-1-2; Sigma), 1:2,000 (vol/vol); and anti-T7 (Novagen), 0.2 μg/ml. Following four washes with 10 mM Tris (pH 8), 150 mM NaCl, and 0.05% Tween, bound antibody was detected with horseradish peroxidase-conjugated secondary antibodies and by enhanced chemiluminescence with Supersignal (Pierce) according to the manufacturer's instructions.

Flat-cell assay. Flat-cell assays were performed basically as described previously (59). Briefly, SAOS-2 cells grown on six-well plates were transfected with 1.0 μg of pCMV-Neo-Bam or pCMV-RB together with the indicated amounts of pSG5L-HA-EID-1 (or mutants thereof) and placed under G418 selection. The surviving cells were stained with crystal violet, and the number of flat cells in 10 high-powered fields was counted.

FACS and cell cycle analysis. Fluorescence-activated cell sorting (FACS) was done essentially as previously described (59). Briefly, subconfluent SAOS-2 cells grown in 100-mm dishes were transfected with 4 μg of pCD19 and 1 μg of pCMV-RB together with the indicated amount of either pSG5L-HA-E2F1 or pSG5L-HA-EID-1. Seventy-two hours later, the cells were resuspended and stained with fluorescein isothiocyanate-conjugated anti-CD19 antibody (Caltag) and propidium iodide. Samples were analyzed by two-color FACS with a FACScan (Becton Dickinson).

Luciferase reporter assays. For the TetR fusion protein transactivation assay, subconfluent SAOS-2 cells were transiently transfected in six-well plates in duplicate with 500 ng of pCMX-βGal, 1 μg of pUHC-13-3 reporter plasmid, and the indicated amounts of pSG5-TetR-EID-1. Sufficient parental pSG5 was added so that each reaction mixture contained the same amount of pSG5 backbone. Forty-eight hours after transfection, the cells were lysed. Luciferase activity and β-galactosidase (β-Gal) activity in the cell extracts were determined as described elsewhere (4).

For GRα transactivation experiments, subconfluent SAOS-2 cells were transfected as above with 500 ng of pCMX-βGal, 1.0 μg of pMMTV-GRE-luciferase reporter, 200 ng of pRS-hGRα, and the indicated amounts of pcDNA3-T7-EID-1. Sufficient parental pcDNA3 was added so that each reaction mixture contained the same amount of pcDNA3 backbone. When the DNA precipitates were removed, dexamethasone was added to a final concentration at 10^{-6} M. Cell lysates were prepared 24 h later.

For MyoD transactivation experiments, C2C12 cells were transiently transfected in six-well plates, in duplicate, with 500 ng of pCMX-βGal, 500 ng of pCMV-MyoD, 1.0 μg of pMCK-luciferase reporter, and the indicated amount of pcDNA3-T7-EID-1. Sufficient parental pcDNA3 was added so that each reaction mixture contained the same amount of pcDNA3 backbone. Cell extracts were prepared 24 h following the removal of DNA precipitates.

For Gal4-p300 transactivation experiments, 40% confluent U-2OS cells were transiently transfected in six-well plates in duplicate with 500 ng of pCMX-βGal, 1 μg of pGal4-luciferase reporter plasmid, and the indicated amounts of pcDNA3-T7-EID-1. Sufficient parental pcDNA3 was added so that each reaction mixture contained the same amount of pcDNA3 backbone. Cell extracts were prepared 24 h following the removal of DNA precipitates.

Mammalian two-hybrid assay. SAOS-2 cells were transiently transfected in six-well plates, in duplicate, with 500 ng of pCMX-βGal, 1.0 μg of pUHC-13-3 reporter, and 1.0 or 2.0 μg of pSG5-TetR-EID-1 along with the indicated amount of pCMV-VP16-p300 or pCMX-VP16L-RB. Luciferase and β-Gal assays were done as described above.

HAT assays. His-EID-1 was produced in *E. coli* and purified with Ni-nitrilotriacetic acid agarose (Qiagen) under native conditions according to the manufacturer's instructions. The purified proteins were dialyzed against 1× buffer A (50 mM Tris [pH 8], 50 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM EDTA, 10 mM butyric acid).

Purified Flag-CBP produced in insect cells with a baculovirus (a generous gift of Jim Direnzo and Myles Brown) or p300-HA recovered in anti-hemagglutinin (anti-HA) immunoprecipitates from transfected 293T cells was incubated with His-EID-1 for 10 min on ice. Acetylation reactions were performed in 30 μl of 1× buffer A containing 5 μg of purified histones (Boehringer Mannheim) and 125 nCi of [^{3}H]acetyl-coenzyme A (Amersham) at 30°C for 30 min.

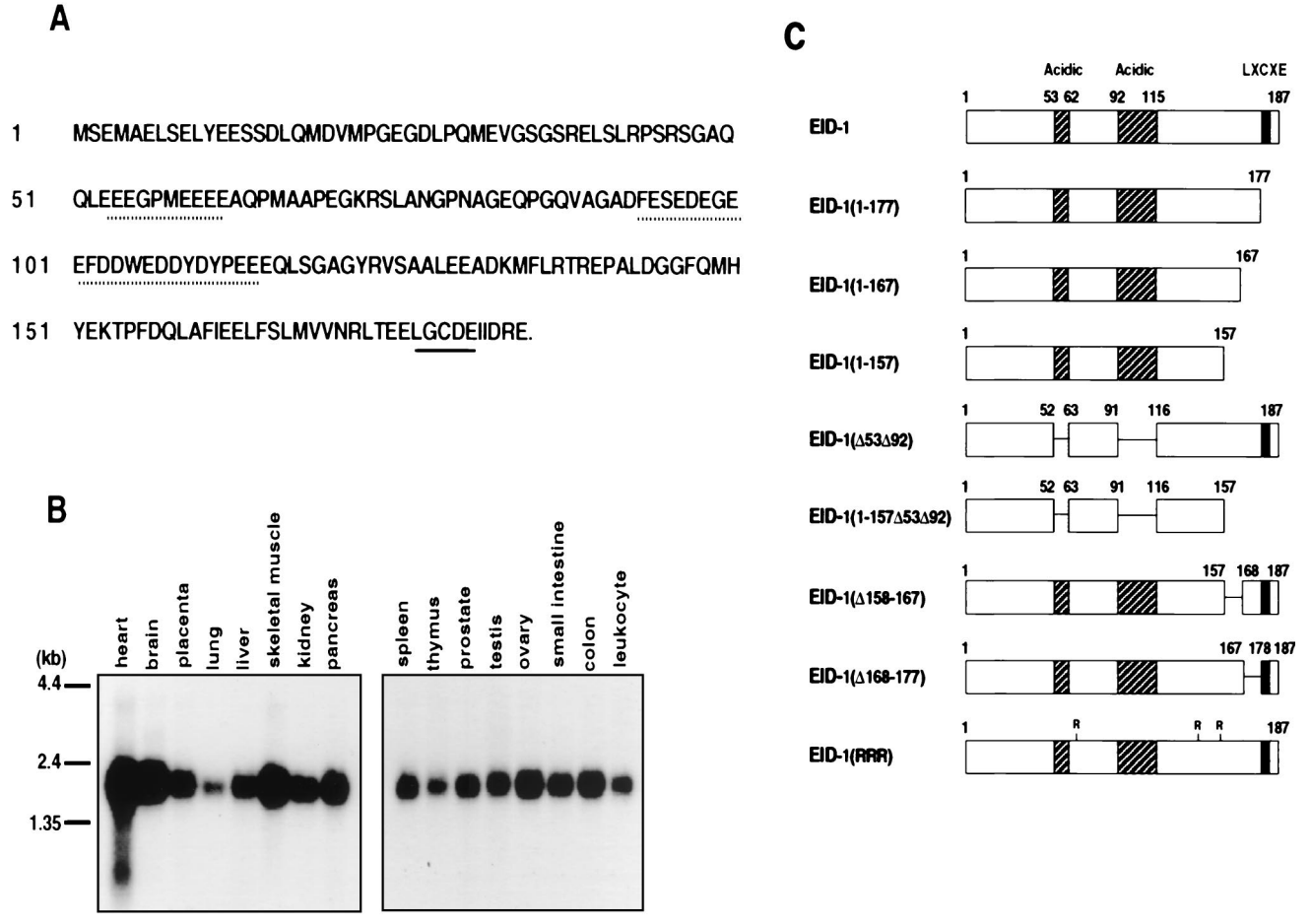

FIG. 1. Expression of EID-1. (A) Conceptual open reading frame of EID-1 cDNA. The pRB-binding motif (LXCXE) is underlined in black. Two acidic clusters are underlined with a dotted line. (B) Northern blot of RNA from indicated tissues with radiolabeled EID-1 cDNA probe. (C) Schematic of EID-1 mutants used in this study.

