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Basic Helix-Loop-Helix Proteins Bind to *TrkB* and $p21^{Cip1}$ Promoters Linking Differentiation and Cell Cycle Arrest in Neuroblastoma Cells

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Differentiation of precursor into specialized cells involves an increasing restriction in proliferative capacity, culminating in cell cycle exit. In this report we used a human neuroblastoma cell line to study the molecular mechanisms that coordinate cell cycle arrest and neuronal differentiation. Exposure to retinoic acid (RA), a differentiation agent in many cell types, causes an upregulation of neurotrophin receptor *TrkB* and the cyclin kinase inhibitor $p21^{Cip1}$ at a transcriptional level. Full transcriptional activation of these two genes requires canonical E-box sequences found in the *TrkB* and $p21^{Cip1}$ promoters. As reported for other E-box-regulated promoters, ectopic expression of E47 and E12 basic helix-loop-helix (bHLH) proteins enhances RA-dependent expression of *TrkB* and $p21^{Cip1}$, whereas the inhibitory HLH Id2 exerts opposite effects. In addition, ectopic expression of E47 and NeuroD, a neuronal bHLH protein, is able to activate *TrkB* transcription in the absence of RA. More importantly, we show that E47 and NeuroD proteins bind the *TrkB* and $p21^{Cip1}$ promoter sequences in vivo. Since they establish a direct transcriptional link between a cell cycle inhibitor, $p21^{Cip1}$, and a neurotrophic receptor, *TrkB*, bHLH proteins would play an important role in coordinating key events of cell cycle arrest and neuronal differentiation.

Cell differentiation is a coordinated process that comprises cell cycle exit and a specific gene expression program to determine tissue identity. Many studies have provided knowledge about the molecules involved in cell cycle arrest and the key regulatory transcription factors that control tissue specific gene expression (22, 27).

The basic helix-loop-helix (bHLH) proteins are transcription factors that regulate gene expression to promote cell differentiation and tissue-specific cellular functions (54). For instance, NeuroD and neurogenins (Ngn1 and Ngn2) are tissue-specific bHLH proteins involved in neurogenesis (28). These tissue-specific proteins form dimers with other ubiquitously expressed bHLH transcription factors called E proteins, which bind to the canonical E-box sequence CANNTG and include HEB, E2-2, and the *E2A* gene products, E12 and E47 (28). In addition, the activity of bHLH proteins as transcription factors is negatively regulated by the structurally related Id proteins (inhibitors of DNA binding and/or differentiation) (37). Id proteins possess the HLH domain, through which they form dimers, mainly with E proteins. As a result, E proteins cannot form functional heterodimers with the tissue-specific bHLH factors, leading to inhibition of differentiation (37). Id proteins are involved not only in cell differentiation control but also in the regulation of cell proliferation. Based on in vivo (20, 27, 33) and in vitro (13, 19, 42) experiments, two mechanisms have been proposed to explain how Id proteins contribute to cell cycle entry. One mechanism involves the downregulation of cyclin-dependent kinase inhibitors at a transcriptional level, where Id proteins would interfere with bHLH-driven expression of $p16^{Ink4a}$, $p27^{Kip1}$, and $p21^{Cip1}$ (27, 33). The other proposed mechanism involves Id protein interaction with the tumor suppressor retinoblastoma protein (pRb). Id2 has been shown to bind the unphosphorylated pRb through interaction between the HLH region and the pocket domain of the respective proteins, resulting in the release of E2F (13, 20).

Neuroblastoma is a pediatric solid tumor derived from crest precursor cells (3). Cell lines and tumors from patients with a poor prognosis are associated with expression of the neurotrophin BDNF (for brain-derived neurotrophic factor) and its tyrosine kinase receptor TrkB (2, 35). Several studies indicate that BDNF increases neuroblastoma cell survival, neurite extension, and cell invasion and protects cells from chemotherapy (6, 14, 25, 29, 32). This indicates that, aside from being a marker of poor prognosis, BDNF and TrkB play a role in the biology of neuroblastoma tumors. Thus, understanding the molecular mechanisms that regulate *TrkB* expression may provide tools to block the ability of BDNF to rescue cells from chemotherapy (14, 47). Little is known about the mechanisms by which *TrkB* is regulated. Alternative splicing within the *TrkB* intracellular domain has been shown to generate isoforms with truncated or full kinase domain (18), which may have distinct signaling capabilities and cellular responses to the neurotrophins (45). With regard to the existence of regulatory elements in the *TrkB* promoter, a recent report demonstrates that in the developing rat brain, thyroid hormone downregulates *TrkB* expression through a novel response element located downstream of the transcription initiation site (41).

Retinoic acid (RA) plays important roles in neural developmental (43), and retinoid therapy significantly improves the survival in patients with acute promyelocytic leukemia or neuroblastoma tumors. In a previous study (5) we have shown that

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in SH-SY5Y cells, a human neuroblastoma cell line, RA treatment induces a neuronal differentiation process, with a concomitant G_1 arrest and the accumulation of hypophosphorylated pRb forms, increased expression of *TrkB* and other neuronal markers, and extension of neuritic processes. Moreover, RA treatment yields a homogeneous population of differentiated cells, which strictly depends on BDNF for their survival, thus behaving in this respect as primary neurons (5).

Here we show that transcriptional activation of $p21^{Cip1}$ by RA depends on E-box sequences. Full activation of $p21^{Cip1}$ is simultaneous to the activation of *TrkB* expression, which also depends on canonical E-box sequences. E2A proteins enhance the expression of *TrkB* and $p21^{Cip1}$, whereas Id2 exerts opposite effects, and ectopic expression of E47 and NeuroD is able to activate *TrkB* transcription in the absence of RA. More important, we show for the first time that E47 and NeuroD proteins bind both the *TrkB* and the $p21^{Cip1}$ promoters. Thus, these bHLH proteins establish a direct transcriptional link between molecules involved in the regulation of cell cycle and neuronal differentiation, which should help to tightly coordinate these two processes.

MATERIALS AND METHODS

Cell culture. SH-SY5Y neuroblastoma cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 2 mM L-glutamine, 20 U of penicillin/ml, 20 mg of streptomycin/ml, and 15% fetal calf serum (Gibco). To induce cell cycle arrest and differentiation, all-trans-RA (Sigma) was added to a final concentration of 10 μM. The medium was changed every 3 days. For data regarding the expression of *GAP43* (see Fig. 6B), cells treated for 2 days with RA were washed three times with DMEM (without serum) and incubated for 6 h with 20 ng of BDNF/ml in DMEM (without serum).

Plasmid constructions. The *Id2* expression plasmid was constructed by cloning the BamHI-HindIII fragment containing the complete human *Id2* coding sequence into pcDNA3 (Invitrogen). The expression plasmids *pCMV-E12* and *pCMV-E47* containing the full-length cDNAs encompassing the respective coding regions of human *E12* and *E47* into pcDNA3 vector were a generous gift from M. Nakamura (9). The $p21^{Cip1}$ luciferase reporter construct carries a 2.3-kb fragment from positions −2296 to +22 relative to the transcription start site of the human $p21^{Cip1}$ promoter into the KpnI-XhoI site of pGL3-Basic (pGL3b) vector (Promega). A 1.9-kb fragment from positions −1888 to +30 relative to the main transcription start site of human *TrkB* promoter was inserted into the KpnI-BamHI site of pGL3b, generating the *TrkB* luciferase reporter construct. *TrkB* and $p21^{Cip1}$ promoter E-box and RARE mutants were produced by site-directed mutagenesis by using the transformer site directed mutagenesis kit (Clontech). The construct with the $p21^{Cip1}$ E-box region contains sequences from positions −212 to +30 of the $p21^{Cip1}$ promoter. Three E-box consensus sequences (TCTGGGCCATCTGCTGATCC) were cloned into the pGL3 promoter (pGL3p) vector (Promega) to create the synthetic E-box reporter construct. To create FLAG-E47(1-651) and FLAG-NeuroD(1-356) expression vectors, the indicated coding regions were amplified from cDNA templates by PCR and inserted into the BamHI/EcoRI site of a modified pcDNA3 plasmid containing three copies of the FLAG epitope. Detailed procedures used to generate these constructions are available upon request.

