Activation of the Phosphatidyl-Inositol-3 Kinase/Akt signaling pathway by Retinoic Acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells

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Running title:
PI3K/Akt signaling required for RA-induced differentiation

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SUMMARY

Retinoic Acid (RA) induces neural differentiation of SH-SY5Y neuroblastoma cells. We show that the mRNA levels of the differentiation-inhibiting bHLH transcription factors *ID1*, *ID2* and *ID3* are downregulated during RA-induced differentiation of SH-SY5Y cells. The levels of ID proteins decreased in parallel to the observed transcriptional repression. The expression of other bHLH genes changed during RA-induced differentiation: expression of neuroblast-specific *ASCL1* (HASH-1) gene was promptly reduced after RA treatment, whereas expression of differentiation-promoting genes *NEUROD6* (NEX-1, HATH-2) and *NEUROD1* was increased. Treatments with TPA, another inducer of neuroblastoma cell differentiation, also resulted in coordinated downregulation of ID gene expression, underscoring the role of ID genes in differentiation. Downregulation of ID gene expression by RA involves a complex mechanism, since full transcriptional repression required newly synthesized proteins and signaling by the Phosphatidylinositol-3 kinase. RA treatment activates the Phosphatidylinositol-3 kinase/Akt signaling pathway, resulting in increased Phosphatidylinositol-3 Kinase activity in extracts from RA-treated cells and a rapid increase in phosphorylation of Akt in Ser473. Inhibition of Phosphatidylinositol-3 kinase by LY294002 impaired RA-induced differentiation, as assessed by morphological and biochemical criteria. We propose that RA, by activating the Phosphatidylinositol-3 kinase/Akt signaling pathway, plays an important role in the regulation of neuronal cell survival.

INTRODUCTION

Cell differentiation is a complex process regulated by an interplay between intrinsic cellular programs, cell-cell and cell-substrate interactions and a plethora of soluble extra-cellular signaling molecules, including hormones, growth factors, cytokines, trophic factors, morphogens, etc. Retinoic Acid (RA)[1], the biologically active form of vitamin A, plays important role in early embryonic development and in the generation of several organs and systems, like it is the case of the nervous system (1-3, for review). *In vitro*, RA also plays a prominent role in regulating the transition from the proliferating precursor cell to the post-mitotic differentiated cell, and there are many examples in the literature of distinct cell types whose differentiation is under the control of RA (4-10).

Two types of receptors, the RARs and the RXRs (11) mediate RA signaling. These receptors belong to the superfamily of the Nuclear Hormone Receptors, which function as ligand-regulated transcription factors, and act modifying the transcriptional activity of specific genes (12, 13, for review). To elucidate the molecular mechanisms through which RA induces differentiation of neural cells, we have analyzed the changes in gene expression associated with RA-induced differentiation using human neuroblastoma cells as a model system. Neuroblastoma is a childhood tumor that arises from neural crest neuroectodermal cells (14, 15, for review). Despite their tumoral origin, neuroblastoma cell lines can be induced to differentiate *in vitro* by several agents, including RA (7, 16-18). *In vitro* differentiated neuroblastoma cells have a neuronal phenotype, as judged by their morphology and the expression of biochemical and functional neuronal markers. Therefore, neuroblastoma cells could be considered as an useful model system to study the initial phases of neuronal differentiation (19). RA derivatives are being employed in the therapy of neuroblastoma, and neuroblastoma patients treated with RA have increased survival rates (20, 21). Nevertheless, little is known about the molecular basis of the therapeutic effects of RA on neuroblastoma tumors.

Here we show that RA treatment of SH-SY5Y human neuroblastoma cells leads to changes in the expression levels of bHLH transcription factors, a gene family involved in the regulation of lineage commitment and cell differentiation in many cell types (22, for review). The products of ID genes act as dominant-negative inhibitors of differentiation by avoiding the binding of other bHLH transcription factors to their cognate DNA elements named E-boxes. RA treatment of SH-SY5Y neuroblastoma cells leads to a coordinated downregulation of the differentiation-inhibiting *ID1*, *ID2* and *ID3* genes. In addition, the expression of the neuroblast-specific bHLH transcription factor *ASCL1* (HASH-1) is rapidly reduced, and the mRNA levels of the differentiation-promoting bHLH factors *NEUROD6* and *NEUROD1* are increased. These results support the idea that RA treatment may induce differentiation by altering the balance between differentiation-inhibiting and differentiation-promoting bHLH factors.

The mechanism through which RA downregulates the ID gene expression appeared to be complex, since repression required newly synthesized proteins and the activity of Phosphatidyl-inositol-3 Kinase (PI3K). RA treatment rapidly activates the PI3K/Akt signaling pathway, which is a requisite for neuroblastoma cell differentiation, since treatment with PI3K specific inhibitor LY294002 impaired RA-induced differentiation. The results described here show evidence of novel actions of RA contributing to the induction of neural cell differentiation. In addition, the activation of PI3K/Akt signaling pathway by RA gives support for a heretofore unrecognized role of RA on the regulation of survival in neural cells, that provides a link between cell differentiation and cell survival.

EXPERIMENTAL PROCEDURES

Cell Culture and treatments.

SH-SY5Y human neuroblastoma cells were cultured in RPMI1640 medium with 10% fetal calf serum and 0.15 mg/ml gentamycin sulfate in a humidified incubator at 37ºC with 5% CO_2. The medium was replaced every 3 days and the cells were split before they reached confluence. RA (all-trans retinoic acid), TPA (phorbol 12-myristate, 13-acetate), cycloheximide and LY294002 (all from Sigma) were dissolved in ethanol or DMSO and added to the culture medium at the indicated concentrations.

DNA probes

Most of the DNA probes were inserts from EST-containing plasmids obtained from IMAGE Consortium (LLNL) (23). The IMAGE ID numbers for the different probes are 148057 (*ID3*), 235131 (*ID1*), 203370 (*ID2*), 1032880 (*ID4*), 1569301 (*ASCL1*), 171864 (*NEUROD6*), 2518343 (*NEUROD1*), 610197 (*GAP43*), and 232714 (*BCL2*). *RARB* probe consisted in a 1.4 Kb DNA fragment containing the human RARβ2 cDNA (24). *MYC* probe consisted of a 1.3 Kb genomic fragment containing human *MYC* exon 3 (25). Probes for *NPY*, *ACTB* and *PPIA* were generated by RT-PCR from RNA from SH-SY5Y cells. *NPY* probe was a 403 bp fragment, including nucleotides 5-407 of *NPY* cDNA (26). *ACTB* (actin beta) probe was a 183 bp fragment, encompassing nucleotides 287-470 of the *ACTB* cDNA (27). *PPIA* (peptidylprolyl isomerase A, cyclophilin A) probe was a 415 bp fragment, including nucleotides 101-516 of the *PPIA* cDNA (28).