RESULTS

Identification and cloning of EID-1. We performed yeast two-hybrid assays with a bait consisting of the Gal4p DNA-binding domain fused to the smallest fragment of pRB [pRB (379-864)] that can both induce a G_1/S block and promote differentiation following reintroduction into $RB^{-/-}$ cells (27, 28, 33, 53; P. D. Adams and W. G. Kaelin, Jr., unpublished data). These assays were performed with the *HIS3* reporter gene, which encodes an enzyme involved in histidine biosynthesis (His3p). This reporter gene allows titratable selection using 3-AT, an inhibitor of His3p. Using a fetal brain cDNA library, we identified 12 clones that encoded potential pRB interactors. Each clone conferred resistance to high levels of 3-AT, suggesting that they mediated strong interactions with Gal4-RB. Restriction analysis and direct sequencing revealed that eight of the clones contained overlapping fragments of the same cDNA, hereafter called EID-1.

A 1.6-kb EID-1 cDNA contig was generated using these clones in addition to expressed sequence tags present in available databases. The conceptual open reading frame for EID-1 contains 187 amino acid residues and an in-frame stop codon 15 bases upstream of the initiator ATG (Fig. 1A and data not shown). EID-1 contains two acid patches (Fig. 1A) and a C-terminal pRB-binding motif (LXCXE, where X equals any amino acid residue) first identified in viral oncoproteins such as E1A and simian virus 40 large T antigen (Fig. 1A). With Northern blot assays, we detected ubiquitous expression of an approximately 2.0-kb EID-1 mRNA with the highest levels detected in heart and skeletal muscle (Fig. 1B). An EID-1 expressed sequence tag was previously mapped to chromosome 15q25 (National Center for Biotechnology Information UniGene database).

EID-1 binds to pRB. In the first set of experiments, wild-type pRB and a tumor-derived pRB mutant (pRBΔexon 22) were translated in vitro in the presence of [^{35}S]methionine and incubated with GST–EID-1 fusion proteins that had been immobilized on glutathione-Sepharose. Wild-type pRB, but not pRBΔexon 22, bound to GST–EID-1 (Fig. 2A, compare lanes 3 and 8). Furthermore, pRB did not bind to GST–EID-1 C-terminal truncation mutants that lacked the LXCXE sequence (Fig. 2A, lanes 4 and 5).

Next, mammalian cells were transfected to produce HA-tagged pRB, T7-tagged EID-1, or both. Cell extracts were prepared and immunoprecipitated with an anti-T7 monoclonal antibody. The immunoprecipitates (Fig. 2B, lanes 2, 4, and 6), as well as aliquots of the cell extracts from which they were derived (Fig. 2B, lanes 1, 3, and 5, respectively) were immunoblotted with an anti-HA antibody. As expected, pRB migrated as a family of polypeptides due to differential phosphorylation (Fig. 2B, lanes 1 and 5). The fastest migrating form of

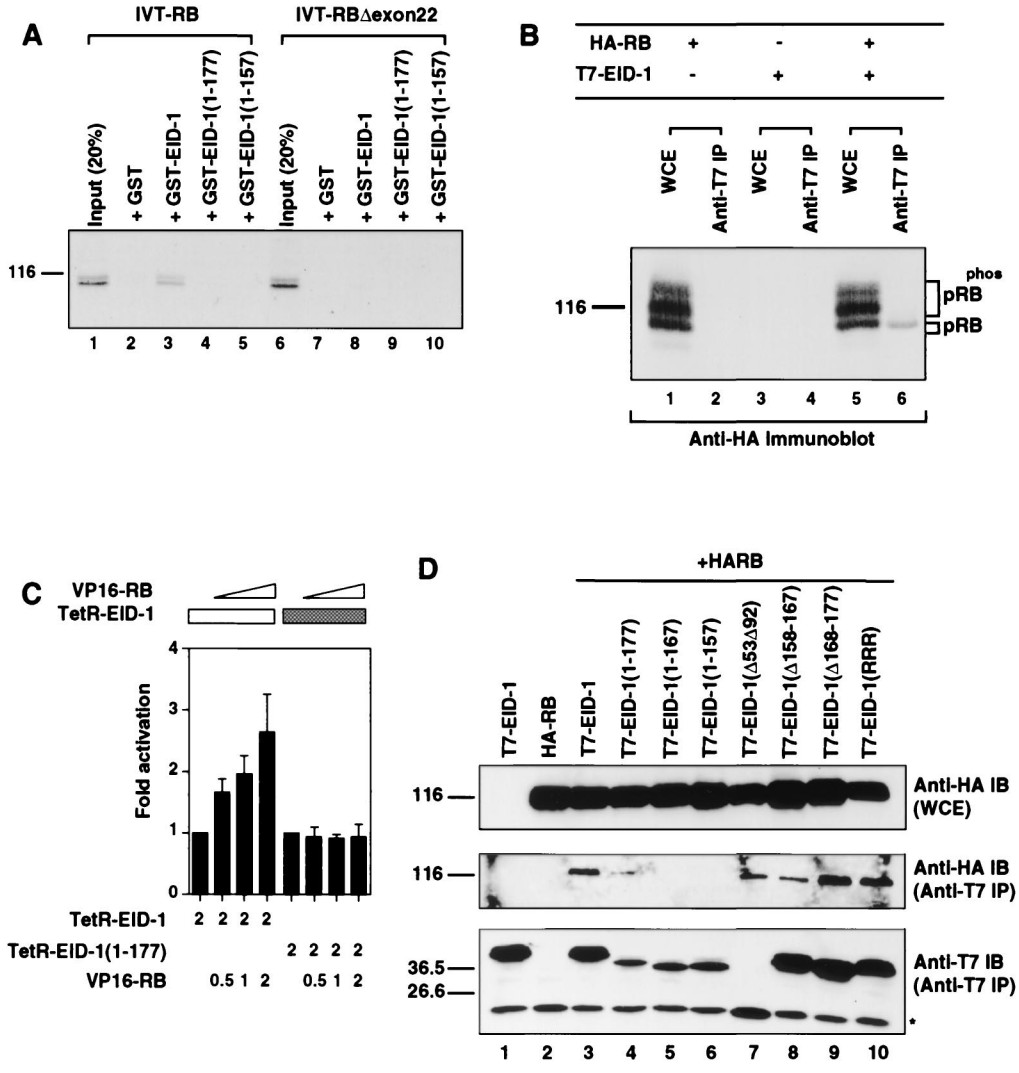

FIG. 2. EID-1 binding to pRB. (A) The indicated immobilized GST–EID-1 proteins were incubated with ^{35}S-radiolabeled wild-type RB (lanes 2 to 5) or a tumor-derived pRB mutant (Δexon22) in vitro translate (lanes 7 to 10). Specifically bound proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by fluorography. Twenty percent of the input proteins was loaded directly in lanes 1 and 6. (B) U-2OS cells were transfected to produce HA-tagged pRB (lanes 1 and 2), T7 epitope-tagged EID-1 (lanes 3 and 4), or both (lanes 5 and 6). Cell extracts were prepared and immunoprecipitated with anti-T7 antibody (lanes 2, 4, and 6) or loaded directly (lanes 1, 3, and 5) prior to anti-HA immunoblot analysis. (C) SAOS-2 cells were transiently transfected with a β-Gal control reporter plasmid, a luciferase reporter plasmid containing TetR binding sites, and plasmids encoding the indicated TetR–EID-1 and VP16-RB fusion proteins in the amounts shown at the bottom of the graph (in micrograms). Cell extracts were prepared, and luciferase activity, corrected for β-Gal activity, was measured. Corrected luciferase values were expressed as fold activation relative to that of TetR–EID-1 and TetR–EID-1(1-177) alone. (D) U-2OS cells were transfected so as to produce T7–EID-1 alone (lane 1), HA-RB alone (lane 2), or HA-RB with the indicated EID-1 mutants (lanes 3 through 10). Cell extracts were prepared and immunoblotted with anti-HA (top). In parallel, an aliquot of each extract was immunoprecipitated with anti-T7 antibody prior to immunoblot analysis with anti-HA (middle) or anti-T7 (bottom). Note that the Δ53Δ92 EID-1 mutant comigrates with the antibody light chain (asterisk).

pRB, corresponding to un(der)phosphorylated pRB, was coimmunoprecipitated with EID-1 (Fig. 2B, compare lanes 5 and 6). This interaction was specific as it required the presence of both HA-pRB and T7–EID-1 (Fig. 2B, compare lane 6 to lanes 2 and 4). Furthermore, T7–EID-1 did not coimmunoprecipitate with HA-pRBΔexon22 when tested in parallel (data not shown).

