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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 development (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
in SH-SY5Y cells, a human neuroblastoma cell line, RA treatment induces a neuronal differentiation process, with a concomitant G<sub>1</sub> 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<sup>Cip1</sup> by RA depends on E-box sequences. Full activation of p21<sup>Cip1</sup> 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<sup>Cip1</sup>, 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<sup>Cip1</sup> 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<sub>2</sub> in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 2 mM l-glutamine, 20 U of penicillin/ml, 20 μg 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<sup>Cip1</sup>-luciferase reporter construct carries a 2.3-kb fragment from positions −2296 to +2, relative to the transcription start site of the human p21<sup>Cip1</sup> promoter into the KpnI-Xhol 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<sup>Cip1</sup> promoter E-box and RARE mutants were produced by site-directed mutagenesis by using the transformant site-directed mutagenesis kit (Clontech). The construct with the p21<sup>Cip1</sup>-E-box region contains sequences from positions −212 to +30 of the p21<sup>Cip1</sup> promoter. Three E-box consensus sequences (TGTCGCCCATCTGCTGATCC) 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 (sc-153; Santa Cruz Biotechnology), α-Cdk1 (06-194; Upstate), α-cyclin D1 (06-137; Upstate), α-cyclin E (06-459; Upstate), α-p21<sup>Cip1</sup> (05-345; Upstate), α-p53 (05-224; Upstate), α-p27<sup>Kip1</sup> (610241; Transduction Laboratories), α-tubulin (T5168; Sigma), α-p16<sup>ink4a</sup> (NA55; Oncogene Research Products), α-p73<sup>α</sup> (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<sup>Cip1</sup> (Hs00177881_m1 MGB probe with FAM reporter label), human TrkB (Hs00177888_m1 MGB probe with VIC reporter label), and human GAPDH (Hs99999905_m1 MGB probe with VIC reporter label). Samples were run and analyzed with a 7900 HT real-time detection system (Bio-Rad). Northern blot analysis 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<sup>4</sup> 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. After 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 DNsase 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 pelleted and suspended in lysis buffer (50 mM Tris-HCl [pH 8.1], 0.5 mM EDTA, 1% Triton X-100) and fixed for 10 min at room temperature. Cells were then treated with 50 μg of DNsase I (Roche)/ml at 37°C for 30 min, incubated with a mouse monoclonal α-e47 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.
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 p27Kip1 and a tenfold increase in p21Cip1 protein levels, whereas the amount of p57Kip2 remained constant. The only inhibitor of the INK family that we were able to detect in SH-SY5Y cells was p18INK4c, which remained also constant during RA treatment. As seen in Fig. 1B and C, a comparable increase in Cdk4- and Cdk2-bound p21Cip1 was also observed in RA-treated cells. On the other hand, although the level of p27Kip1 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 p21Cip1 levels observed after treatment with RA.

**Regulation of TrkB and p21Cip1 expression by RA treatment.** Our finding that p21Cip1 expression is activated by RA agrees with previous studies with other cell types (11). It is known that the p21Cip1 promoter has a functional RA-responsive element localized between bp −1212 and −1194 (11). If we assume that the observed induction of p21Cip1 was due to the binding of the RA receptor (RAR) to the RA response element (RARE) in the p21Cip1 promoter, we would expect to have an early response of p21Cip1 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 p21Cip1 expression. Figure 2A and B shows that p21Cip1 expression increased in a stepwise manner. During the first 6 h of RA treatment, the expression of p21Cip1 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 p21Cip1 expression levels, and remained constant during the remaining period analyzed. Changes in p21Cip1 protein followed the levels of the p21Cip1 transcript, suggesting that p21Cip1 is upregulated mainly by transcriptional mechanisms when cells are subjected to RA treatment. As observed in other differentiation models in which a p21Cip1 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 p21Cip1 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 p21Cip1 expression. Thus, the first increase of p21Cip1 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 p21Cip1 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

**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
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\textsuperscript{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\textsuperscript{Cip1} and TrkB. The first induction of p21\textsuperscript{Cip1} expression could be directly mediated by the RA receptor as an early event, whereas the mechanisms involved in the second burst of p21\textsuperscript{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\textsuperscript{Cip1}**. To understand the mechanism by which RA induces TrkB expression at the same time that full induction of p21\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{Cip1} expression, normalized luciferase activities obtained with a p21\textsuperscript{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\textsuperscript{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\textsuperscript{Cip1} promoter cause strong effects in the

![FIG. 2. Effects of RA in a time course experiment. (A) Expression levels of p21\textsuperscript{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\textsuperscript{Cip1} and TrkB analyzed by real-time RT-PCR in three independent experiments. Average values and standard deviations are shown. GAPDH was coamplified in the same RT-PCR as an internal control.](http://mbc.asm.org/)
transcriptional response to RA, it suggests the existence of cooperative effects between the RA receptor and bHLH proteins on the full p21Cip1 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 p21Cip1 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 p21Cip1 and TrkB promoters essential for RA to cause a full transcriptional activation.