Northern Blot

Total RNA was obtained from PBS-washed cell monolayers by the method of Chomczynski and Sacchi (29). RNA (15-20 µg per lane) was denatured, electrophoresed through 1% agarose-formaldehyde gels and transferred to nylon membranes (NytranN45, Schleicher and Schuell) following standard techniques. DNA probes were labeled at high specific activity with [α^{32}P]-dCTP (Amersham-Pharmacia Biotech, spec. act.>3000 Ci/mmol) by multiprime (RadPrime labeling system, Life Technologies). Hybridization was carried out overnight at 65°C according to Church and Gilbert (30). Filters were washed with 0.5xSSC, 0.1% SDS, first at room temperature for 10 min, and then for 30 min at 65ºC. Membranes were exposed to X-ray films at -70ºC with intensifying screens.

Nuclear Run-on transcription assay.

Preparation of nuclei. About 1-5x10^6 cells were lysated at 4ºC in 20 mM Tris-HCl, pH 8.0, 0.3 M Sucrose, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM Spermidine, 0.15 mM Spermine, 0.5 mM β-mercapto-ethanol and 0.1% Nonidet P-40. After washing with cold buffer B (50 mM HEPES-NaOH pH 8.0, 5 mM $MgCl_2$, 0.5 mM DTT, 1 µg/ml Bovine Serum Albumin, 25 % Glycerol), nuclei were pelleted by centrifugation and carefully resuspended in the same buffer B. Aliquots of 50 µl containing 2-10x10^6 nuclei were made, snap frozen in liquid nitrogen and stored at -80ºC.

Preparation of the filters. 5 µg of linearized plasmids containing cDNA probe fragments (*ID3, BCL2* and pBS-KS) or 1 µg of purified PCR fragments (*ACTB, PPIA*) were denatured with NaOH and neutralized by adding 10xSSC. Samples were applied to Nytran N45 membranes (Schleicher and Schuell) with the aid of a slot-blot apparatus (Minifold II, Schleicher and Schuell), and the DNA was fixed to the membrane by UV radiation.

Run-on Transcription. Transcription reactions were set with 50 µl of Transcription mix (50 mM Hepes-NaOH, pH 8.0, 2 mM $MgCl_2$, 2mM $MnCl_2$, 1 mg/ml Bovine Serum Albumin, 300 mM NH_4Cl), 5 µl of 10 mM CTP, ATP, GTP mix, 100 µCi [α-^{32}P]-UTP (Amersham Pharmacia Biotech, spec. act. 800 Ci/mmol), 40 u. RNAsin (Promega) and 50 µl of nuclei (2-10x10^6) in buffer B. After incubation for 20 min at 30ºC, nuclear DNA was digested with 10 u of RQ1 DNAse (Promega), and the labeled RNA purified according to Chomczynski and Sacchi (29). The RNA in the aqueous phase was precipitated twice with Isopropanol to eliminate the unincorporated nucleotide, and the RNA pellet was resuspended in DEPC-treated water. The incorporated radioactivity is estimated by measuring an aliquot in a scintillation counter (^{32}P-Cerenkov). Normally, 1-5x10^6 cpm/reaction are obtained.

Hybridization. Filters were pre-hybridized in 2 ml of 200 mM sodium phosphate, pH 7.2, 1 mM EDTA, 7% SDS, 45% Formamide, 250 µg/ml yeast tRNA for 2-3 h at 42ºC, in a rotary oven. Equal amounts of labeled RNA probe obtained for each condition in 100 µl of DEPC-treated water were mixed with 1 vol of 99% Formamide, denatured at 65ºC, and chilled out on ice. The denatured probe was mixed with 2 ml of fresh hybridization solution, and added to the filters. Filters are hybridized for at least 40 h at 42ºC, and washed with 0.5xSSC, 0.1% SDS for 30 min at the hybridization temperature. Membranes were exposed to X-ray films at -70ºC with intensifying screens.

Western blot.

Cell monolayers were washed with PBS, and lysated in RIPA buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM NaF, 1% TritonX100, 1% Deoxycholate, 0.1% SDS, 1mM sodium orthovanadate, 10 µg/ml Leupeptin, 10 µg/ml aprotinin, 100 µM PMSF). The extract was cleared by centrifugation at 4ºC and the protein content was determined with a detergent-compatible BioRad DC protein assay. Equal amounts of extracts were loaded to acrylamide SDS-PAGE gels according to Laemmli (31), and the electrophoresed proteins were electro-transferred to Nitrocellulose membranes (HybondECL, Amersham Pharmacia Biotech). Membrane was blocked with PBS, 0.1% Tween20 and 5% non-fat dry milk for at least 1 h. Membrane was incubated with the primary antibody in the above solution at 4ºC for 16 h. After washing with PBS-0.1% Tween20, the filter was incubated with horseradish peroxidase-conjugated secondary antibody in PBS, 0.1% Tween20 and 5% non-fat dry milk for 1 h at room temperature. Luminescent signal development with ECL (Amersham Pharmacia Biotech) was performed as recommended by the manufacturer. Filters were exposed to X-ray films in the dark. Antibodies against p110β subunit of PI3K, Akt, α-tubulin, ID1, ID2 and ID3 were purchased from Santa Cruz Biotechnology. Antibody against phosphorylated Akt was obtained from Cell Signal Technologies. Horseradish-peroxidase-conjugated secondary antibodies were obtained from Amersham Pharmacia Biotech, Jackson ImmunoResearch and Santa Cruz Biotechnology.

Electric Mobility Shift assay (EMSA)

Cell pellets were frozen in liquid nitrogen and cells lysated in 10 mM HEPES, pH 7.9; 0.1 mM EDTA, 5% Glycerol, 400 mM NaCl, 1 mM DTT, 0.1 mM PMSF. The lysate was cleared by centrifugation and the protein content determined with the BioRad Protein assay. A double-stranded oligonucleotide E-box probe corresponding to the sequence present in the muscle creatine kinase enhancer was used for EMSA (MCKE; 5': TCGACCCAA**CACCTG**CTGCCGTACCAGCT-3', the E-box sequence shown in bold characters). The DNA probe was labeled by filling of the protruding ends with Klenow enzyme and [α-^{32}P]-dCTP. The protein extracts (5 µg) were incubated in 12 µl of 20 mM HEPES pH 7.9; 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 20 % glycerol, 0.1% Triton X-100, containing 200 µg of bovine serum albumin, 2 µg of poly (dI-dC) and 50x10^3 cpm of the labeled MCKE probe. After 10 min incubation, the samples were loaded to a 6% poly-acrylamide gel in 0.5xTBE, and the gel run at 300 V for 2 h at 4°C. Finally, the dried gel was exposed to X-ray films.

PI3K assay.