We also performed mammalian two-hybrid assays with a reporter plasmid containing binding sites for the Tet repressor DNA-binding domain (TetR) upstream of a minimal promoter and plasmids encoding TetR–EID-1 and the VP16 transactivation domain fused to pRB (VP16-RB) (Fig. 2C). Since TetR–EID-1 itself can activate transcription (see below), the signal obtained with the reporter in the presence of TetR–EID-1 or TetR–EID-1(1-177) alone was set to a value of 1 in these assays. VP16-RB led to a dose-dependent increase in reporter activity in the presence of TetR–EID-1. This effect was specific because TetR–EID-1(1-177), which lacks the LXCXE motif, was seemingly inert in this assay. Comparable production of the TetR fusion proteins following transient transfection was confirmed by anti-TetR immunoblot analysis (data not shown).

Finally, mammalian cells were transfected to produce HA-tagged pRB and T7-tagged EID-1 mutants (Fig. 2D; see also Fig. 1C). pRB coimmunoprecipitated with wild-type EID-1 (Fig. 2D, lane 3) but not with EID-1(1-167) or EID-1(1-157) (Fig. 2D, lanes 5 and 6). Surprisingly, we reproducibly detected weak binding of pRB to EID-1(1-177) in this assay, despite the

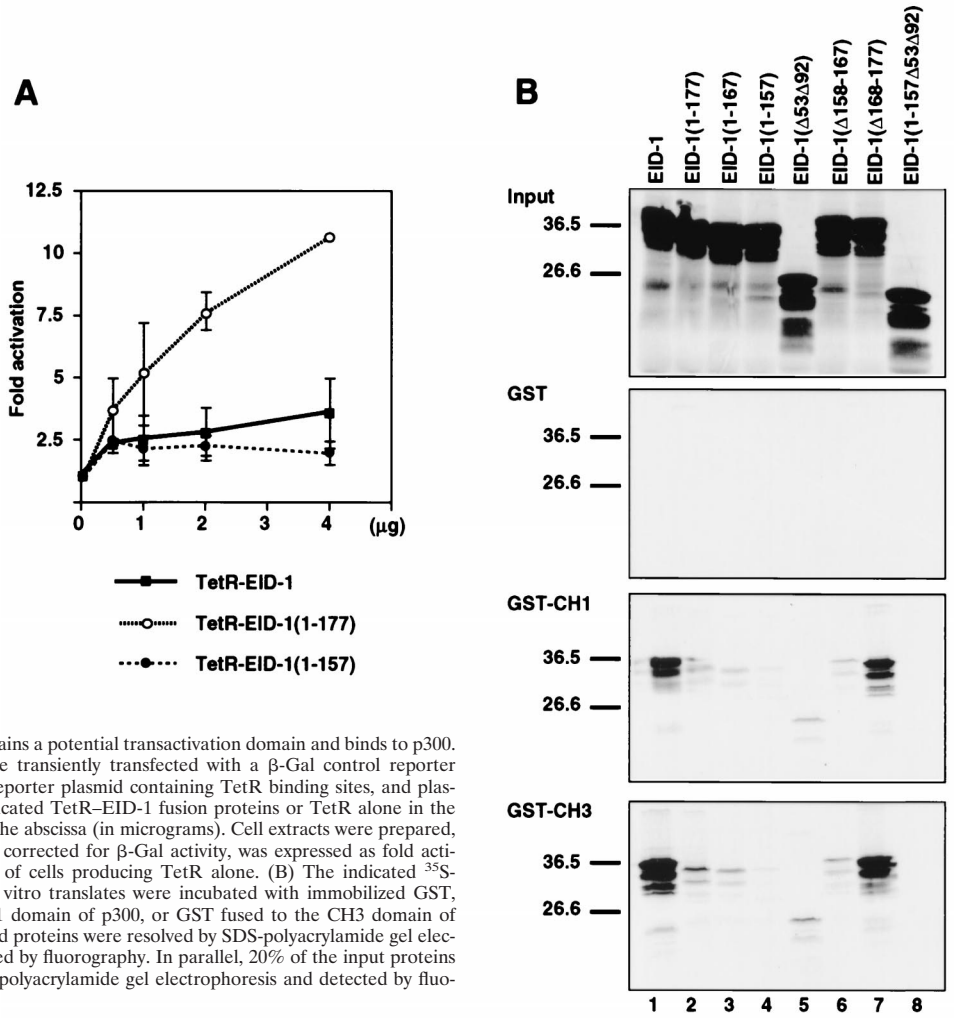

FIG. 3. EID-1 contains a potential transactivation domain and binds to p300. (A) SAOS-2 cells were transiently transfected with a β-Gal control reporter plasmid, a luciferase reporter plasmid containing TetR binding sites, and plasmids encoding the indicated TetR–EID-1 fusion proteins or TetR alone in the amounts shown along the abscissa (in micrograms). Cell extracts were prepared, and luciferase activity, corrected for β-Gal activity, was expressed as fold activation relative to that of cells producing TetR alone. (B) The indicated ^{35}S-radiolabeled EID-1 in vitro translates were incubated with immobilized GST, GST fused to the CH1 domain of p300, or GST fused to the CH3 domain of p300. Specifically bound proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by fluorography. In parallel, 20% of the input proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by fluorography (top).

fact that this mutant lacks an intact LXCXE sequence and did not score positively in the mammalian two-hybrid assay and GST pull-down assay. Nonetheless, the residual pRB-binding activity of EID-1(1-177) may account for some of the observations described below. Taken together, these results suggest that EID-1 can physically interact with pRB (see also Discussion).

EID-1 has a potential transactivation domain. pRB is a nuclear protein and many of the pRB-binding proteins identified to date play roles in transcription (66). To this end, EID-1 or various mutants thereof were fused to TetR and scored for their ability to activate transcription from the luciferase reporter plasmid described above (Fig. 3A). TetR–EID-1 led to a modest, but reproducible, dose-dependent increase in reporter activity whereas TetR–EID-1(1-157) did not. Interestingly, TetR–EID-1(1-177), which lacks the LXCXE motif and binds poorly to pRB (Fig. 2), was a much better transcriptional activator than TetR–EID-1. This suggests that pRB, or a related pocket protein, might inhibit transcriptional activation by EID-1 in this assay. Production of the various TetR fusion proteins was confirmed by anti-TetR immunoblot analysis (data not shown).

EID-1 binds to p300. Although these experiments did not prove that EID-1 is a transcriptional activator in vivo, they did prompt us to ask whether EID-1 can bind to coactivator molecules such as p300 and CBP (22, 63, 69). To test this possibility, EID-1 and selected mutant derivatives thereof, were translated in vitro in the presence of [^{35}S]methionine and incubated with immobilized GST fusion proteins containing either the CH1 or CH3 protein binding domains of p300. Wild-type EID-1 bound to both CH1 and CH3 in this assay (Fig. 3B). Similarly, wild-type EID-1 bound to full-length p300 in mammalian two-hybrid assays analogous to the pRB-binding assay described above (data not shown). Of note, EID-1(1-177) bound to p300 less well than wild-type EID-1 in these two assays (Fig. 3B and data not shown) and yet was a more potent activator than wild-type EID-1 (Fig. 3A). This again might reflect differential binding of EID-1(1-177) and wild-type EID-1 to pRB or a related pocket protein.

Most of the mutations tested, with the exception of the deletion of amino acid residues 168 to 177 (Fig. 3B, lane 7), led to a decrease in p300 binding. This suggests that there are multiple p300 contact sites within EID-1 and/or that p300 binding is sensitive to conformational changes in EID-1. EID-1 deletion mutants lacking either the acidic patches [EID-1 (Δ53Δ92)] or residues 158 to 187 [EID-1(1-157)] displayed diminished, but not absent, p300-binding capability in vitro, whereas a mutant in which these deletions were combined [EID-1(1-157Δ53Δ92)] did not detectably bind to p300 (Fig. 3B). These three mutants, and the knowledge of their pRB- and p300-binding properties, proved to be informative in the biological assays described below.