Flow cytometry analysis. Cells were harvested by treatment with trypsin and resuspension in 1 ml of cold PBS, followed by incubation with 1 ml of 0.1% Triton X-100, 50 μg of propidium iodide/ml, and 50 μg of RNase/ml for 30 min on ice before analysis on an EPICS XL flow cytometer (Coulter).

Western blotting and immunoprecipitation. Cells were washed with PBS three times, lysed in 2% sodium dodecyl sulfate (SDS), 125 mM Tris-HCl (pH 6.8), sonicated and boiled for 5 min. The protein concentration was determined by a Micro- DC protein assay (Bio-Rad). Immunoblot analysis was carried out as previously described (5). The following antibodies were used at the dilutions recommended by the manufacturers: anti-Cdk4 (α-Cdk4; 06-139; Upstate), α-Cdk2 (06-505; Upstate), α-Cdk1 (06-194; Upstate), α-cyclin D1 (06-137; Upstate), α-cyclin E (06-459; Upstate), α-$p21^{Cip1}$ (05-345; Upstate), α-p53 (05-224; Upstate), α-$p27^{Kip1}$ (610241; Transduction Laboratories), α-tubulin (T5168; Sigma), α-$p18^{Ink4c}$ (NA55; Oncogene Research Products), α-$p57^{Kip2}$ (65021A; Pharmingen), α-pRB (554136; Pharmingen), and α-E47 (sc-763; Santa Cruz Biotechnology). Cdk2 and Cdk4 were immunoprecipitated as described previously (31).

RNA extraction, real-time RT-PCR, and Northern analysis. Total cellular RNA preparation and reverse transcription (RT) were carried out as described previously (4). Quantitative real-time PCR analysis was performed with Applied Biosystems TaqMan Universal PCR Master Mix according to manufacturer instructions. Triplicate reactions for each sample were done in a 96-well optical reaction plate for human $p21^{Cip1}$ (4318364T MGB probe with FAM reporter label), human *TrkB* (Hs001778811 m1 MGB probe with FAM reporter label), and human *GAPDH* (4326317E MGB probe with VIC reporter label). Samples were run and analyzed with a Icycler iQ real-time detection system (Bio-Rad). Northern blot assay was performed as previously reported (10).

Luciferase assays. Transfection was accomplished with Lipofectamine 2000 (Invitrogen). Unless otherwise indicated, luciferase assays during RA treatment were performed as follows: SH-SY5Y cells were plated in medium with 10 μM RA, in triplicate, at 6.5×10^4 cells in 24-well plates for 3 days and then transfected and maintained in RA for 48 to 96 h before luciferase activity was determined with the Dual Luciferase reporter assay system (Promega). Firefly luciferase activity was made relative to *Renilla* luciferase activity. Routinely, 1.1 μg of firefly luciferase reporter plasmid and 0.1 μg of pRL-TK plasmid (Promega), which contains the *Renilla* luciferase gene as an internal control, were used to determine normalized gene expression values. To assess effects due to E12, E47, or Id2, 0.2 μg of corresponding expression plasmids or empty vector was added in each transfection assay. Activation was estimated by the luciferase activity in RA-treated cells relative to untreated cycling cells. The results shown are the means and standard deviations of three independent transfection experiments.

Immunofluorescence. Immunofluorescence was performed as previously described with minor modifications (5). Cells were treated with 10 μM RA for 4 days and then cotransfected with pEGFP-N1 (Clontech) and *Id2* expression plasmid or pcDNA3 with Lipofectamine 2000. At 24 h after transfection, cells were incubated with 10 μM bromodeoxyuridine (BrdU) for 4 h at 37°C, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Cells were then treated with 50 μg of DNase I (Roche)/ml at 37°C for 30 min, incubated with a mouse monoclonal α-BrdU antibody (1:20 dilution; Molecular Probes) for 1 h at room temperature, and subsequently incubated with an anti-mouse Alexa-546-labeled antibody (1:200 dilution; Molecular Probes) for 45 min at room temperature. Cells were counterstained with DAPI (4′,6′-diamidino-2-phenylindole) before examination under a fluorescence microscope, and a minimum of 1,000 cells were evaluated for each transfection experiment.

ChIP assay. Formaldehyde was added at a final concentration of 1% directly to SH-SY5Y cells either during cycling or after 5 days of RA treatment. Fixation proceeded at 37°C for 10 min and was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were rinsed twice with ice-cold PBS, collected into lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% SDS) with a protease inhibitor cocktail (Sigma), incubated on ice for 10 min, and sonicated to shear the chromatin to an average size of ~600 bp, followed by centrifugation for 30 min. Supernatants were collected and diluted 1:10 in buffer A (16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS, protease inhibitor cocktail) and precleared with protein A-Sepharose beads for 4 h at 4°C. Samples of total chromatin were taken at this point as positive control samples for PCR (input chromatin). Immunoprecipitation was performed overnight at 4°C with 5 μg of α-E47 antibody (sc-763, Santa Cruz Biotechnology, a polyclonal α-E-47 antibody that also cross-reacts with E12). Although a successful chromatin immunoprecipitation (ChIP) experiment has been recently reported with a monoclonal α-E47 antibody on the pT alpha gene promoter (49), the polyclonal antibody used gave higher signal-noise ratios under our conditions (data not shown). DNA-protein complexes were collected with protein A-Sepharose beads and washed four times: once with 50 mM Tris-HCl (pH 8.0)–2 mM EDTA–0.2% Sarkosyl, twice with 100 mM Tris-HCl (pH 9.0)–500 mM LiCl–1% NP-40–1% sodium deoxycholate, and once with Tris-EDTA buffer. Precipitates were extracted twice with lysis buffer. Eluates were pooled, diluted 1:10 in buffer A, and immunoprecipitated again as described above. Final eluates were adjusted to 0.3 M NaCl and 50 μg of RNase A/ml, followed by incubation overnight at 65°C. Samples were then extracted with phenol-chloroform, and precipitated overnight with ethanol in the presence of 20 μg of glycogen as carrier. Immunoprecipitated material was used as a template for PCR amplification. The primers 5′-TGATTAGAAAGTAGGA CCGC and 5′-CTGTCTTGTCAGTGAACCTT were used to amplify a 337-bp region of the *TrkB* promoter; the primers 5′-CGAAGTCAGTTCCTTGTGGA

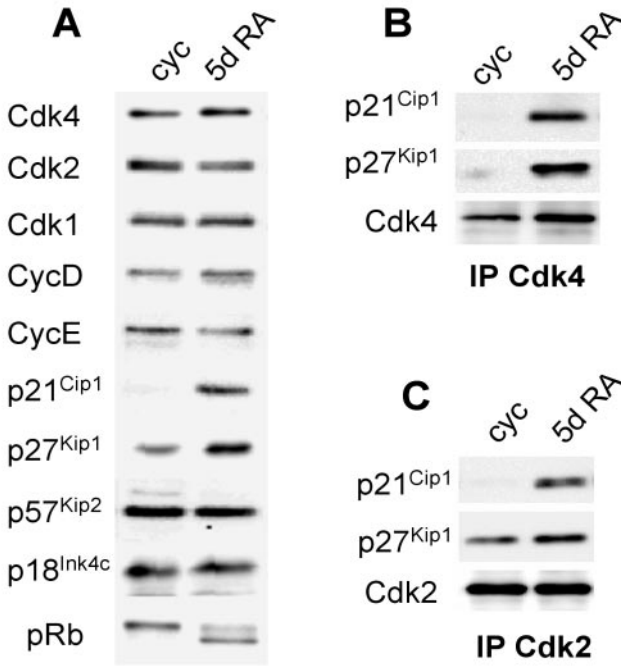

FIG. 1. RA modifies expression levels of genes involved in cell cycle control in SH-SY5Y cells. Cells either cycling (cyc) or treated for 5 days with RA (5d RA) were analyzed. (A) Western blot of cell cycle regulatory proteins; (B and C) $p21^{Cip1}$ and $p27^{Kip1}$ levels in Cdk4 and Cdk2 immunoprecipitates, respectively.

and 5′-ACATCCCGACTCTCGTCAC were used to amplify a 292-bp region of the *p21^Cip1* promoter. The primer set used to amplify the promoter region of *L27* was as described previously (4).