Cell monolayers were washed with ice-cold PBS, and cells were scraped off in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 1mM sodium orthovanadate, 10µg/ml aprotinin, 10 µg/ml leupeptin, 0.1 mM PMSF and lysated by sonication. The extracts were cleared by centrifugation and total protein amount was measured (BioRad Protein Assay). Kinase reactions were set by mixing 20 µl of cell extract in lysis buffer (containing up to 40 µg of total protein), 20 µl of 0.5 mg/ml L-α-phosphatidyl-inositol (Sigma) in 10 mM HEPES pH 7.4, 0.1 mM EGTA and 10 µl of 50 mM HEPES pH 7.4, 25 mM $MgCl_2$ containing 10 µCi of [γ-^{32}P]-ATP (Amersham Pharmacia Biotech, spec. act 3000 Ci/mmol). When required, negative control reactions included 30 µM LY 294002. Reactions were incubated 10 min at room temperature, and were finished by adding 60 µl of 2 M HCl. Lipids were extracted with chloroform-methanol (1:1), and the organic phase loaded into a thin layer chromatography plate (Merck silica gel 60) pre-treated with 1% potassium oxalate in water. Chromatography was run in chloroform: methanol: 4M ammonia (9:7:2) until the front reached 80% of the plate. After drying, plates were exposed to X-ray films at -70ºC with intensifying screens. Quantification of the spots was made in a FUJI BAS phosphorimager.

RESULTS

The expression of ID bHLH transcription factors is downregulated during RA-induced differentiation of SH-SY5Y human neuroblastoma cells.

To characterize the molecular mechanisms through which RA triggers differentiation in SH-SY5Y cells, we analyzed differentially expressed genes using the Ordered Differential Display-PCR technique (32). Among several differentially expressed sequences obtained, one was identical to the 3' end of human *ID3* mRNA. The profile obtained in the differential display gel showed elevated expression of *ID3* in untreated cells, that was sustained after 8h of treatment with 1 μM RA but dropped sharply after 24 h of RA treatment (data not shown). Northern blot experiments confirmed the downregulation of *ID3* mRNA during RA-induced differentiation (Fig. 1A), and nuclear run-on transcription experiments demonstrated a severe reduction of the transcription rate of *ID3* gene accounting for the observed RA-induced downregulation (Fig. 1B). As different members of the ID gene subfamily of bHLH factors have been involved in the regulation of differentiation in many cell types (33, 34), we investigated whether other ID genes are expressed in SH-SY5Y neuroblastoma cells and how RA treatment could modify their expression levels. As shown in Figure 1C, *ID1* was expressed in SH-SY5Y cells at levels comparable to those of *ID3*. *ID2* mRNA levels appeared to be lower, since it was detected after longer exposures of the blots. *ID4* mRNA was not detected in SH-SY5Y cells. Treatment of SH-SY5Y cells with 1 μM RA resulted in downregulation of *ID1* and *ID2* expression, in parallel to the observed for *ID3*. Thus, RA treatment led to a coordinated downregulation of ID genes. The levels of ID1, ID2 and ID3 proteins in SH-SY5Y cells were severely reduced during RA treatment, in parallel to the observed changes in mRNA expression, as shown by western blot with specific antibodies (Fig. 1D).

RA treatment modified the expression pattern of bHLH transcription factors involved in neuronal differentiation.

ID proteins bind to and sequester ubiquitous E-proteins, avoiding complex formation with tissue-specific bHLH proteins and precluding heterodimer binding to E-box DNA elements. Treatment of SH-SY5Y cells with RA resulted in an increase of the protein complexes bound to an E-box element in gel-shift experiments (Fig. 2A), compatible with the observed decrease in the amounts of inhibitory ID proteins. We have analyzed the expression of bHLH genes involved in neuronal commitment and differentiation during the course of RA-induced neuronal differentiation of SH-SY5Y cells. As shown in figure 2B, the mRNA levels of *ASCL1*, a gene involved in determination of the neuronal fate expressed in proliferating neuroblasts (35), were reduced rapidly after RA treatment. On the other hand, expression of two members of the *atonal* subfamily of neurogenic bHLH factors was detected. The mRNA levels of *NEUROD6* (NEX-1, Hath-2) are very abundant and increased rapidly after RA administration. Small amounts of *NEUROD1* transcript were detected induced by RA. The mRNAs for other bHLH factors like neurogenin and *ATOH1* were not detected (data not shown). As marker for neuronal differentiation and E-box-regulated gene we have assayed the expression of *GAP43*. Levels of *GAP43* transcript were very low in untreated cells, and increased during RA-induced differentiation, reaching maximal expression after 24 h of treatment. In summary, differentiation induced by RA led to dramatic changes in the expression patterns of bHLH transcription factors in SH-SY5Y cells. In addition to downregulation of ID genes, RA treatment repressed the transcription of *ASCL1*, a neuroblast-specific gene, and activated the expression of at least two proneural genes from the *atonal* subfamily, *NEUROD6* and *NEUROD1*. In parallel, the transcription of a bHLH-regulated, neuron-specific gene like *GAP43* is upregulated.

ID genes are downregulated during TPA-induced differentiation of SH-SY5Y cells.

Phorbol esters are Protein Kinase C activators that have been reported to induce neural differentiation in several neuroblastoma cell lines (16). We have investigated whether the expression levels of the ID genes are modified during TPA-induced differentiation of SH-SY5Y cells. As shown in Figure 3, the levels of *ID3*, *ID1* and *ID2* mRNAs are maintained after 6 h of treatment with 100 nM TPA, but sharply reduced between 6 and 24 h of differentiation, with a similar kinetics to that observed with RA treatment. In addition, TPA induced transiently *ID4* expression. Low levels of *ID4* mRNAs could be detected after 6 h of treatment, but were reduced later during TPA-induced differentiation. The expression of the major ID genes is thus coordinately downregulated during differentiation induced by two disparate compounds like RA, a signal acting through a Nuclear Hormone Receptor, and TPA, an activator of Protein Kinase C, underscoring the relevance of the downregulation of ID genes for terminal differentiation of neuroblastoma cells.

Downregulation of the ID genes by RA requires newly synthesized proteins and Phosphatidyl-inositol 3-kinase activity.

Next we investigated the molecular mechanisms of RA-induced coordinated downregulation of the ID genes. The fact that downregulation of the ID genes expression takes place between 6-24 h after administration of RA, with a considerable delay in respect to other downregulated genes like *MYC* (see Fig.1A), does not argue in favor of a direct regulation of the transcription of ID genes by ligand-activated RAR/RXR heterodimers. In addition, RA and TPA, two agents that normally have opposite effect on gene transcription, have similar effects on the expression of the major ID genes, supporting an indirect transcriptional regulation mechanism. Treatment of SH-SY5Y cells with cycloheximide, an inhibitor of protein synthesis, prevented downregulation of *ID1* and *ID3* expression by RA, without affecting transcriptional activation of genes directly activated by RA, like *RARB*. (Fig. 4A), supporting the idea that newly synthesized proteins are required for repression of ID genes by RA.