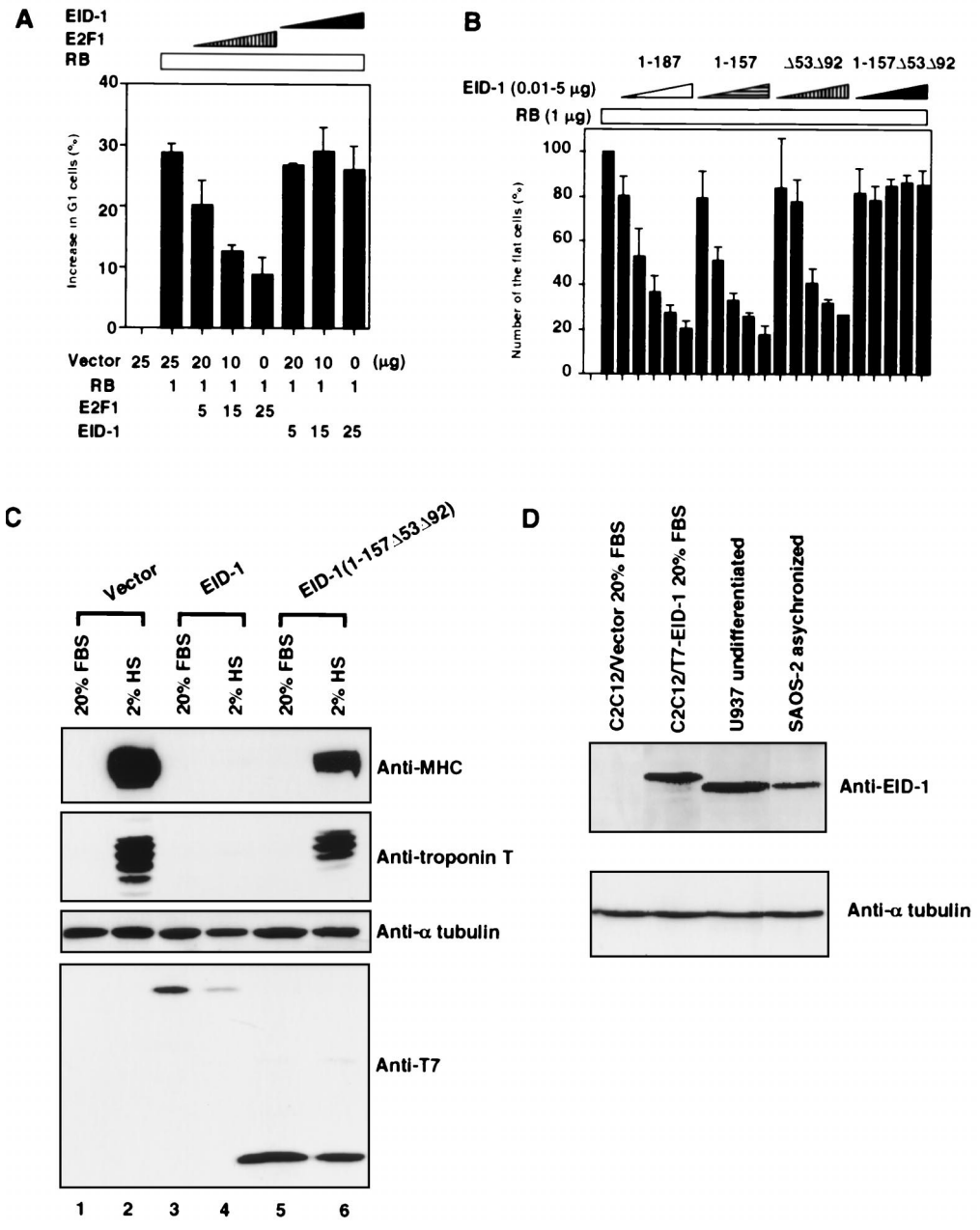

FIG. 4. EID-1 blocks differentiation but does not override a pRB-induced G_1/S block. (A) SAOS-2 cells were transfected with a plasmid encoding wild-type RB and plasmids encoding either E2F1 or EID-1 in the amounts shown at the bottom of the graph. The percentage of transfected cells in G_1 phase was determined by FACS and was expressed as the absolute increase in the percentage of cells in G_1 phase relative to that of mock transfectants. (B) SAOS-2 cells were transfected with a neomycin resistance plasmid encoding pRB and increasing amounts (10 ng, 100 ng, 1 μg, 3 μg, and 5 μg) of plasmids encoding the indicated EID-1 proteins depicted by the triangles. After 14 days of G418 selection, the number of flat cells per 10 high-powered fields was determined and expressed relative to the number of flat cells observed with pRB alone. (C) Stable murine C2C12 subclones producing T7–EID-1, T7–EID-1(1-157Δ53Δ92), and an empty vector transfectant, were grown under conditions that do (2% horse serum) or do not (20% FBS) promote differentiation. Cell extracts were prepared and immunoblotted for the indicated proteins. (D) Anti-EID-1 and anti-α tubulin immunoblot analysis of the indicated cell lines. The anti-EID-1 antibody does not react with murine EID-1.

EID-1 blocks differentiation but not a pRB-induced cell cycle arrest. To begin to address the function of EID-1, we first asked whether EID-1 could modulate any of pRB's biological activities. pRB can repress E2F-responsive promoters and induce a G_1/S block following reintroduction into certain RB-defective tumor cells (15, 39). We next transfected SAOS-2 $RB^{-/-}$ osteosarcoma cells with a plasmid encoding wild-type pRB in the presence or absence of plasmids encoding epitope-tagged versions of either E2F1 or EID-1 (Fig. 4A). As expected, wild-type pRB led to an increase in the number of SAOS-2 cells in G_0/G_1, and this effect was diminished in the presence of increasing amounts of E2F1. In contrast, coproduction of EID-1 had no measurable effect on the ability of pRB to induce a G_1/S block despite the fact that EID-1 was produced at higher levels than E2F1 as determined by immunoblot analysis with an antibody directed against the shared epitope tag (data not shown).

pRB can also promote differentiation in a variety of systems.

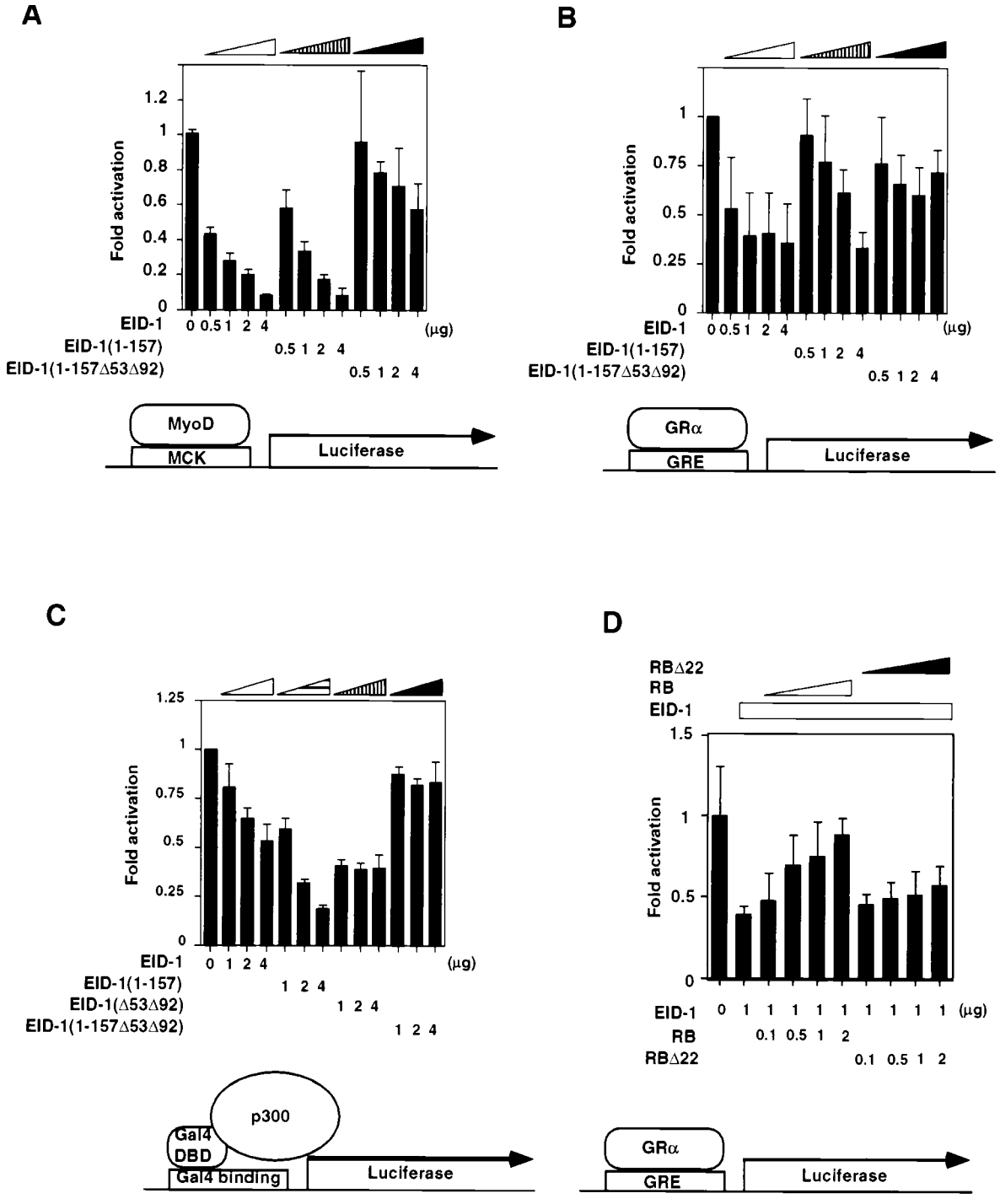

FIG. 5. EID-1 inhibits transcription factors that utilize p300 as a coactivator. (A) C2C12 myoblasts were transfected with a β-Gal control reporter plasmid, a luciferase reporter plasmid containing the MCK promoter, a plasmid encoding MyoD, and plasmids encoding the indicated EID-1 proteins in the amounts shown at the bottom of the graph. (B) SAOS-2 cells were transfected with a β-Gal control reporter plasmid, a luciferase reporter plasmid containing GREs, a plasmid encoding GRα, and the indicated EID-1 proteins in the amounts shown at the bottom of the graph. (C) U-2OS cells were transfected with a β-Gal control reporter plasmid, a luciferase reporter plasmid containing Gal4 DNA-binding sites, a plasmid encoding Gal4-p300, and plasmids encoding the indicated EID-1 proteins in the amounts shown (in micrograms) along the abscissa. (D) SAOS-2 cells were transfected with a β-Gal control reporter plasmid, a luciferase reporter plasmid containing GREs, a plasmid encoding GRα, and plasmids encoding EID-1 and the indicated pRB proteins in the amounts shown (in micrograms) at the bottom of the graph. Cell extracts were prepared and luciferase activity, corrected for β-Gal activity, was measured and expressed as fold activation relative to that of cells that did not ectopically produce EID-1.