E2A proteins have been found to stimulate p21Cip1 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 p21Cip1 under the same experimental conditions. SH-SY5Y cells were incubated for 3 days with RA and cotransfected with TrkB or p21Cip1 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 p21Cip1 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 p21Cip1 promoters by RA. Moreover, we observed that cells subjected to Id2 overexpression incorporated significantly more BrdU.

FIG. 3. Transcriptional activation of p21Cip1 and TrkB by RA requires E-box elements. (A) Schematic representation of the p21Cip1 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 p21Cip1 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 p21Cip1 promoter, and a shorter fragment of the p21Cip1 promoter containing the E-box elements (p21Cip1 E-box region) in cells treated with RA for 5 days. Normalized expression levels were obtained as in panel B.
E2A and Id2 proteins modulate TrkB and p21Cip1 promoter activity in RA-treated cells. (A) TrkB (■) and p21Cip1 (□) 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-SYSY 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 RAtreated cells.** To test whether E2A proteins bind to the promoter region of TrkB in vivo, we performed ChIP assays with a polyclonal \( \alpha \)-E47 antibody that also cross-reacts with E12 protein in RA-treated and untreated cycling cells. As shown in Fig. 5A, the \( \alpha \)-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 \( \alpha \)-E47 antibody on the \( p21^{cip1} \) promoter in SH-SYSY 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 a 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-SYSY 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 \( \alpha \)-E47 antibody in a first immunoprecipitation round and to \( \alpha \)-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...
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 p21Cip1 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 p21Cip1 and TrkB in the absence of RA. Since the p21Cip1 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-
relative 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 p21Cip1 at a transcriptional level; (ii) full transcriptional activation of these two genes require the E-box sequences found in the TrkB and p21Cip1 promoters; (iii) E47 and E12 proteins enhance the expression of TrkB and p21Cip1, whereas Id2 exerts opposite effects; and (iv) E2A and NeuroD proteins bind the TrkB and p21Cip1 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, p21Cip1, and a neurotrophic receptor, TrkB, bHLH proteins would play a key role in the coordination between cell cycle arrest and neuronal differentiation.

Upregulation of p21Cip1 expression as an early event induced by RA has been reported by different groups (23, 46), and RA transcriptionally upregulates p21Cip1 through an RA-responsive element in the promoter region of p21Cip1 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 p21Cip1 promoter by RA. In addition, we have found by ChIP assays that RARα, the all-trans-RAR receptor, binds directly to the RAR element in the p21Cip1 promoter when SH-SY5Y cells are treated with RA (unpublished results). Thus, the initial changes in p21Cip1 expression in SH-SY5Y cells may be directly mediated by the RA receptor as an early event.

We have shown that p21Cip1 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 G1 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 p21Cip1 (and p27Kip1 to a lesser extent). Elevation of p21Cip1 levels by RA has been found also in other neuroblastoma cell lines, and ectopic expression of p21Cip1 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 p21Cip1 is mediated by bHLH proteins and requires the E-box elements of the p21Cip1 promoter. We have shown that TrkB expression is also upregulated in SH-SY5Y cells simultaneously with the second burst of p21Cip1 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 p21Cip1, exit from the cell cycle, and a subsequent upregulation of neurotrophin receptors (TrkA, TrkB, TrkC, and p75NTR). 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 p21Cip1 promoter in vivo. The relative efficiencies of ChIP with the α-E47 antibody for both TrkB and p21Cip1 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 p21Cip1 promoters, suggesting that this tissue-specific bHLH protein could be involved in regulating TrkB and p21Cip1 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 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 p16Ink4a and p15Ink4b (27). Further, Id2-/- mammary epithelial cells are defective in proliferation during pregnancy and exhibit elevated levels of p21Cip1 and p27Kip1 expression (33). According to this, overexpression of Id2 in RA-treated SH-SY5Y cells was able to prevent activation of the p21Cip1 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(NTR) 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-
yaltable pRB mutant is able to activate TrkB and p21<sup>Cip1</sup> 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<sup>Cip1</sup> 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<sup>Cip1</sup> 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<sup>Cip1</sup> 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<sup>Cip1</sup>, 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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