Since Phosphatidyl-inositol 3-Kinase (PI3K) signaling pathway has been involved in differentiation of many cell types, we have studied the effect of LY294002, a specific inhibitor of PI3K, on RA-induced repression of ID gene expression. As shown in figure 4B, treatment with LY294002 prevented RA-induced full downregulation of *ID3* and *ID1* gene expression in SH-SY5Y cells, without affecting the induction of other genes directly activated by RA (data not shown). These results indicate that repression of ID genes by RA requires a signaling pathway involving PI3K.

Activation of the PI3K/Akt signaling pathway by RA is required for neuronal differentiation of SH-SY5Y cells.

RA treatment of SH-SY5Y cells resulted in sustained activation of PI3K as demonstrated by the corresponding *in vitro* kinase assay performed with total cellular extracts. (Fig. 5A). Treatment with RA for 24 h resulted in a four-fold increase in PI3K activity, which lasted up to 48 h. This increase was abrogated when the assay was performed in the presence of LY294002 (Fig. 5B). This sustained increase of PI3K activity correlated with an increase in the levels of the catalytic subunit of the enzyme, p110β, as shown by western blot with specific antibodies (Fig. 5C). This increase appears to be consequence of an effect of RA on the stability of the protein, since a parallel increase in p110β mRNA was not detected (data not shown). Akt kinase is one of the major targets of PI3K. Treatment of SH-SY5Y neuroblastoma cells with RA resulted in rapid phosphorylation of the Akt kinase in Ser473 (Fig. 5D). Increased phosphorylated Akt immunoreactivity was detected as soon as after 30 min of RA treatment, and increased further to reach a maximum after 2-3 h of RA treatment. The level of Ser473-Akt phosphorylation obtained by RA treatment was comparable to that observed by heat-shock treatment (45°C, 30 min), a known inducer of Akt phosphorylation in SH-SY5Y cells (36).

Next we have addressed whether the inhibition of PI3K by its specific inhibitor LY294002 would impair RA-induced differentiation. This was evaluated both morphologically,

by estimating the percentage of cells bearing long neurites, and biochemically, by analyzing the transcriptional activation of the neuron-specific gene *GAP43*. RA-induced morphological differentiation, shown by an increase in the percentage of cells bearing long neurites, was inhibited by addition of LY294002 (Fig. 6A). Treatment of the cells with the PI3K inhibitor resulted in a moderate increase in *GAP43* mRNA basal level. However further transcriptional activation of *GAP43* by RA was prevented by treatment with LY294002 (Fig. 6B). Transcriptional activation of the anti-apoptotic gene *BCL2* was also impaired by treatment with LY294002 (Fig. 6C). Transcription of *BCL2* is strongly activated during RA-induced differentiation. Again a moderate increase of *BCL2* mRNA basal level could be observed in LY294002-treated cells. However, the inhibitor prevented RA-induced upregulation of *BCL2*. Taken together, these results indicate that activation of PI3K/Akt signaling pathway by RA is required for RA-induced cellular differentiation and for the transcription genes involved in the regulation of cell survival.

DISCUSSION

The members of the bHLH family of transcription factors are involved in the control of commitment, differentiation and development of many different tissues and organs, including the nervous system (22, 37, 38, for a review). This control is often the result of the balance between differentiation-promoting and differentiation-inhibiting bHLH factors. ID genes encode dominant-negative bHLH proteins that are supposed to inhibit differentiation by counteracting the actions of differentiation-promoting bHLH proteins (33, 34, for a review). Here we show that ID gene expression is coordinately downregulated during RA-induced differentiation of SH-SY5Yneuroblastoma cells, and that ID protein levels follow a parallel decrease. In many cell types and experimental systems the expression of ID genes could be associated to active proliferation, and ID genes mRNA levels are downregulated during differentiation. Conversely, ectopic expression of ID genes leads to increased proliferation and to a block in differentiation (33, 34). Reduced expression of *ID1* and *ID3* in double knockout mutant mice leads to impaired development of the nervous system, with premature cell differentiation and aberrant expression of proneural bHLH genes (39). ID proteins bind to and sequester ubiquitous E-proteins, avoiding complex formation with tissue-specific bHLH proteins and activation of E-box-regulated genes involved in cell differentiation. Downregulation of ID gene expression by RA treatment of neuroblastoma cells would increase the amount of *free* bHLH factors ready to form functional heterodimers and bind to E-box elements. This idea is supported by the RA-dependent increased complex formation on an E-box element shown in the gel-shift experiment (Fig.2A), although this experiment is not conclusive. In addition, RA treatment leads to an increase in the expression of bHLH factors from the *atonal* subfamily (like *NEUROD1* and *NEUROD6*), that eventually may contribute to counteract the inhibition of DNA binding and the block in differentiation imposed by ID factors. The expression of factors from the *atonal* subfamily might be sufficient to initiate the differentiation process, since ectopic expression of this class of bHLH factors has been reported to be sufficient to produce neural differentiation of ectoderm cells (40) and

neuroblastoma cells (41). The actions of these transcription factors would be also responsible for the transcriptional activation of neuron-specific genes, like *GAP43,* encoding a protein present in the growth cones of post-mitotic neurons, whose transcription is regulated through E-box sequences present at its promoter (42). On the other hand, the expression of *ASCL1* (HASH-1), the human orthologue of mouse *MASH1*, is rapidly reduced during RA treatment. *MASH1* is expressed in developing sympathetic neuronal precursors and is crucial for neuronal fate determination and development of olfactory neurons, sympathetic ganglia and some enteric and parasympathetic neuron populations (43, 44). During human fetal development, *ASCL1* is expressed in sympathetic neuroblasts and its expression decays at the onset of the formation of sympathetic ganglia and paraganglia (35). *ASCL1* is expressed in neuroblastoma cell lines and a majority of primary neuroblastoma tumors (35, 45-47). Our results are in good agreement with recent reports showing a decrease in *ASCL1* mRNA levels during neuroblastoma cell differentiation (45, 47, 48), suggesting that downregulation of *ASCL1* may be necessary to promote neuronal differentiation of neuroblastoma cells. Taken together, our results suggest that the differentiation signal elicited by RA could be transduced to the proneural bHLH network of transcription factors, via the downregulation of the ID gene expression. The reduction in the levels of differentiation-inhibiting ID factors induced by RA, would allow the differentiation-promoting *atonal* bHLH transcription factors *NEUROD1* and *NEUROD6* act as effector genes of the differentiation signal initiated by RA, and these actions would result in the transcriptional activation of neuron-specific genes, like has been shown for *GAP43*.