For ChIP with tagged proteins (see Fig. 5D), cells were treated with 10 μM RA for 2 days and then transfected with pcDNA3 (as a control) or expression vectors FLAG-E47 and FLAG-NeuroD with Lipofectamine 2000. At 72 h after transfection, cells were fixed for ChIP assay as described above with some modifications. Lysates were first immunoprecipitated with α-E47 antibody (sc-763; Santa Cruz Biotechnology), and the eluates were subjected to a second round of immunoprecipitation with α-Flag M2 agarose beads (F3165; Sigma). The primers 5′-GTTACGGTTTGTCACCCGAC and 5′-TTCCCAGATCGTGGAT TCAC were used to amplify a 260-bp region of the *TrkB* promoter. To amplify the promoter region of *p21^Cip1* and *L27*, we used the same primer set as described above.

RESULTS

RA triggers mechanisms to promote cell cycle arrest. In a previous study (5), we reported that RA induces cell cycle arrest and promotes differentiation in SH-SY5Y cells. To study the mechanism triggered by RA to induce these two processes, we monitored the expression of genes involved in the regulation of cell cycle progression and differentiation. Accordingly with the observed withdrawal from the cell cycle, RA treatment for 5 days caused an accumulation of the hypophosphorylated forms of pRb (6) (Fig. 1A), suggesting that cyclin D- and cyclin E-associated kinase activities could be downregulated. However, SH-SY5Y cells maintained invariable levels of D- and E-type cyclins after RA treatment compared to untreated cycling cells (Fig. 1A). The amount of the catalytic subunits Cdk4 and Cdk2 was not affected either by RA treatment, indicating that RA does not regulate these Cdk activities at transcriptional or translational levels in SH-SY5Y cells. We next decided to test whether the accumulation of hypophosphorylated pRB during RA-induced cell cycle arrest could be mediated by Cdk inhibitors. Regarding the KIP family of Cdk inhibitors, RA treatment caused a twofold increase in $p27^{Kip1}$ and a tenfold increase in $p21^{Cip1}$ protein levels, whereas the amount of $p57^{Kip2}$ remained constant. The only inhibitor of the INK family that we were able to detect in SH-SY5Y cells was $p18^{INK4c}$, which remained also constant during RA treatment. As seen in Fig. 1B and C, a comparable increase in Cdk4- and Cdk2-bound $p21^{Cip1}$ was also observed in RA-treated cells. On the other hand, although the level of $p27^{Kip1}$ associated with Cdk2 was unaffected by RA treatment, the amount bound to Cdk4 showed an important increase, a finding which suggests the existence of unequal redistribution mechanisms driven by RA treatment. Taken together, these results suggest that the accumulation of hypophosphorylated pRb species in SH-SY5Y cells could be due to the direct inhibition of cyclin-Cdk complexes mediated by the increase of $p21^{Cip1}$ levels observed after treatment with RA.

Regulation of *TrkB* and *p21^Cip1* expression by RA treatment. Our finding that $p21^{Cip1}$ expression is activated by RA agrees with previous studies with other cell types (11). It is known that the *p21^Cip1* promoter has a functional RA-responsive element localized between bp −1212 and −1194 (11). If we assume that the observed induction of *p21^Cip1* was due to the binding of the RA receptor (RAR) to the RA response element (RARE) in the *p21^Cip1* promoter, we would expect to have an early response of *p21^Cip1* expression, since it has classically been described for signaling mechanisms directly mediated by nuclear receptors such as RAR (39). To test this possibility, we performed a time course experiment to determine the kinetics of *p21^Cip1* expression. Figure 2A and B shows that *p21^Cip1* expression increased in a stepwise manner. During the first 6 h of RA treatment, the expression of *p21^Cip1* showed an early although moderate (~2-fold) induction. A second induction burst was observed at between 24 and 48 h of RA treatment, which caused an overall increase of 10-fold in *p21^Cip1* expression levels, and remained constant during the remaining period analyzed. Changes in $p21^{Cip1}$ protein followed the levels of the *p21^Cip1* transcript, suggesting that *p21^Cip1* is upregulated mainly by transcriptional mechanisms when cells are subjected to RA treatment. As observed in other differentiation models in which a *p21^Cip1* induction has been described (1, 34, 55), the levels of p53 remained constant (Fig. 2B), and the protein was localized in the cytoplasm during RA treatment (data not shown), making the direct participation of p53 in the transcriptional upregulation of *p21^Cip1* by RA treatment unlikely. In a time course analysis, we found that pRb phosphorylation decreased only after 48 h of treatment with RA (Fig. 2B), correlating with the second burst of *p21^Cip1* expression. Thus, the first increase of *p21^Cip1* expression was unable to inhibit pRb phosphorylation significantly. Moreover, as seen in Fig. 2C, the proportion of cells in S phase started to decrease only after 2 days in RA, suggesting that cell cycle arrest would only be achieved when cells reached high levels of *p21^Cip1* expression, as a consequence of the second upregulation step.

It has been reported that RA induces the expression of *TrkB* in SH-SY5Y cells, making them responsive to BDNF (15). *TrkB* is a neuron-specific gene that codes for the receptor of