In the next set of experiments, SAOS-2 cells were transfected with a pRB expression plasmid that confers resistance to G418 in the presence of plasmids encoding wild-type or mutant EID-1. Following 14 days of drug selection, the number of flat cells, indicative of osteoblastic differentiation, was determined (49, 59). Coproduction of either wild-type EID-1 or an EID-1 mutant lacking its acidic clusters (Δ53Δ92) led to an approximately 80% decrease in the number of flat cells (Fig. 4B).

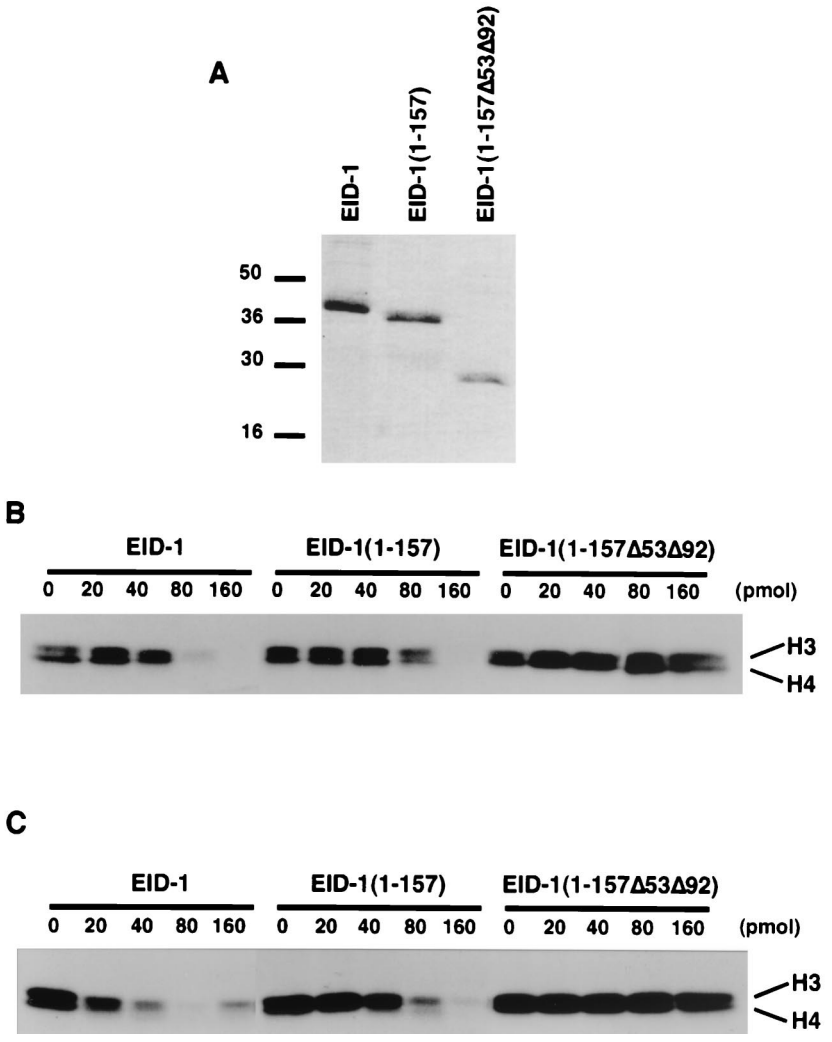

FIG. 6. EID-1 inhibits p300 and CBP HAT activity. (A) The indicated EID-1 proteins were produced in *E. coli* as His fusion proteins and purified by nickel chromatography. Aliquots were resolved by SDS-polyacrylamide gel electrophoresis and detected by Coomassie blue staining. (B and C) In vitro HAT assays were performed with p300 immunoprecipitated from cells (B) or recombinant CBP (C) in the presence of increasing amounts of the indicated EID-1 proteins. Acetylated histones H3 and H4 were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography.

EID-1(1-157) also blocked differentiation, suggesting that this effect was not due solely to titration of pRB. These effects were specific as they were not observed with an EID-1(1-157) mutant that lacked the two acidic clusters [EID-1(1-157Δ53Δ92)]. All of the EID-1 mutants in these studies were produced at comparable levels as determined by immunoblot analysis (data not shown).

pRB has been implicated in myogenic differentiation (24, 47, 55, 77). In the next set of experiments, C2C12 myoblasts were stably transfected so as to produce wild-type EID-1 or EID-1(1-157Δ53Δ92) (Fig. 4C). The levels of wild-type EID-1 ectopically produced in these cells approximated those seen in undifferentiated U937 leukemia cells and tumor cell lines such as SAOS-2 (Fig. 4D). Clones transfected with empty vector or producing EID-1(1-157Δ53Δ92) formed myotubes (data not shown) and expressed late markers of muscle differentiation such as myosin heavy chain and troponin T (Fig. 4C) upon shift to differentiation media, whereas clones producing wild-type EID-1 did not. In these cells, EID-1 did not inhibit the production of early myogenic markers such as MyoD, myogenin, and p21 (data not shown). Similar findings are described in the accompanying manuscript (45). Note also the decrease in ectopically produced wild-type EID-1 under differentiation conditions (Fig. 4C, lane 4), discussed in greater detail below.

EID-1 blocks the function of transcription factors implicated in differentiation. Promotion of differentiation by pRB has been linked to its ability to activate transcription in concert with certain fate-determining proteins such as MyoD (58). To find out whether EID-1 might inhibit the activity of such proteins, C2C12 myoblasts were transiently transfected with plasmids encoding EID-1 along with a MyoD expression plasmid and a MyoD-responsive reporter plasmid containing the muscle creatine kinase (MCK) promoter. Both EID-1 and EID-1(1-157) inhibited transcription of the MCK promoter in a dose-dependent fashion (Fig. 5A). Similar effects were observed when SAOS-2 cells were transfected with plasmids encoding the glucocorticoid receptor GRα, which also cooperates with pRB (59, 62), and a reporter plasmid containing glucocorticoid response elements (GREs) (Fig. 5B). Both MyoD and GRα utilize p300 to activate transcription (17, 18, 52, 54, 76). To find out whether EID-1 could block p300 function, U-2OS cells were transfected with a reporter plasmid