In addition, we have shown that treatment with TPA, another of the compounds that induce neuroblastoma cell differentiation (16), also results in the coordinated downregulation of ID gene expression. The fact that two distinct differentiating stimuli, like RA and TPA, that normally act through very different molecular mechanisms and generally have opposite transcriptional effects (49, 50), result in coordinated downregulation of ID gene expression underscores the role of ID gene in differentiation. This fact, together with the delayed kinetics of

downregulation, implies that the molecular mechanisms underlying ID gene coordinated repression might be complex. Coordinated downregulation of ID genes by RA appears to be indirect and requires newly synthesized proteins. In addition, we show that the PI3K/Akt signaling pathway is involved in RA-induced transcriptional downregulation of ID genes, since treatment with LY294002, a specific inhibitor of PI3K, prevented full repression of the ID genes by RA. The signaling pathway of PI3K/Akt is involved in regulating differentiation in many cell types. The similarities between the differentiation process in myoblasts and in neuroblastoma cells are striking. In both cases transcription factors from the bHLH family are involved, and activation of NF-κB and Nitric Oxide production take place in differentiating myoblasts (51), as well as in differentiating neuroblastoma cells (52, 53), suggesting that the molecular events regulating cell differentiation in both cell types might be similar. As activation of the PI3K/Akt pathway plays a crucial role in myoblast differentiation, and inhibition of PI3K activity by LY294002 abrogates differentiation (54), we considered the possibility that this signaling pathway could be involved in neuroblastoma cell differentiation. We have shown that RA treatment leads to a strong (four-fold) activation of PI3K activity in SH-SY5Y cells. Activation is detected after 24 h RA treatment and is maintained up to 48 h of differentiation. The observed increase of PI3K catalytic subunit p110β protein level during RA treatment might collaborate to this rather sustained activation of PI3K. Nevertheless, this increase in p110β protein level appears to be originated by a post-translational effect, since the amount of p110β mRNA remains unchanged. A similar effect on p110β during mouse adipocyte differentiation has been reported (55). The protein kinase Akt is one of the targets of PI3K. Here we show that RA treatment leads to a rapid activation of Akt, and phosphorylation of Akt in Ser473 could be detected as soon as after 30 min of RA treatment. Akt kinase plays an important role in the regulation of cell death and survival (56, 57, for review). In addition, our results indicate that activation of the PI3K/Akt signaling pathway by RA is required for RA-induced differentiation, since inhibition of PI3K by

its specific inhibitor LY294002 resulted in a block of differentiation, according to morphological and biochemical criteria. Neurite extension, that became evident in RA-treated SH-SY5Ycells, is not observed when neuroblastoma cells were treated with RA in the presence of LY294002. In addition, transcriptional activation of the neural marker gene *GAP43* did not took place in the course of RA-induced differentiation when PI3K was inhibited. Taken together, these results indicated that activation of the PI3K/Akt signaling pathway by RA is necessary for RA-induced differentiation of SH-SY5Y neuroblastoma cells. However, we do not believe that activation of this signaling pathway could be sufficient for differentiation. Recently, it has been shown that SH-SY5Y cells transiently transfected with a constitutively active form of PI3K did not become differentiated, and only a rather modest neurite outgrowth could be detected[2]. This idea is also supported by the fact that treatment with IGF-I, a potent activator of PI3K, does not induce differentiation of SH-SY5Y cells, but act as a mitogen. However, IGF-I cooperates strongly with RA or TPA in inducing cell differentiation (17, 58), effect that could be now better understood taking into account the results shown in this paper. An important question that arises from the results shown here is the nature of the molecular mechanism by which RA activates PI3K. The typical activators of PI3K act through tyrosine kinase receptors, like is the case for IGF, PDGF, FGF, neurotrophins etc., and some of these factors promote neuroblastoma cell differentiation *in vitro* (17, 18). As the usual action of RA is transcriptional regulation, we initially thought that RA might induce the expression of a tyrosine kinase receptor, and we have examined several candidate genes (data not shown). However, on the basis of the rapid RA-induced phosphorylation of Akt, we put forward the idea that RA may activate PI3K through an atypical extra-genomic action of its receptor. A growing number of extra-genomic actions of Nuclear Hormone Receptors have been reported (59, 60, for review). It has been reported that estradiol activates PI3K through extra-genomic actions of its receptor ER in endothelial cells (61-63), in cortical neurons in culture (64), and in a cell line derived from a mammary tumor (65). In

addition, activation of PI3K by vitamin D3, through an extra-genomic action of VDR has been reported to be involved in myeloid cell differentiation (66).

The results shown here support a heretofore unrecognized role for RA in the regulation of apoptosis and survival in neural cells. In neuroblastoma cells, RA-induced transcriptional activation of the anti-apoptotic gene *BCL2* and increased resistance to apoptosis-inducing drugs has been reported (67-69). Effects of RA on the promotion of cell survival have been also reported for primary cultures of neuroblasts from spinal cord (70, 71), and for neurons derived from neural stem cells *in vitro* (72). Our results provide a molecular mechanism explaining the effects of RA on neural cell survival. Integration of the genomic and extra-genomic actions of RA results in coupling of cell differentiation and survival. On one hand, RA acts regulating the transcription of specific genes involved in cell differentiation. On the other hand, RA activates through an extra-genomic action the PI3K/Akt signaling pathway, which is involved in the regulation of survival. However, both types of actions do not occur independently, but are intertwined. Activation of the PI3K/Akt signaling pathway by RA also contributes to the transcriptional regulation of genes crucial for differentiation, as we have shown for the coordinated downregulation of the ID genes. Reciprocally, RA may contribute to cell survival by activating the transcription of genes encoding receptors for neurotrophic factors, like trk, trkB and RET[3] (73-76). The PI3K/Akt signaling pathway is involved in the regulation of both differentiation and survival during RA-induced differentiation of neuroblastoma cells. However, this does not appear to be the case in PC12 pheochromocytoma cells, another well-characterized model system for neural differentiation, since regulation of differentiation and survival by NGF occurs via two independent signaling pathways. Differentiation requires activation of RAS/ERK pathway and survival requires the activation of PI3K/Akt pathway (77, 78).

The coordinated regulation of cell differentiation and survival by RA may play an important role in the context of neuronal cell generation, in which an excess of precursor cells are produced to ensure that all the required nervous connections take place. With this *neurotrophic*

strategy (79, for review), those cells that establish contact with their target cells will receive from them neurotrophic survival factors, whereas those cells that do not succeed in finding their targets will die through apoptosis. The coupling between cell differentiation and survival elicited by RA may result in avoiding a premature cell death, allowing survival of the newly differentiated cell for a limited time period, until the contacts with its target cells could be established.

RA and other synthetic retinoids are used frequently in the therapy of neuroblastoma tumors (20, 21), and other cancer types (80, for review). Another question arising from the results described here is that activation of the PI3K/Akt signaling pathway by RA leading to an increase of the resistance to cell death could be an unfavorable effect for cancer therapy. This could be especially important if RA treatment must be combined with therapies based on induction of cell death, like chemo-or radiotherapy. A possible solution for this problem would be the generation of synthetic retinoids lacking the ability to activate the PI3K/Akt pathway, which could have advantages as anticancer therapy.