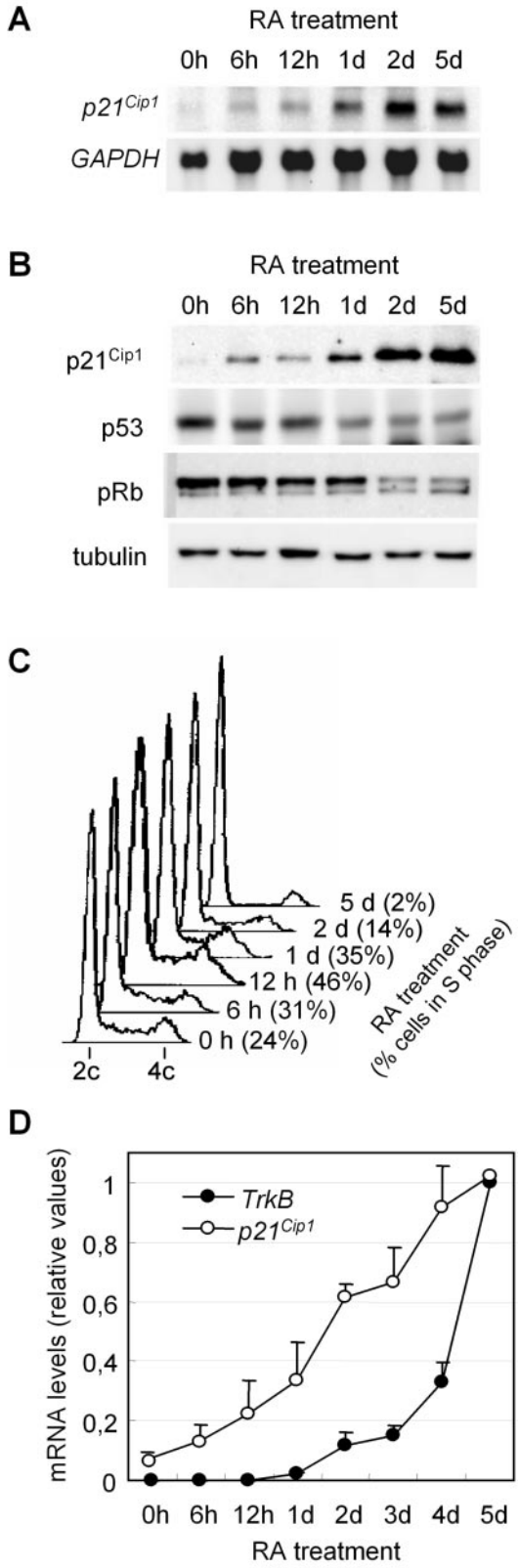

FIG. 2. Effects of RA in a time course experiment. (A) Expression levels of $p21^{Cip1}$ analyzed by Northern blotting. GAPDH mRNA is shown as loading control. (B) Western blot of p21 and p53. The state of phosphorylation of pRB is shown at the bottom. Westerns blots were reprobed with antibody against tubulin as loading control. (C) DNA content distributions during RA treatment. Percentages of cells in S phase during RA treatment are shown. (D) Expression levels of $p21^{Cip1}$ and *TrkB* analyzed by real-time RT-PCR in three independent experiments. Average values and standard deviations are shown. *GADPH* was coamplified in the same RT-PCR as an internal control.

neurotrophin BDNF and is expressed during the final steps of neuronal differentiation. However, the molecular mechanisms involved in the transcriptional activation of *TrkB* during neuronal differentiation are unknown. Since we were also interested in studying the coordination of cell cycle arrest and differentiation induced by RA treatment, we monitored the expression levels of *TrkB* by real-time RT-PCR in a time course experiment. Figure 2D shows that the upregulation of *TrkB* expression takes place 48 h after RA treatment, clearly later than the first burst of $p21^{Cip1}$ induction. Since the *TrkB* promoter sequence does not show any canonical RA-receptor binding sites, these data suggest that RA may use different early and late mechanisms to induce expression of $p21^{Cip1}$ and *TrkB*. The first induction of $p21^{Cip1}$ expression could be directly mediated by the RA receptor as an early event, whereas the mechanisms involved in the second burst of $p21^{Cip1}$ and the induction of *TrkB* expression could be intrinsically different from those causing the early response.

E2A proteins mediate transcriptional activation of *TrkB* and $p21^{Cip1}$. To understand the mechanism by which RA induces *TrkB* expression at the same time that full induction of $p21^{Cip1}$ occurs, we sought to determine whether other known DNA binding elements, different from the RAR site, could participate in this transcriptional regulation. It is known that bHLH proteins are involved in the upregulation of $p21^{Cip1}$ transcription during muscle and enteroendocrine cell differentiation (36). It has been shown by electrophoretic mobility shift assay that bHLH proteins bind the E-box elements located within 180 bp upstream from the start site of the $p21^{Cip1}$ promoter (42). On the other hand, the human *TrkB* promoter sequences show three canonical E-box sequences located at bp −16, −236, and −820 relative to the transcription start site of *TrkB*. To test whether E-box sequences could mediate full induction of $p21^{Cip1}$ and *TrkB* gene expression during RA treatment, luciferase-based reporter constructs containing wild-type and mutant versions of the respective promoter regions (Fig. 3A) were assayed by transfection in SH-SY5Y cells. As expected, normalized luciferase activities obtained from wild-type $p21^{Cip1}$ and *TrkB* promoters showed a fivefold increase when RA-treated versus untreated cycling cells were compared (Fig. 3B). Supporting a direct role of RA on $p21^{Cip1}$ expression, normalized luciferase activities obtained with a $p21^{Cip1}$ promoter mutant lacking the RA-receptor binding site remained nearly constant after RA treatment. More importantly, point mutations targeting the E-box sequences almost abolished the activation caused by RA in both $p21^{Cip1}$ and *TrkB* promoters (Fig. 3B). Thus, E-box sequences seem to play an essential role in the transcriptional response of these two genes during RA treatment. Supporting this idea, Fig. 3C shows that a luciferase-based reporter construct driven by three copies of a synthetic E-box sequence showed a twofold activation by RA treatment. Since point mutations in either the RARE or E-box sequences of the $p21^{Cip1}$ promoter cause strong effects in the