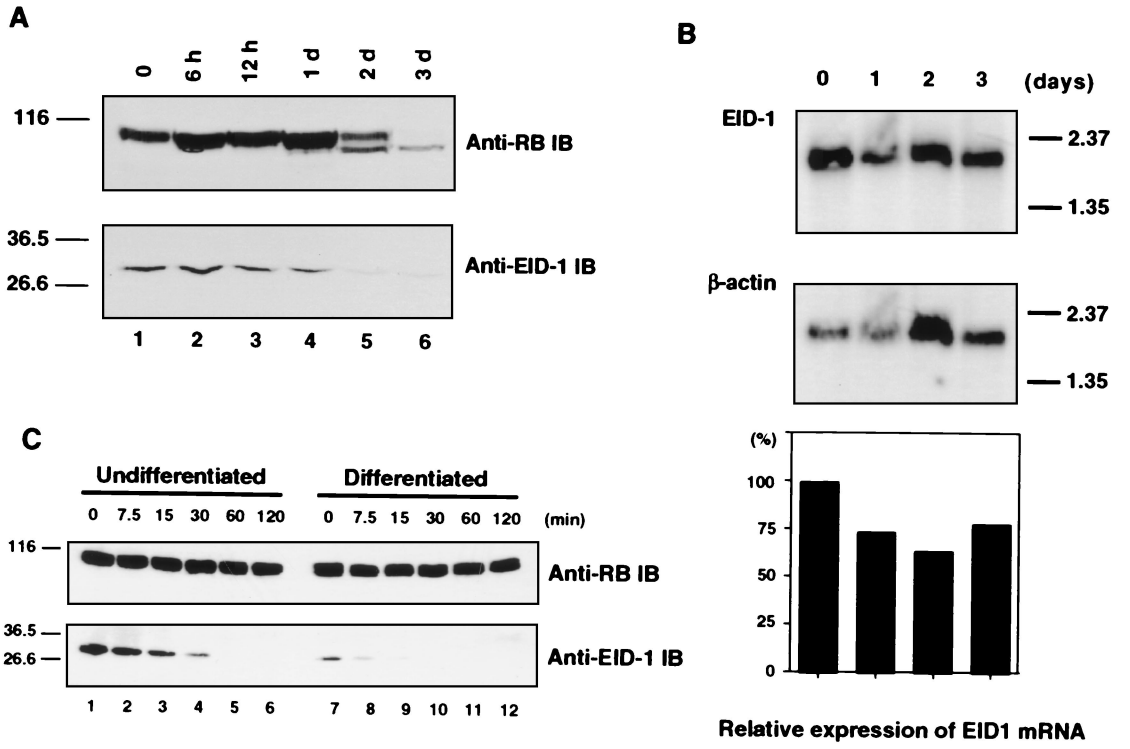

FIG. 7. EID-1 is degraded during differentiation. U937 leukemia cells were induced to differentiate by the addition of TPA. At the indicated time points, protein and mRNA were isolated and subjected to immunoblot (A) and Northern blot (B) analysis with the indicated antibodies and cDNA probes. EID-1 mRNA abundance was normalized to β-actin in the graph shown in panel B. (C) Undifferentiated (lanes 1 to 6) and differentiated (lanes 7 to 12) U937 cells were treated with 20 μg of cycloheximide per ml. At the indicated time points thereafter, cell extracts were immunoblotted with anti-RB and anti-EID-1 antibodies. All lanes contained comparable amounts of protein as determined by the Bradford method.

containing Gal4p DNA-binding sites and a plasmid encoding the Gal4p DNA-binding domain fused to full-length p300 in the presence of plasmids encoding wild-type or mutant EID-1. Wild-type EID-1 inhibited transactivation by p300 (Fig. 5C). EID-1 mutants which lacked either the EID-1 C terminus [EID-1(1-157)] or acid clusters [EID-1(Δ53Δ92)] were even more potent in this regard. These effects were specific because EID-1(1-157Δ53Δ92) was inert in this assay and none of the EID-1 variants tested measurably affected the activity of β-Gal reporter plasmid that was included in each transfection mixture to normalize for transfection efficiency.

In the next set of experiments, the GRE assay was repeated with a fixed amount of EID-1. Coproduction of GRα with wild-type pRB, but not a tumor-derived pRB mutant (pRBΔexon22), led to reporter activities that approximated those observed in the absence of EID-1 (Fig. 5D). This, together the data shown in Fig. 5B, shows that pRB and EID-1 antagonize one another in cells.

EID-1 inhibits p300 and CBP HAT activity. Transcriptional activation by p300 and CBP is due, at least in part, to HAT (22, 69). To ask how EID-1 blocks transcription and differentiation, EID-1, EID-1(1-157), and EID-1(1-157Δ53Δ92) were produced in bacteria and affinity purified (Fig. 6A). These proteins were then added to in vitro HAT assays performed with immunoprecipitated p300 (Fig. 6B) or recombinant CBP produced in insect cells (Fig. 6C). EID-1 and EID-1(1-157) led to a dose-dependent decrease in p300 or CBP HAT activity, whereas EID-1 (1-157Δ53Δ92) did not. Thus, inhibition of p300 or CBP HAT by EID-1 correlated with its ability to block transcription and prevent differentiation.

EID-1 is downregulated during differentiation. To find out whether EID-1 might normally play a role in differentiation, U937 leukemia cells were induced to differentiate with TPA. As expected from earlier studies, treatment of these cells with TPA led to the conversion of pRB from a more slowly migrating, hyperphosphorylated form to the more rapidly migrating, un(der)phosphorylated form (Fig. 7A, top). This coincided with these cells assuming a monocytoid appearance and becoming adherent to plastic (data not shown). In parallel, anti-EID-1 immunoblot analysis confirmed that EID-1 was downregulated during the differentiation of these cells (Fig. 7A, bottom). The band labeled EID-1 in Fig. 7A comigrates with recombinant EID-1 and is recognized by multiple independent anti-EID-1 monoclonal antibodies (data not shown). Northern blot analysis showed that transcription of EID-1 is minimally affected upon differentiation of U937 cells (Fig. 7B). In contrast, anti-EID-1 immunoblot analysis at various time points after cycloheximide treatment showed that EID-1, which is already short lived (half-life, 15 to 30 min) in undifferentiated U937 cells, becomes even more unstable following differentiation (half-life, <7.5 min) (Fig. 7C). Similarly, pulse-chase experiments with radiolabeled methionine indicated an EID-1 half-life of ~30 min in undifferentiated cells and ~7.5 min in differentiated cells (data not shown). Thus, the turnover of EID-1 is increased as U937 cells differentiate.

EID-1 is a ubiquitinated protein that is degraded upon cell cycle exit. To find out whether the degradation of EID-1 during leukemic differentiation was due to cell cycle exit, WI38 human fibroblasts were serum starved into quiescence (Fig. 8B). As expected, pRB was converted to its fastest-migrating, un(der)phosphorylated form under these conditions as determined by anti-RB immunoblot analysis (Fig. 8A, top). An anti-EID-1 immunoblot analysis of these cells confirmed an approx-

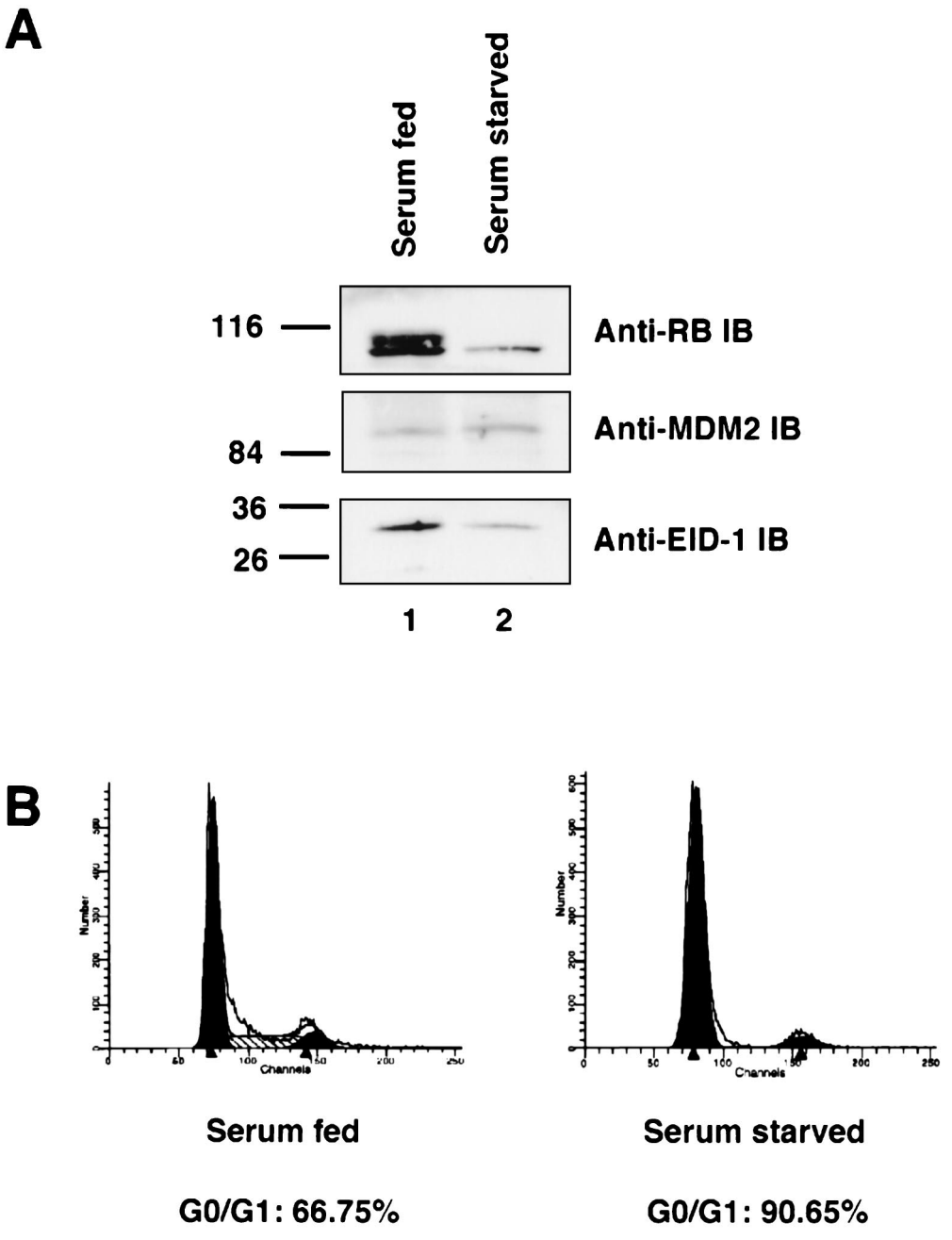

FIG. 8. Downregulation of EID-1 upon cell cycle exit. (A) WI38 fibroblasts grown in the presence (lane 1) or absence (lane 2) of serum were lysed and immunoblotted with anti-RB (top) or anti-EID-1 (bottom) antibodies. Both lanes contained comparable amounts of protein as determined by the Bradford method and confirmed by anti-MDM2 immunoblot (middle). (B) Cells grown as described in the legend to panel A were stained with propidium iodide, and cell cycle distribution was determined by FACS.

imately 50% decrease in levels of EID-1 protein compared to asynchronously growing cells.