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FOOTNOTES

[1] The abbreviations used are: RA, all-trans Retinoic Acid; RAR, Retinoic Acid Receptor; RXR, 9-cis Retinoic Acid Receptor; bHLH, basic domain helix-loop-helix transcription factor; PI3K, Phosphatidyl-inositol-3 Kinase; TPA, Phorbol 12-myristate 13-acetate; PBS, Phosphate-buffered saline; DEPC, Diethyl-pyrocarbonate; PMSF, Phenyl-methyl-sulfonyl-fluoride; PAGE, Poly-acrylamide gel electrophoresis; EMSA, Electric mobility shift assay; MCKE, muscle creatinine kinase enhancer; NF-κB, Nuclear factor κB; ER, Estrogen Receptor; VDR, Vitamin D Receptor; PIP3: Phosphatidyl-inositol (3, 4, 5)-triphosphate.

[2] F. Wandosell, personal communication.

[3] López-Carballo G. et al., work in preparation.

LEGEND TO FIGURES

Figure 1.

Expression of ID genes is downregulated during RA-induced differentiation.

(A) Northern blot Analysis. Each lane contains 15 µg of total RNA from SH-SY5Y neuroblastoma cells treated with 1 µM RA for the times indicated in the figure. The blot was sequentially hybridized with [^{32}P]-labeled DNA probes specific for the mRNAs for RARβ (*RARB*, 3 Kb), c-myc (*MYC*, 2.5 kb), *ID3* (1.2 Kb), and cyclophilin A (prolyl-peptidyl-isomerase A, *PPIA*, 1 Kb) as internal loading control.

(B) Run-on assay of ID3 transcription rate. Nuclei from SH-SY5Y neuroblastoma cells treated for 24 h with vehicle (-) or 1 µM RA (+) were used to generate [^{32}P]-labeled run-on transcription probes. These probes were hybridized to nylon membranes containing 5 µg of *ID3* probe plasmid DNA. As controls the filter also contained 5 µg of *BCL2* probe plasmid DNA and 1 µg of purified actin β (*ACTB*) and cyclophilin A (*PPIA*) probes generated by PCR. Filters included 5 µg of pBluescript-KS (pBS-KS) as specificity control.

(C) Coordinated downregulation of ID genes by RA. Northern blot Analysis. Each lane contains 15 µg of total RNA from SH-SY5Y neuroblastoma cells treated with 1 µM RA for the times indicated in the figure. The blot was sequentially hybridized with [^{32}P]-labeled DNA probes specific for the mRNAs for *ID3* (1.2 Kb), *ID1* (1.2 Kb) and *ID2* (1.4 Kb). The 28S ribosomal RNA band in the blot stained with methylene blue is shown as internal loading control.

(D) Western blot analysis. Each lane contains 20 µg of protein extract from SH-SY5Y neuroblastoma cells treated with 1 µM RA for the times indicated in the figure. The blot was sequentially incubated with specific antibodies raised against ID3, ID1 and ID2 proteins. As loading control, the membrane was incubated finally with antibodies against α-tubulin (α-tub).

Figure 2.

The expression pattern of bHLH transcription factors involved in neuronal differentiation is modified during RA treatment

(A) Electric mobility shift assay (EMSA). A [^{32}P]-labeled double-stranded oligonucleotide probe containing the E-box element present in the Muscle Creatin Kinase Enhancer was incubated with 5 µg of protein extract from SH-SY5Y neuroblastoma cells treated with 1 µM RA for the times indicated. The complexes formed were resolved in a 6% poly-acrylamide gel run in 0.5xTBE.

(B) Northern blot. Each lane contains 15 µg of total RNA from SH-SY5Y neuroblastoma cells treated with 1 µM RA for the times indicated. The blot was sequentially hybridized with [^{32}P]-labeled DNA probes specific for the mRNAs for *ASCL1* (2.6 Kb), *NEUROD6* (1.4 Kb) *NEUROD1* (4.2 Kb) and *GAP43* (1.4 Kb). The 28S ribosomal RNA band in the blot stained with methylene blue is shown as internal loading control.

Figure 3.

Downregulation of ID genes during TPA-induced differentiation

Northern blot. Each lane contains 15 µg of total RNA from SH-SY5Y neuroblastoma cells treated with 100 nM TPA for the times indicated. The blot was sequentially hybridized with [^{32}P]-labeled DNA probes specific for the mRNAs for *ID3* (1.2 Kb), *ID1* (1.2 Kb), *ID2* (1.4 Kb), *ID4* (4.0, 2.8, and 1.7 Kb), *NPY* (1 Kb) and *MYC* (2.5 Kb). The 28S ribosomal RNA band in the blot stained with methylene blue is shown as internal loading control.

Figure 4

Coordinated downregulation of the ID genes by RA requires newly synthesized proteins and the activity of the Phosphatidyl-inositol 3-kinase.

(A) Northern blot. Each lane contains 15 µg of total RNA from SH-SY5Y neuroblastoma cells treated with 1 µM RA for the times indicated (left panel) or pre-treated for 30 min with cycloheximide (10 µg/ml) and then treated with 1 µM RA in the presence of the inhibitor for the times indicated (right panel). The blot was sequentially hybridized with [^{32}P]-labeled DNA probes specific for the mRNAs for *ID3* (1.2 Kb), *ID1* (1.2 Kb), and *RARB* (3 Kb). The 28S ribosomal RNA band in the blot stained with methylene blue is shown as internal loading control.

(B) Northern blot. Each lane contains 15 µg of total RNA from SH-SY5Y neuroblastoma cells treated with 1 µM RA for the times indicated (left panel) or pre-treated for 30 min with LY294002 (10 µM) and then treated with 1 µM RA in the presence of the inhibitor for the times indicated (right panel). The blot was sequentially hybridized with [^{32}P]-labeled DNA probes specific for the mRNAs for *ID3* (1.2 Kb), and *ID1* (1.2 Kb). The 28S ribosomal RNA band in the blot stained with methylene blue is shown as internal loading control.

Figure 5

Activation of the Phosphatidyl-inositol 3-kinase/Akt signaling pathway by RA

(A) Phosphatidyl-inositol 3-kinase Assay. Phosphatidyl-inositol 3-kinase assay was performed with L-α-phosphatidyl-inositol, [γ-^{32}P]-ATP and 40 µg whole cell protein extract from SH-SY5Y cells treated with 1 µM RA for the times indicated in the figure. As specificity control, a parallel assay was performed in the presence of 30 µM LY294002. The figure shows a detail of the autoradiogram of the thin layer chromatography plate, demonstrating a RA-induced increase in the generation of Phosphatidyl-inositol (3, 4, 5)-triphosphate (PIP3). The experiment was repeated five times with different amounts of protein extracts, and similar results were obtained. The figure shows a representative experiment.

(B) Quantification of the assay shown in (A). The figure shows the quantification of the radioactivity present in the spots shown in (A). PI3K activity is shown as percent of the activity obtained for the untreated control extract.