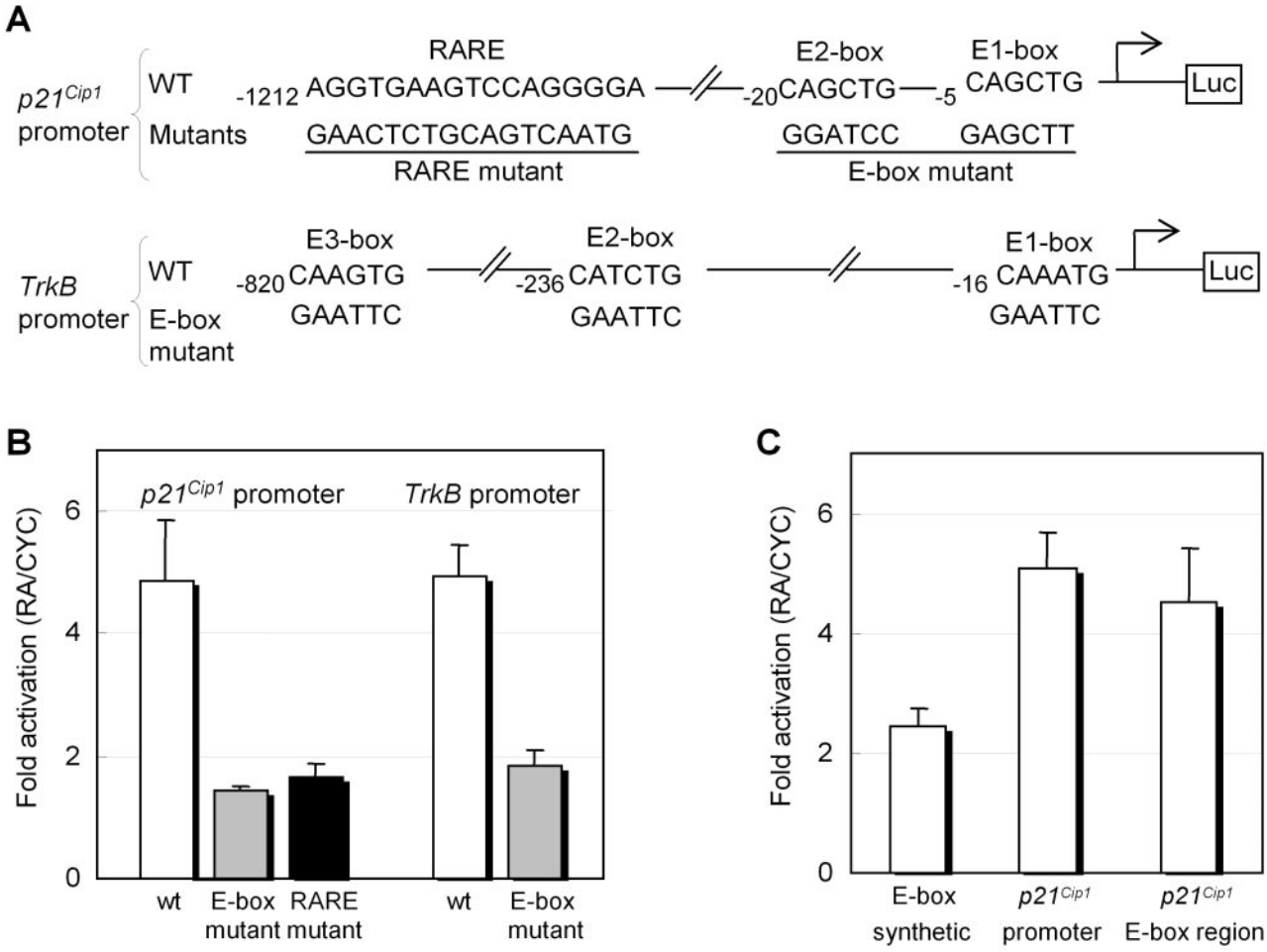

FIG. 3. Transcriptional activation of *p21*^{Cip1} and *TrkB* by RA requires E-box elements. (A) Schematic representation of the *p21*^{Cip1} and *TrkB* promoter fusions to luciferase used in the present study (see Materials and Methods), showing wild-type and mutated E-box and RARE regulatory sequences. (B) Transcriptional activation of the *p21*^{Cip1} and *TrkB* promoter constructs by RA. Normalized expression levels produced by the respective promoter constructs after 5 days of RA treatment were made relative to those obtained in cycling cells. Average values and standard deviations obtained from three independent transfection experiments are shown. (C) Transcriptional activation driven by a three synthetic E-box construct (E-box synthetic), the wild-type *p21*^{Cip1} promoter, and a shorter fragment of the *p21*^{Cip1} promoter containing the E-box elements (*p21*^{Cip1} E-box region) in cells treated with RA for 5 days. Normalized expression levels were obtained as in panel B.

transcriptional response to RA, it suggests the existence of cooperative effects between the RA receptor and bHLH proteins on the full *p21*^{Cip1} promoter. This cooperative effect should involve other elements besides RARE and E-box elements, since a construct that only contains the E-box region of the *p21*^{Cip1} promoter is almost as active as the wild-type promoter (Fig. 3C). Taken together, these data suggest that E-box sequences may be the common elements in *p21*^{Cip1} and *TrkB* promoters essential for RA to cause a full transcriptional activation.

E2A proteins have been found to stimulate *p21*^{Cip1} expression in several cell lines, and it has been shown that E-box sequences mediate this activation (9, 42). On the other hand, Id proteins are negative regulators of bHLH proteins, and it has been shown recently that RA treatment causes a transcriptional downregulation of *Id* genes in SH-SY5Y cells (24). To determine whether these proteins regulate the expression of *TrkB*, we examined the effects of Id2 and the *E2A* gene products, E12 and E47, on *TrkB* promoter activity. In parallel, we checked the expression of *p21*^{Cip1} under the same experimental conditions. SH-SY5Y cells were incubated for 3 days with RA and cotransfected with *TrkB* or *p21*^{Cip1} transcriptional reporter constructs, with the addition of *Id2* or *E2A* expression plasmids (*pCMV-E12* or *pCMV-E47*), and then normalized luciferase activities were determined after 4 days of culture with RA (Fig. 4A). Overexpression of E12 and E47 further increased RA-dependent activation of the *TrkB* promoter by a factor of 2. Similarly, the *p21*^{Cip1} promoter was further activated by the ectopic expression of E12 and E47, although the magnitude of activation was lower compared to the *TrkB* promoter. Figure 4A also shows that Id2 overexpression clearly impaired the transcriptional activation of *TrkB* and *p21*^{Cip1} promoters by RA. Moreover, we observed that cells subjected to *Id2* overexpression incorporated significantly more BrdU

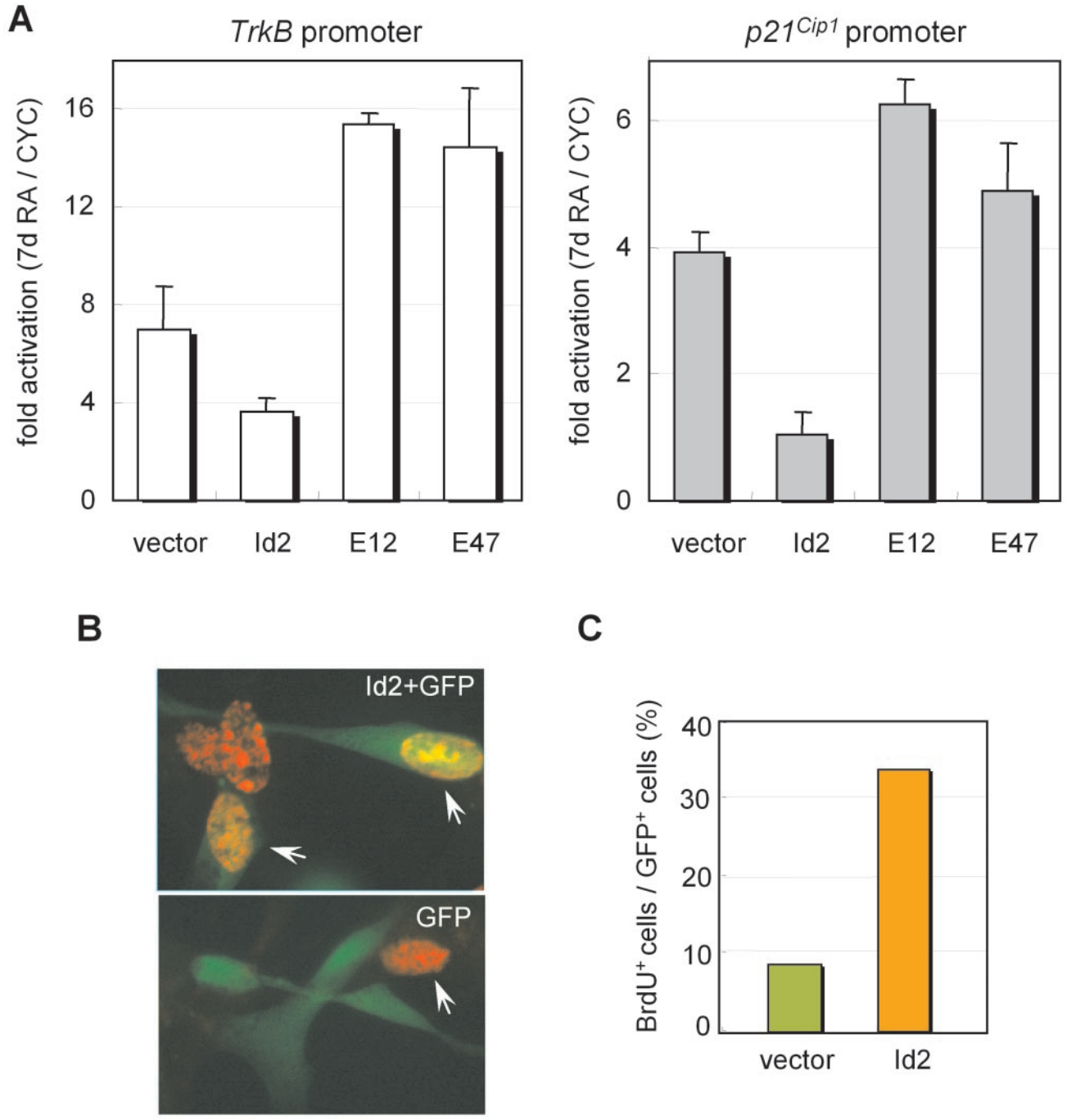

FIG. 4. E2A and Id2 proteins modulate *TrkB* and *p21^{Cip1}* promoter activity in RA-treated cells. (A) *TrkB* (□) and *p21^{Cip1}* (▨) promoter constructs used in Fig. 3 were cotransfected with *E12*, *E47*, and *Id2* expression vectors or the empty vector. Normalized expression levels produced by the respective promoter constructs after 7 days of RA treatment were made relative to those obtained in cycling cells. Average values and standard deviations obtained from three independent transfection experiments are shown. (B) BrdU incorporation as shown by immunofluorescence after 5 days of treatment with RA. Cells were cotransfected with a transfection reporter GFP plasmid and the *Id2* expression vector (top panel) or the empty vector (bottom panel). Although some BrdU incorporation was observed in GFP-negative cells in both panels (see arrow in lower panel), cells positive for both BrdU incorporation and GFP expression were much more frequent if the *Id2* expression plasmid was added (arrows in upper panel). (C) Quantification of the BrdU incorporation shown in panel B. As indicated in Materials and Methods, cells were counterstained with DAPI, and a minimum of 1,000 cells were evaluated in each transfection. The graph shows the percentages of BrdU-positive cells relative to GFP-positive cells.