Ubiquitination plays an important role in the regulated destruction of proteins (11, 14, 42). To this end, U-2OS cells were transfected with T7 epitope-tagged EID-1, or mutants thereof, along with Myc epitope-tagged ubiquitin. Reciprocal coimmunoprecipitation experiments confirmed that wild-type EID-1 becomes ubiquitinated in these cells (Fig. 9A and B). Furthermore, ubiquitination of EID-1 was linked to the integrity of its C-terminal p300- and pRB-binding region (Fig. 9B). An EID-1 mutant that lacked its three potential lysine ubiquitin acceptor sites [EID-1(RRR)] was, as expected, not ubiquitinated in this assay (Fig. 9B, lane 10).

EID-1 ubiquitination correlates with its ability to bind to MDM2. Both p300 and pRB can bind to MDM2, and MDM2 can function as an E3 ubiquitin ligase (23, 26, 30–32, 41, 73). To find out whether EID-1 could bind to MDM2, U-2OS cells were cotransfected with plasmids encoding T7 epitope-tagged EID-1, or mutants thereof, along with a plasmid encoding MDM2. MDM2 binding to EID-1 was scored by anti-MDM2 immunoblot analysis of anti-T7 immunoprecipitates (Fig. 9C, middle). MDM2 binding to EID-1 correlated with its ability to

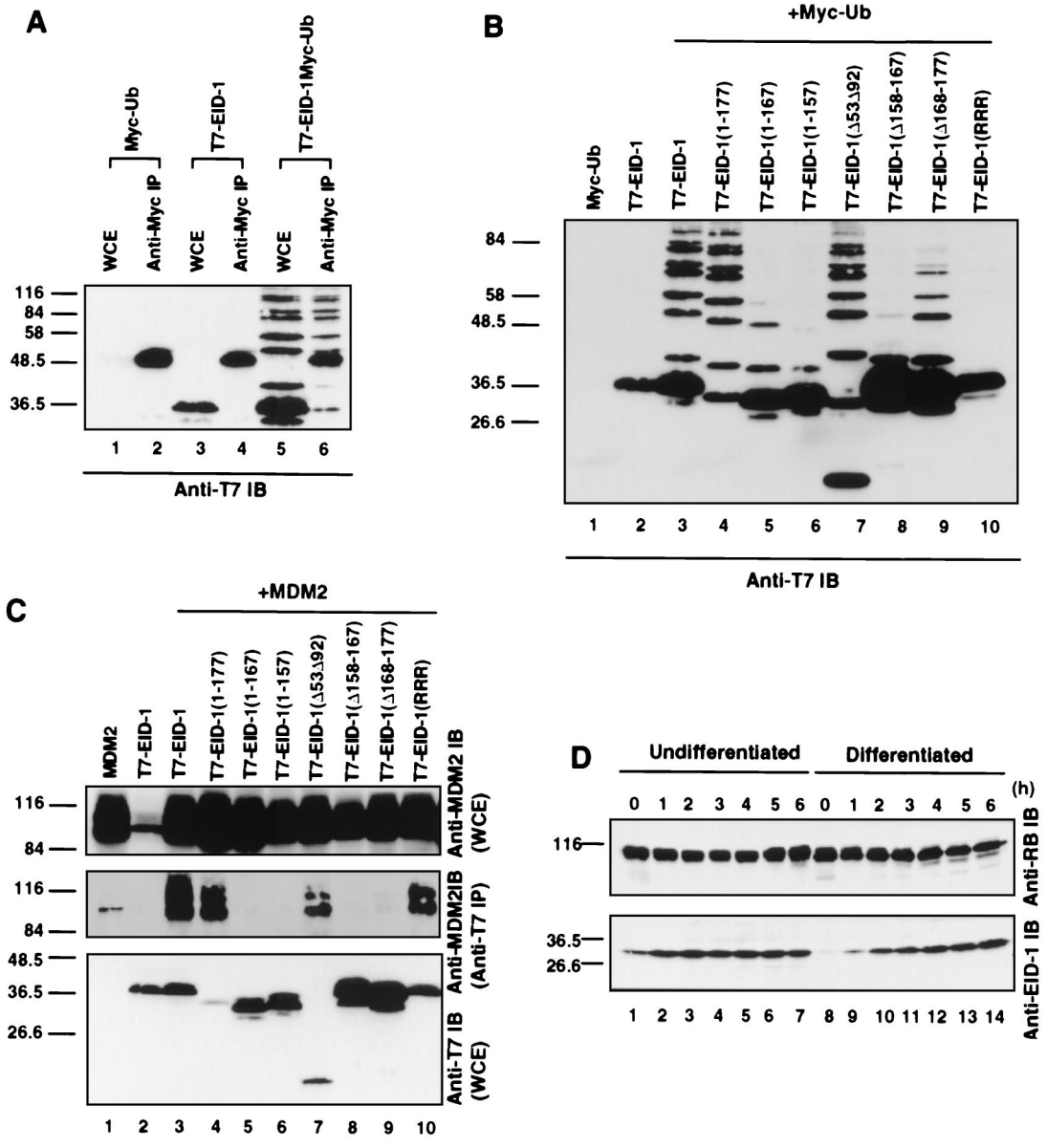

FIG. 9. Degradation of EID-1 occurs via ubiquitin-dependent proteolysis and correlates with MDM2 binding. (A) U-2OS cells were transfected to produce Myc epitope-tagged ubiquitin (lanes 1 and 2), T7 epitope-tagged EID-1 (lanes 3 and 4), or both (lanes 5 and 6). Cell extracts were prepared and immunoprecipitated with anti-Myc antibody (lanes 2, 4, and 6) or loaded directly (lanes 1, 3, and 5) prior to anti-T7 immunoblot analysis. (B) U-2OS cells were transfected to produce Myc-ubiquitin alone (lane 1), T7–EID-1 alone (lane 2), or Myc-ubiquitin with the indicated EID-1 proteins (lanes 3 to 10). Cell extracts were prepared and immunoblotted with anti-T7 antibody. (C) U-2OS cells were transfected to produce MDM2 (lane 1), T7–EID-1 alone (lane 2), or MDM2 with the indicated EID-1 proteins (lanes 3 to 10). Cell extracts were prepared and immunoblotted with anti-MDM2 antibody (top) or with anti-T7 antibody (bottom). In parallel, an aliquot of each extract was immunoprecipitated with anti-T7 antibody prior to immunoblot analysis with anti-MDM2 (middle). (D) Undifferentiated U937 cells (lanes 1 to 6) and U937 cells treated with TPA for 2 days (lanes 7 to 12) were treated with *N*-acetyl-Leu-Leu-norleucinal. At the indicated time points thereafter, cell extracts were immunoblotted with anti-RB and anti-EID-1 antibodies. All lanes contained comparable amounts of protein as determined by the Bradford method.

become ubiquitinated (compare Fig. 9B and C). These experiments suggested that EID-1 was ubiquitinated by MDM2 or an MDM2-like molecule and degraded by the proteasome. In keeping with the latter hypothesis, treatment of U937 cells with the proteasome inhibitor *N*-acetyl-Leu-Leu-norleucinal blocked the degradation of EID-1 following treatment with TPA (Fig. 9D). Similarly, EID-1 is stabilized in a variety of cells following treatment with the proteasome inhibitor MG273 (data not shown). Finally, overproduction of a pRB mutant (1-792) that can bind to EID-1, but not bind to MDM2 (73), stabilized ectopically produced T7-EID-1 (Fig. 10A) as well as endogenous EID-1 (Fig. 10B). These results are consistent with a model wherein destruction of EID-1 is linked to its ability to interact with MDM2 via either p300 or pRB.