(C) Western blot analysis. Each lane contains 20 µg of protein extract from SH-SY5Y neuroblastoma cells treated with 1 µM RA for the times indicated in the figure. The blot was incubated with specific antibodies raised against p110β, the catalytic subunit of the Phosphatidyl-inositol 3-kinase.

(D) Immunodetection of Phosphorylated Akt. Each lane contains 20 µg of protein extract from SH-SY5Y neuroblastoma cells treated with 1 µM RA for the times indicated in the figure. Lane labeled HS contains 20 µg of protein extract from SH-SY5Y neuroblastoma cells submitted to a heat shock (45°C, 30 min). The blot was sequentially incubated with specific antibodies raised against Akt phosphorylated at Ser473 (P-Akt) and against total Akt (Akt).

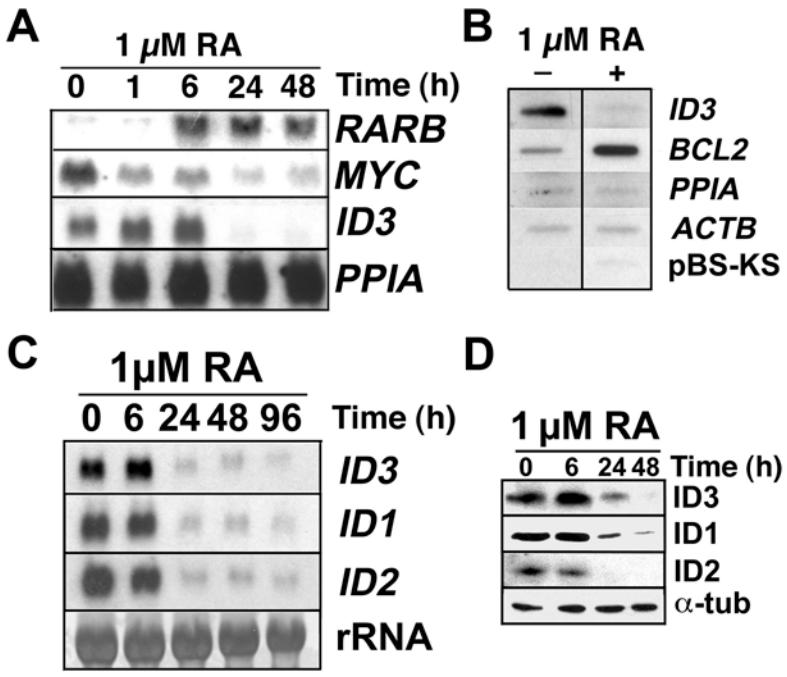
Figure 6

Activation of the PI3K/Akt signaling pathway by RA is required for neuronal differentiation of SH-SY5Y cells.

(A) Morphological differentiation. We considered as morphologically differentiated those cells that bear neurites longer than twice the size of the cell body. The morphology of living SH-SY5Y neuroblastoma cells was examined under the inverted microscope, and the percentage of differentiated cells determined for cells treated with vehicle (Control), treated for 24 h with 10 μM LY 294002 (LY), treated for 24 h with 1 μM RA (RA), and pre-treated for 30 min with 10 μM LY 294002 and treated for 24 h simultaneously with 1 μM RA and 10 μM LY 294002 (RA+LY). A minimum of 100 cells per dish is examined. The graph shows the results (expressed as mean ± standard deviation) of an experiment performed in triplicate.

(B) Northern blot. Each lane contains 15 μg of total RNA from SH-SY5Y neuroblastoma cells treated with 1 μM RA for the times indicated (left panel) or pre-treated for 30 min with LY294002 (10 μM) and then treated with 1 μM RA in the presence of the inhibitor for the times indicated (right panel). For better comparison of basal levels 15 μg of total RNA from cells treated with vehicle was included in the right panel (lane labeled 0) The blots were hybridized with a [^{32}P]-labeled DNA probe specific for the mRNA for *GAP43* (1.4 Kb). The 28S ribosomal RNA band in the blot stained with methylene blue is shown as internal loading control.

(C) Northern blot. Each lane contains 15 μg of total RNA from SH-SY5Y neuroblastoma cells treated with 1 μM RA for the times indicated (left panel) or pre-treated for 30 min with LY294002 (10 μM) and then treated with 1 μM RA in the presence of the inhibitor for the times indicated (right panel). The blots were hybridized with a [^{32}P]-labeled DNA probe specific for the mRNA for *BCL2* (7.6 Kb). The 28S ribosomal RNA band in the blot stained with methylene blue is shown as internal loading control.


A
1 μM RA
0 1 6 24 48 Time (h)
RARB
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ID3
PPIA
B
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BCL2
PPIA
ACTB
pBS-KS
C
1μM RA
0 6 24 48 96 Time (h)
ID3
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ID2
rRNA
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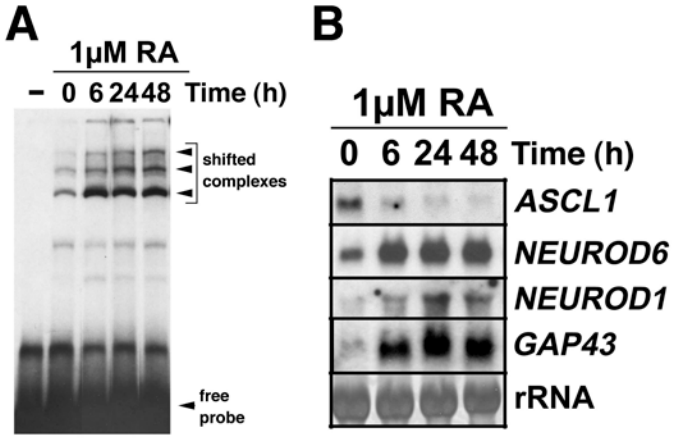
ID3
ID1
ID2
α-tub

Fig. 1


A
1μM RA
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B
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ASCL1
NEUROD6
NEUROD1
GAP43
rRNA

Fig. 2

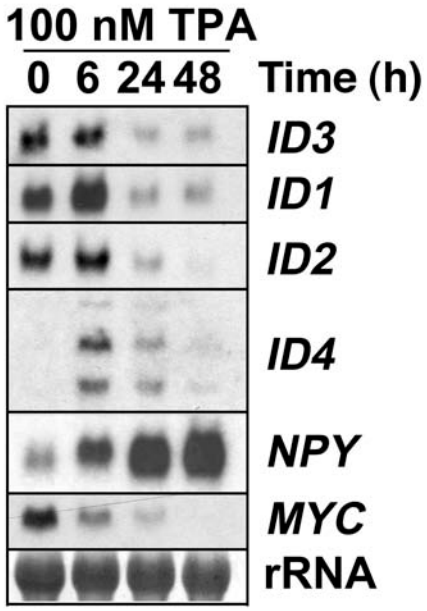
100 nM TPA
0 6 24 48 Time (h)
ID3
ID1
ID2
ID4
NPY
MYC
rRNA