compared to control cells (Fig. 4B and C). These results suggest that the E2A products, E12 and E47, induce *TrkB* and $p21^{Cip1}$ promoter activity in SH-SY5Y cells during RA treatment, whereas, in addition to its role in pRB inhibition, Id2 could act as a positive regulator of the cell cycle machinery by inhibiting bHLH-driven expression.

E2A proteins and NeuroD bind the *TrkB* promoter in RA-treated cells. To test whether E2A proteins bind to the promoter region of *TrkB* in vivo, we performed ChIP assays with a polyclonal α-E47 antibody that also cross-reacts with E12 protein in RA-treated cells and untreated cycling cells. As shown in Fig. 5A, the α-E47 antibody, but not normal immunoglobulin G, was able to immunoprecipitate the *TrkB* promoter sequences in both RA-treated and untreated cells. In addition, immunoprecipitation of the *TrkB* promoter sequences was specific since DNA from the ribosomal gene L27 included as a control was efficiently removed during the double-immunoprecipitation steps of the procedure used for ChIP. After the *TrkB* and *L27* PCR products were normalized to those obtained in input samples, immunoprecipitation of the *TrkB* promoter from RA-treated cell extracts was found to be approximately six times more efficient than that obtained from untreated cell extracts.

Although the E47 protein has been involved in transcriptional regulation of $p21^{Cip1}$ in other models, molecular proof of a direct implication in vivo was still missing. Thus, we also carried out a ChIP assay with the α-E47 antibody on the $p21^{Cip1}$ promoter in SH-SY5Y cells and obtained positive results (Fig. 5A). Normalized immunoprecipitation efficiencies of the $p21^{Cip1}$ promoter were also higher (about three times higher) in RA-treated cell extracts compared to untreated cell extracts (Fig. 5A). Since the levels of E2A proteins were very similar in both RA-treated and untreated cells (Fig. 5B), these data suggest that the presence of E2A proteins on the $p21^{Cip1}$ promoter may be enhanced by RA treatment. The differences in the two promoters regarding the relative increase of ChIP efficiencies in RA-treated compared to cycling cells may be related to the fact that transcriptional control of *TrkB* expression is much tighter compared to $p21^{Cip1}$. To confirm these results, we carried out a ChIP assay in RA treated cells transiently expressing a Flag-tagged E47 protein (see Fig. 5C). As shown in Fig. 5D, both *TrkB* and $p21^{Cip1}$ promoter sequences were clearly enriched compared to control samples of cells transfected with an empty vector.

Different lines of evidence suggest that NeuroD could act as partner of E2A proteins in the upregulation of *TrkB.* First, inner-ear sensory neurons from *NeuroD*-null mice fail to express *TrkB* and *TrkC* (17). Second, RA has been shown to increase *NeuroD* expression in both SH-SY5Y cells (24) and neural stem cells from adult rat hippocampus (46). Since two commercially available antibodies raised against NeuroD did not efficiently immunoprecipitate NeuroD under ChIP conditions (data not shown), we decided to use a Flag-tagged NeuroD protein (see Fig. 5C). In order to minimize the overexpression effects due to transient transfection of the Flag-NeuroD construct, cross-linked cell extracts were subjected to the polyclonal α-E47 antibody in a first immunoprecipitation round and to α-Flag beads in the second immunoprecipitation step. In this way we should only detect promoter sequences not only bound by overexpressed NeuroD but also by endogenous

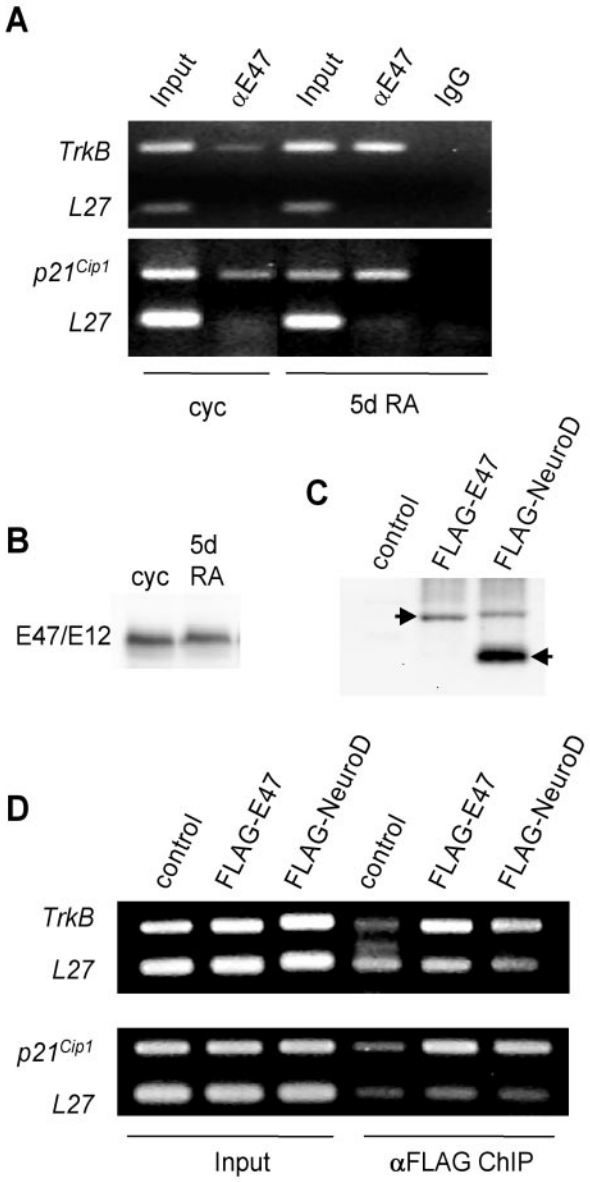

FIG. 5. bHLH proteins bind the *TrkB* and $p21^{Cip1}$ promoters in vivo. (A) Cells either cycling or treated for 5 days with RA were processed for ChIP with a polyclonal α-E47 antibody. A dummy affinity-purified IgG was used as control in extracts obtained from RA-treated cells. Input material and immunoprecipitates were analyzed by PCR with oligonucleotide primers specific for the *TrkB* and $p21^{Cip1}$ promoters (see Materials and Methods). *L27* was coamplified in the same PCR as the internal negative control. (B) E47 levels were determined by Western blot in the same samples taken as in panel A. (C) For ChIP with α-FLAG agarose beads, SH-SY5Y cells were transfected with empty vector as a negative control or vectors encoding FLAG-E47 and FLAG-NeuroD, which were analyzed by Western blotting to detect ectopically expressed proteins. (D) Cells transfected with vectors as in panel C were treated with RA for 5 days prior to the ChIP assay. Lysates were immunoprecipitated with an α-E47 antibody (a polyclonal α-E47 antibody that cross-reacts with E12), and eluates were subjected to a second round of immunoprecipitation with α-FLAG M2 agarose beads (see Materials and Methods for details). *L27* was coamplified in the same PCR as the internal control.