DISCUSSION

We isolated a novel protein, EID-1, that interacts biochemically and functionally with pRB and p300 or CBP. An accompanying manuscript describes the isolation of this protein by a second group using a strategy similar to our own (45). We found that EID-1 blocks differentiation in several models. This

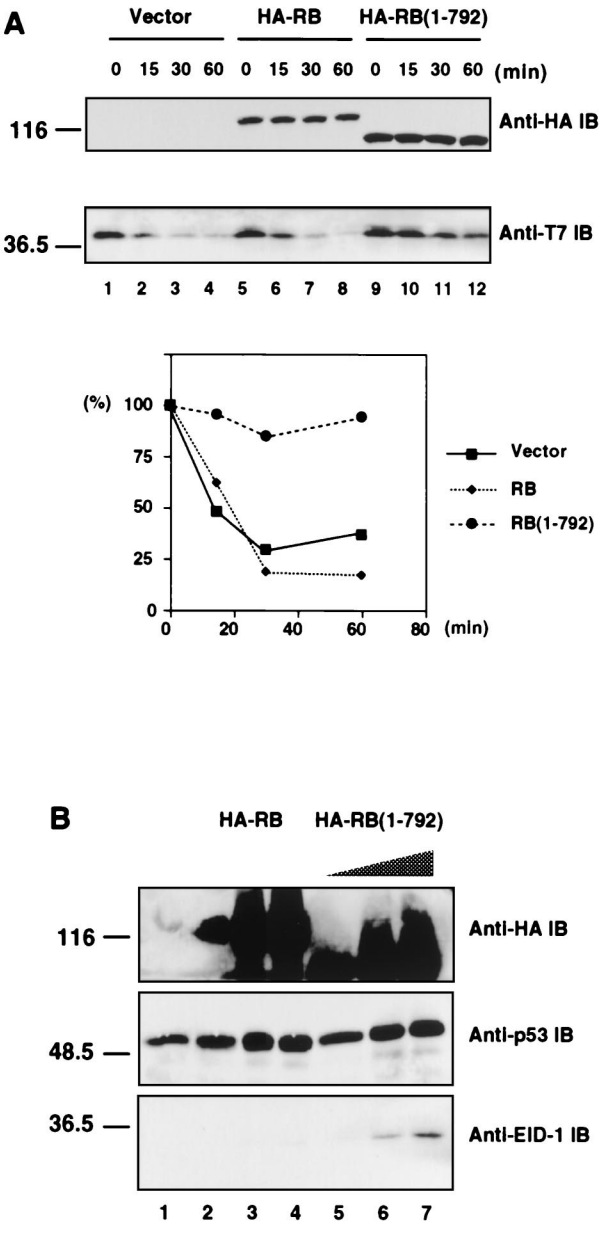

FIG. 10. EID-1 turnover blocked by a pRB mutant that cannot bind to MDM2 (A) U-2OS cells were transfected with a plasmid encoding HA-RB, HA-RB(1-792), or empty vector, as indicated, along with a plasmid encoding T7–EID-1. At the indicated time points following the addition of cycloheximide, cell extracts were prepared and immunoblotted with anti-HA or anti-T7 antibody. Densitometry data for the anti-T7 blot is shown. (B) U-2OS cells were transfected with a plasmid encoding HA-RB, HA-RB(1-792), or empty vector, as indicated, along with a plasmid encoding CD19. Transfected cells were captured on anti-CD19 magnetic beads, lysed, and immunoblotted with anti-HA, anti-p53, or anti-EID-1 antibody.

activity correlates with its ability to inhibit transcriptional activation by proteins such as MyoD that utilize p300 and CBP as coactivators. Furthermore, we showed that EID-1 directly blocks p300 and CBP HAT activity. EID-1 is ubiquitinated and rapidly degraded as cells exit the cell cycle, provided its C-terminal pRB- and p300-binding domain is intact. Thus, EID-1 is poised to couple cell cycle exit to the execution of a differentiation program.

We have thus far been unable to detect complexes of pRB and EID-1 in untransfected cells. One possibility is that the isolation of EID-1 in our pRB two-hybrid screen was fortuitous. This seems unlikely, however, for several reasons. First, eight of eight clones that we recovered in our screen encoded fragments of EID-1. When tested with the two-hybrid screen, EID-1 bound to pRB with higher affinity than known pRB interactors such as E2F1 and E7 (data not shown). Biochemical and structural studies show that E7 possesses a core 9mer motif with the sequence DLYCYEQLN that is necessary and sufficient for high-affinity pRB-binding (underlined are critical residues that directly contact pRB) (35, 44). This sequence is well conserved in EID-1.

There are several reasons why detection of endogenous pRB–EID-1 complexes in cells may be inherently difficult. First, EID-1, like other pocket-binding LXCXE proteins, binds exclusively to the un(der)phosphorylated form of pRB. Secondly, EID-1 is a short-lived protein of low abundance. More importantly, EID-1 is rapidly degraded as cells exit the cell cycle. Thus, under the conditions where pRB becomes un(der) phosphorylated, EID-1 disappears. Indeed, as described below, these two phenomena may be linked. In this regard, we find that overproduced EID-1 is more stable than endogenous EID-1, perhaps because one or more cellular proteins required for its degradation become limiting. Our attempts to stabilize pRB–EID-1 complexes with proteasomal inhibitors have thus far been unsuccessful (data not shown). A caveat, however, is that these agents block degradation but not ubiquitination. It is possible that EID-1 cannot bind to pRB once it has been ubiquitinated.

In functional assays, pRB and EID-1 antagonize one another. pRB, but not tumor-derived mutants, prevents EID-1 from inhibiting transcription. Conceivably, this activity relates to the earlier observation that pRB cooperates with certain transcription factors and promotes differentiation. Conversely, forced production of EID-1 inhibits pRB-dependent differentiation. Importantly, an EID-1 mutant that cannot form stable complexes with pRB [EID-1(1-157)] retains this property. This would place EID-1 downstream of pRB in a differentiation control pathway.

Several lines of evidence suggest that inhibition of p300- and CBP-dependent transcriptional activation by EID-1 does not merely reflect nonspecific transcriptional squelching. Firstly, EID-1 had no significant effect on the CMV promoter used as an internal control in our experiments. Secondly, EID-1(1-157) more potently inhibited p300- and CBP-dependent transactivation than did wild-type EID-1 and yet did not, itself, act as a transcriptional activator at any concentration tested when fused to Tetr. Finally, we easily obtained stable SAOS-2 and C2C12 clones producing high levels of EID-1 (data not shown). This last observation argues against a nonspecific "toxic" effect by EID-1.

Instead, our biochemical studies suggest that EID-1 inhibits p300 and CBP HAT activity. Importantly, both wild-type EID-1 and EID-1(1-157) inhibited HAT activity in vitro and blocked differentiation. In contrast, EID-1(1-157Δ53Δ92) was defective for both of these activities, suggesting that these two properties are linked. It is noteworthy that EID-1(1-157) retained the ability to inhibit p300 and CBP HAT despite a diminished (but not absent) ability to bind to these two proteins. In this way, EID-1 resembles E1A and TWIST (5, 25).

Several lines of evidence lead us to hypothesize that pRB and/or p300 play a role in targeting EID-1 for ubiquitin-dependent proteolysis upon cell cycle exit. First, only those EID-1 mutants that measurably bound to p300 or pRB were ubiquitinated in cells. Second, both p300 and pRB can bind to MDM2 (23, 32, 73), which is a known E3 ubiquitin ligase (30, 31). The results of our coimmunoprecipitation assays are con-

sistent with the hypothesis that p300 and/or pRB serve as adapters that recruit MDM2 to EID-1. Finally, degradation of EID-1 was temporally related to dephosphorylation of pRB during leukemic cell differentiation. Conversely, overproduction of a pRB mutant that can bind to EID-1, but cannot bind to MDM2, stabilized EID1.

EID-1 bears some functional similarity to HBP1 (61, 68). HBP1 inhibits myogenic differentiation and can inhibit pRB-dependent differentiation but cannot override a pRB-induced cell cycle block (68). In contrast to EID-1, however, HBP1 requires an intact LXCXE motif for these activities (68). Indeed, our data raise the possibility that the above-noted effects of HBP1 were due to displacement of EID-1 from pRB.

pRB is a more potent inducer of differentiation than p107 and p130 (47, 59). In an adipocyte model, pRB promoted differentiation whereas p107 and p130 inhibited differentiation and antagonized pRB (9; M. Classon and E. Harlow, personal communication). It will be important to determine whether p107 and p130 physically interact with EID-1 and, if so, whether the functional consequences of such interactions differ from that described here for pRB.

Both the pRB family and p300 and CBP are targeted for inactivation during viral transformation. The majority of human tumors harbor mutations which, directly or indirectly, perturb pRB function (58, 60, 71). Deletions and translocations of p300 and CBP have recently been identified in some solid tumors such as gastric and colon carcinomas as well as in leukemias (20, 46). EID-1 may act at a nodal point that couples the activity of the pRB pathway to the p300-CBP pathway. Deregulation of EID-1 may contribute to the failure of cancer cells to differentiate in vivo. This raises the interesting possibility that EID-1 may, itself, function as an oncogene and be a target of mutations in human cancer.

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