Fig. 3

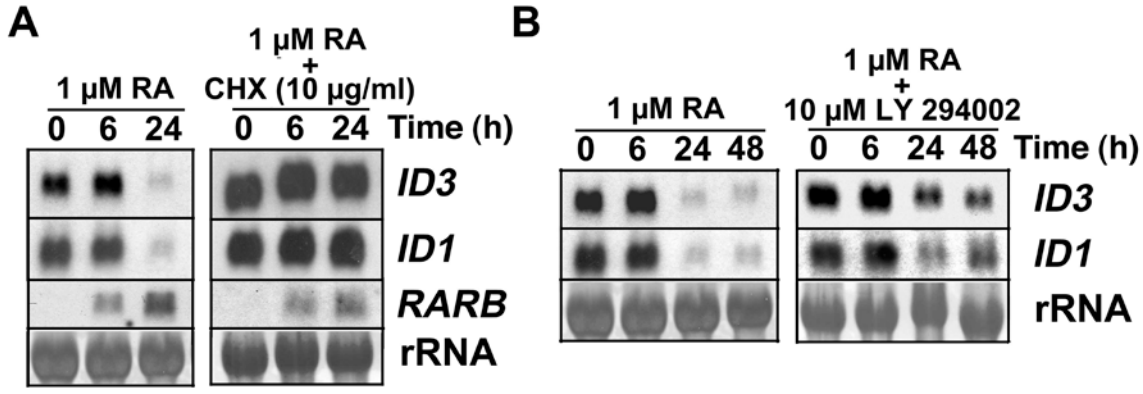
A
1 μM RA
1 μM RA + CHX (10 μg/ml)
0 6 24 0 6 24 Time (h)
ID3
ID1
RARB
rRNA
B
1 μM RA
1 μM RA + 10 μM LY 294002
0 6 24 48 0 6 24 48 Time (h)
ID3
ID1
rRNA

Fig. 4

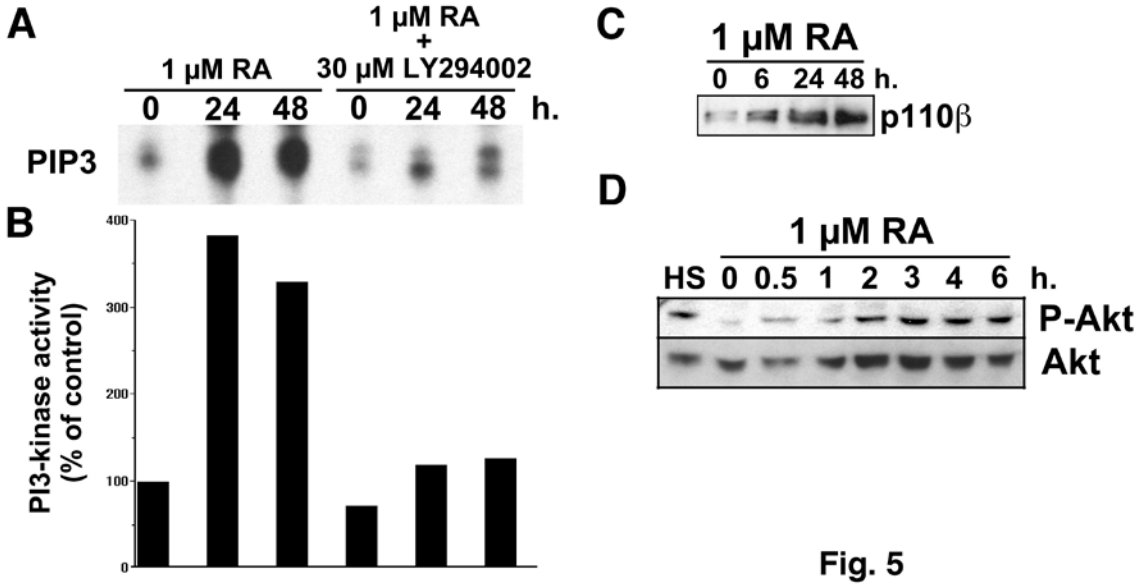
A
1 μM RA
1 μM RA + 30 μM LY294002
0 24 48 0 24 48 h.
PIP3
B
PI3-kinase activity (% of control)
400
300
200
100
0
C
1 μM RA
0 6 24 48 h.
p110β
D
1 μM RA
HS 0 0.5 1 2 3 4 6 h.
P-Akt
Akt

Fig. 5

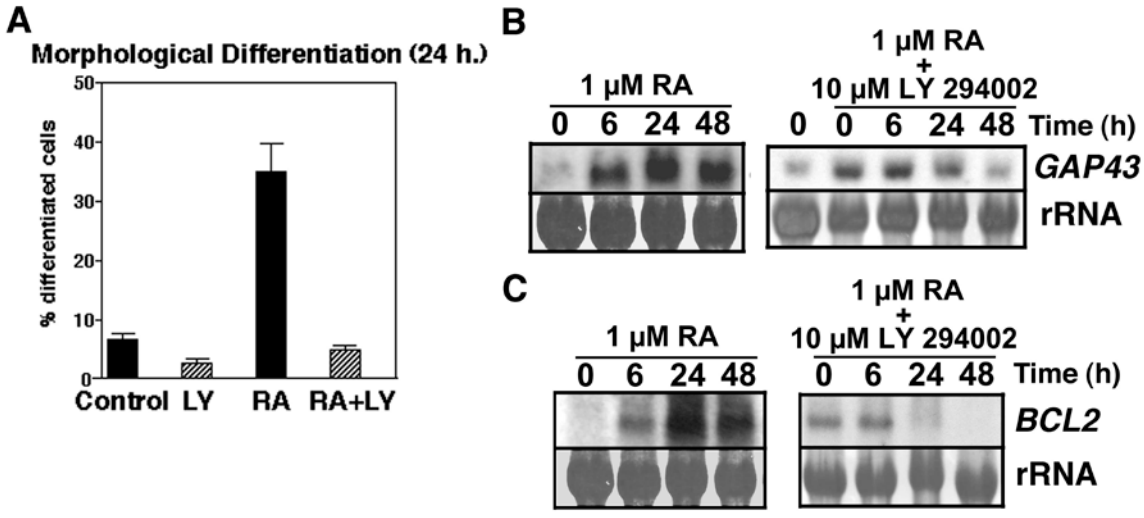
A
Morphological Differentiation (24 h.)
% differentiated cells
50
40
30
20
10
0
Control LY RA RA+LY
B
1 μM RA
1 μM RA + 10 μM LY 294002
0 6 24 48 0 0 6 24 48 Time (h)
GAP43
rRNA
C
1 μM RA
1 μM RA + 10 μM LY 294002
0 6 24 48 0 6 24 48 Time (h)
BCL2
rRNA

Fig. 6