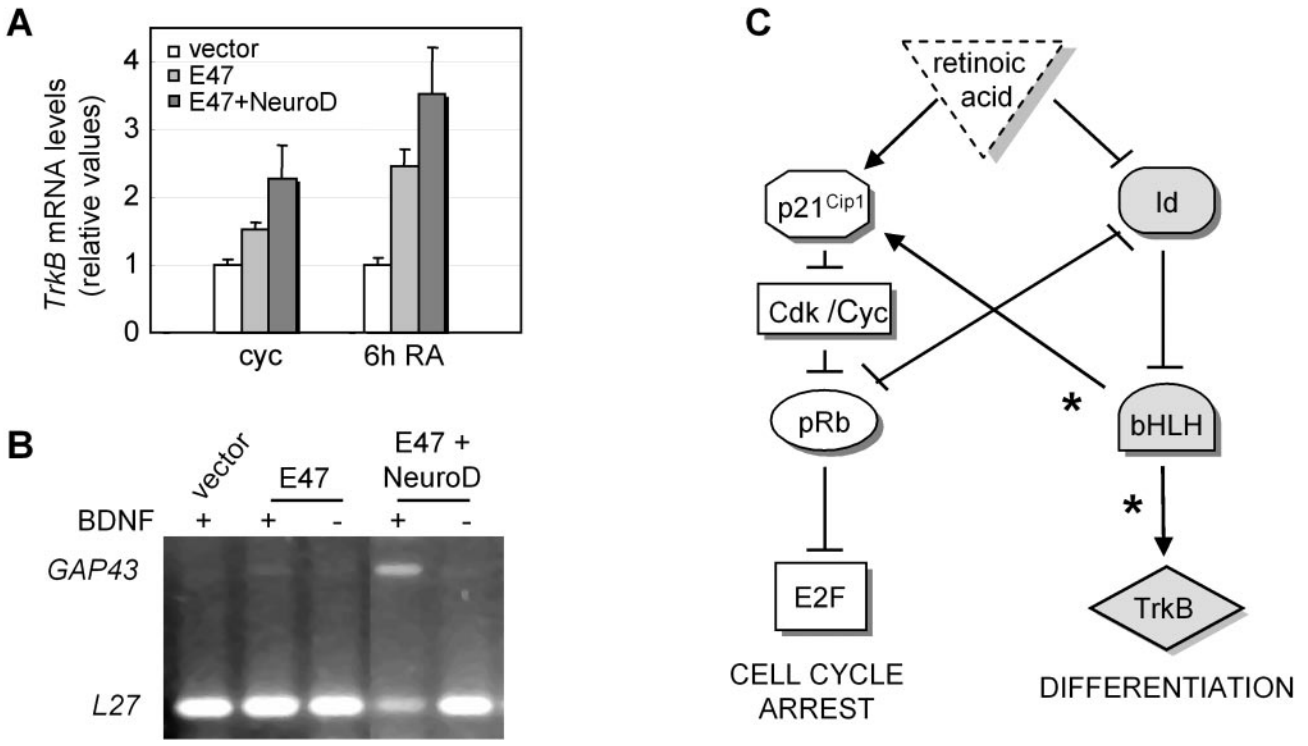

FIG. 6. Induction of endogenous *TrkB* by bHLH proteins. (A) SH-SY5Y cells were transfected with empty vector or vectors encoding E47 and NeuroD. Transfected cells cycling (cyc) or treated for 6 h (6 h RA) with RA were used to analyze the expression levels of *TrkB* by real-time RT-PCR. Average values and standard deviations obtained from three independent transfection experiments are shown. (B) *GAP43* expression analyzed by RT-PCR. Transfected cells as in panel A were treated for 2 days with RA and then stimulated or not with BDNF cultured for 6 h in serum-free medium. *L27* was coamplified in the same PCR as an internal control. The primer set used to amplify the promoter region of *GAP43* and *L27* were as described previously (4). (C) Scheme showing functional interactions between elements involved in controlling cell cycle and differentiation. Asterisks mark major contributions of the present study (see Discussion for details).

levels of E2A proteins. As shown in Fig. 5D, the results obtained by competitive PCR strongly suggest that NeuroD is present with E2A proteins on both the *TrkB* and $p21^{Cip1}$ promoters.

To test the functional relevance of these interactions, we sought to determine whether ectopic expression of E47 and NeuroD could increase the endogenous expression levels of $p21^{Cip1}$ and *TrkB* in the absence of RA. Since the $p21^{Cip1}$ gene is already expressed in cycling cells, small increases over the basal level on a fraction of cells (we routinely obtain 20 to 30% of transfected cells as assayed by green fluorescent protein [GFP] coexpression) would be difficult to detect. Instead, *TrkB* mRNA is almost undetectable in SH-SY5Y cycling cells (see Fig. 2D). For this reason we decided to test by real-time PCR whether *TrkB* expression levels could be upregulated by ectopic E47 and NeuroD in the absence of RA and 6 h after RA addition. As shown in Fig. 6A, a minor but reproducible effect was observed when cycling cells were transfected with E47 or E47 plus NeuroD. Nonetheless, the relative difference increased when cells were treated for 6 h with RA prior to sample collection for real-time PCR, which suggests that RA may exert posttranslational effects on the activity of these bHLH proteins. Finally, Fig. 6B shows that ectopic expression of E47 or E47 plus NeuroD enhanced the transcriptional activation of *GAP43* in a BDNF-dependent manner (4). Taken together, these results strengthen the notion that E2A and NeuroD bHLH proteins are key activators of *TrkB* expression.

DISCUSSION

Terminal differentiation of many tissues and cell types involves an initial phase of growth and division of precursor cells and a subsequent process of differentiation and withdrawal from the cell cycle. Precise regulation of the timing of these processes is very important to ensure the production of a proper number of differentiated cells at an appropriate time. A variety of observations in primary cultures support a role for RA in neuronal differentiation. When hippocampus-derived stem cells are exposed to RA, the expression of neurotrophin receptors is upregulated (46). The sequential addition of RA and BDNF or NT-3 to these cells leads to a significant increase in the number of mature neurons generated. In vitro studies with embryonic stem (ES) cells demonstrated that RA was able to drive ES cell differentiation to a neuronal cell type. As would be expected in vivo, neuronal determination depended on the developmental stage at which they were treated with RA, suggesting that differentiation signals are specifically interpreted as a function of time during development (43). These and other observations (16, 30) suggest that RA may be involved in the acquisition of a neuronal fate and that collabo-

rative effects between RA and neurotrophins may be needed to achieve a fully developed neuronal phenotype. The human neuroblastoma cell line SH-SY5Y has proven to be a valuable model for studying the effects of RA on neuronal differentiation, including extension of neuritic processes, expression of the *TrkB* receptor, and development of an enhanced choline acetyltransferase activity (5, 15, 38).

Here we show that (i) RA activates expression of both *TrkB* and $p21^{Cip1}$ at a transcriptional level; (ii) full transcriptional activation of these two genes require the E-box sequences found in the *TrkB* and $p21^{Cip1}$ promoters; (iii) E47 and E12 proteins enhance the expression of *TrkB* and $p21^{Cip1}$, whereas Id2 exerts opposite effects; and (iv) E2A and NeuroD proteins bind the *TrkB* and $p21^{Cip1}$ promoters; and (v) ectopic expression of these bHLH transcriptional factors is able to activate *TrkB* expression in the absence of RA. Since they regulate expression of both a cell cycle inhibitor, $p21^{Cip1}$, and a neurotrophic receptor, *TrkB*, bHLH proteins would play a key role in the coordination between cell cycle arrest and neuronal differentiation.

Upregulation of $p21^{Cip1}$ expression as an early event induced by RA has been reported by different groups (23, 46), and RA transcriptionally upregulates $p21C^{ip1}$ through an RA-responsive element in the promoter region of $p21^{Cip1}$ during RA-induced monocyte differentiation of U937 cells (23). According to these data we show that the RAR-binding site is important for transcriptional activation of the $p21^{Cip1}$ promoter by RA. In addition, we have found by ChIP assays that RARα, the all-*trans*-RA receptor, binds directly to the RAR element in the $p21^{Cip1}$ promoter when SH-SY5Y cells are treated with RA (unpublished results). Thus, the initial changes in $p21^{Cip1}$ expression in SH-SY5Y cells may be directly mediated by the RA receptor as an early event.

We have shown that $p21^{Cip1}$ expression suffers a second burst of induction after 2 days of RA treatment that is quantitatively much more important than the early increase and causes accumulation of hypophosphorylated pRB with a subsequent G_1 arrest. Our findings suggest that the accumulation of hypophosphorylated pRB forms could be a consequence of the inhibition of Cdk4/cyclin D1 and Cdk2/cyclin E complexes by binding to increasing amounts of $p21^{Cip1}$ (and $p27^{Kip1}$ to a lesser extent). Elevation of $p21^{Cip1}$ levels by RA has been found also in other neuroblastoma cell lines, and ectopic expression of $p21^{Cip1}$ was sufficient to produce a cell cycle arrest in human SK-N-SH-N cells (51), a neuroblastoma cell line closely related to the SH-SY5Y cells used in our study.

Transcriptional activation of $p21^{Cip1}$ is mediated by bHLH proteins and requires the E-box elements of the $p21^{Cip1}$ promoter. We have shown that *TrkB* expression is also upregulated in SH-SY5Y cells simultaneously with the second burst of $p21^{Cip1}$ expression. It is well known that RA is able to increase *TrkB* expression in many neuroblastoma cell lines, including KCNR, LAR-5, and SH-SY5Y cells (15, 26, 38). On the other hand, in adult-derived neuronal stem cell cultures (46), the cellular responses to RA included upregulation of $p21^{Cip1}$, exit from the cell cycle, and a subsequent upregulation of neurotrophin receptors (*TrkA*, *TrkB*, *TrkC*, and $p75^{NGFR}$). Nonetheless, the molecular mechanisms by which RA activates *TrkB* gene transcription were not identified.

Here we show for the first time that E2A proteins bind the *TrkB* promoter in vivo. Consistent with this result, overexpression of E2A proteins clearly increases the *TrkB* promoter activity even in the absence of RA. Moreover, we have also proved the DNA-binding activity of E2A proteins toward the $p21^{Cip1}$ promoter in vivo. The relative efficiencies of ChIP with the α-E47 antibody for both *TrkB* and $p21^{Cip1}$ promoters were slightly higher in RA-treated cells. Considering that the overall protein levels of E2A are very similar in both cycling and RA-treated cell extracts, we propose that RA may modulate the DNA-binding ability of these bHLH proteins, either as a homodimer or a heterodimer with class II bHLH proteins. Although E2A proteins form homodimers and bind to E-box elements in the immunoglobulin heavy-chain enhancer to activate B-cell differentiation (44), E proteins have been found to bind to DNA usually as heterodimers with tissue-specific bHLH proteins (12, 35, 40, 42). Inner-ear sensory neurons depend on NeuroD as a tissue-specific bHLH protein for survival during differentiation, and mice lacking *NeuroD* produced inner-ear sensory neurons that failed to express the neurotrophin receptors *TrkB* and *TrkC*, suggesting that the ability of NeuroD to support neuronal survival may involve the regulation of neurotrophin receptor expression (17). In addition, RA has been shown to increase *NeuroD* expression in both SH-SY5Y cells (24) and neural stem cells from adult rat hippocampus (46). Accordingly, we have detected the presence of ectopically expressed FLAG-NeuroD on both the *TrkB* and $p21^{Cip1}$ promoters, suggesting that this tissue-specific bHLH protein could be involved in regulating *TrkB* and $p21^{Cip1}$ expression in SH-SY5Y cells.

Loss of Id proteins from proliferating zones of the brain, which occurs during early neurogenesis, plays an important role in initiating expression of neuronal genes and differentiation (27). Oligodendrocyte precursor cells forced to overexpress *Id2* exhibit a substantially attenuated ability to differentiate. On the other hand, $Id2^{-/-}$ oligodendrocytes show premature differentiation in mice (53). We have found that, in agreement with its negative role in regulating transcription activity of bHLH proteins, Id2 is also capable of inhibiting the transcription of *TrkB* during RA treatment.

In addition to their role in regulating differentiation, Id proteins play active roles in cell cycle regulation (37). In vivo, neuroblasts derived from $Id1^{-/-}$ $Id3^{-/-}$ fetal brain exit prematurely from the cell cycle, accompanied by elevated expression of $p16^{Ink4a}$ and $p27^{Kip1}$ (27). Further, $Id2^{-/-}$ mammary epithelial cells are defective in proliferation during pregnancy and exhibit elevated levels of $p21^{Cip1}$ and $p27^{Kip1}$ expression (33). According to this, overexpression of Id2 in RA-treated SH-SY5Y cells was able to prevent activation of the $p21^{Cip1}$ promoter, and produced an abnormal S-phase entry.

Several studies have demonstrated that pRB is a key regulator of neuronal development (7). In this regard, the expression of neuronal differentiation markers such as βII tubulin, *TrkA*, *TrkB*, and $p75^{NGFR}$ is significantly decreased in pRb-deficient embryos (21). On the other hand, pRB and Id2 counteract functionally by a direct physical interaction. Id2 is able to bind pRb and abolish its growth-suppressing activity (13, 20), whereas a constitutively active pRB mutant is able to suppress Id2 overexpression-mediated effects in neuron-specific gene expression and apoptosis (48). According to this role in bHLH-mediated gene expression, we have found that a nonphosphor-

ylatable pRb mutant is able to activate *TrkB* and $p21^{Cip1}$ expression in SH-SY5Y cells (unpublished results).

The close relationship between Id proteins and the regulation of cell proliferation and differentiation suggests that these inhibitory proteins may play a key role connecting RA signals to bHLH protein activity. Myc has been involved in transcriptional activation of *Id2* (8, 20), and RA causes a dramatic decrease in Myc levels as a very early response in SH-SY5Y cells (24). Although other factors controlling *Id* gene expression may be involved in human neuroblastoma (50, 52), Myc downregulation could explain the subsequent decrease of Id2 caused by RA in SH-SY5Y cells, thus producing an increase in bHLH protein activity. Ectopic overexpression of E2A proteins in SH-SY5Y cells was not sufficient to cause a clear delay in cell cycle entry in the absence of RA (our unpublished data), suggesting that cell cycle arrest would involve both upregulation of bHLH-mediated $p21^{Cip1}$ transcription and release of hypophosphorylated pRB by Id2 downregulation.

Similar coordination mechanisms involving bHLH proteins in osteoblast differentiation have recently been proposed by Funato et al. (9). Although proof for a direct protein-DNA interaction was not provided, these authors found that E2A proteins induce transcription of $p21^{Cip1}$ and fibroblast growth factor receptor 3 (*FGFR3*), which is important for osteoblast differentiation. In addition, bHLH negative regulators including Id1, Id3, and Twist inhibited the transcriptional activation of both $p21^{Cip1}$ and *FGFR3*.

In conclusion, our findings show that bHLH proteins activate expression of genes that lead to cell cycle arrest and differentiation in a coordinate way. This coordination would be mediated by the fact that bHLH proteins bind the promoter of $p21^{Cip1}$, a cell cycle exit gene, and the promoter of *TrkB*, a gene encoding a key tyrosine kinase receptor for differentiation and survival in the nervous system.

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